Fungi as Biocontrol Agents

Progress, Problems and Potential

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Preface

The development of fungi for the biocontrol of pests, weeds and diseases has received a significant amount of interest in recent years. We have seen the progression of scientific wisdom from reports of potential biocontrol agents (BCAs) under optimized laboratory bioassay conditions (often a naïve and tenuous link) followed by disappointing field trials, to an advanced understanding of the important concepts required to produce a reliable and effective BCA. It is thus surprising that, while research directed to these major targets has a number of common goals, very little attention has previously been given to the integration of research effort. Disciplines such as pathology, genetics, physiology, mass production, formulation and application strategies are essential components in all three targets in making the necessary advances to enable fungal BCAs to become registered and commercialized.

Our aim in organizing the International Symposia at the University of Southampton in 1998 and the University of Wales Swansea in 1999, from which this book emerged, was to attempt to bring together scientists, industry and government agencies involved in all aspects of fungal BCAs for the first time. We believe that these meetings were timely and highly successful. Together they attracted over 700 participants from more than 36 countries to interact, identify common bottlenecks and suggest ways in which these can be overcome to enable progress to be achieved more rapidly. Common themes such as production, formulation and application technologies, biosafety, risk assessment and registration requirements were all covered.

This book has thus adopted a multidisciplinary approach to integrate the state-of-the-art knowledge in key areas of common interest in the development of fungal BCAs of pests, weeds and diseases. We hope that this will encourage further integration and focus on common hurdles that need to be overcome to enable more fungal BCAs to be commercialized and registered. With the significant pressure from consumers and the growing organic market requirements for fungal BCAs, we hope that this book will be beneficial in stimulating the required advances for this to be achieved.

This book is a timely attempt to link scientists from different and complementary disciplines to achieve a unified synthesis. No equivalent has been published for 10 years; the last notable work was that of Whipps and Lumsden (1989, *Biotechnology*

of Fungi for Improving Plant Growth, Cambridge University Press). The work offers an update of progress in the development of fungal BCAs, as well as drawing attention to potential and associated problems, and will integrate theory and practice.

We are grateful to all the contributors for their efforts in enabling this book to come to fruition. We also thank the referees, who include: Horace 'Hank' Cutler, Mike Bidochka, Milton Typas, Stefan Jaronski, Steve Wraight, Steve Lisansky, Doug Strongman, Mark Goettel, Jim Pratt, Sebastian Kiewnick and Mark Jackson.

It is our goal that this book will serve as the current, most comprehensive treatise on the rapidly emerging field of fungal biocontrol and as a useful resource for practitioners, students, regulators, and industrial planners and marketeers.

Tariq M. Butt
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1 Introduction – Fungal Biological Control Agents: Progress, Problems and Potential

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There is increasing interest in the exploitation of fungi for the control of invertebrate pests, weeds and diseases, as evidenced by the number of commercial products available and under development (Tables 1.1–1.3). Fungal biological control is an exciting and rapidly developing research area with implications for plant productivity, animal and human health and food production. This area includes a number of important disciplines, such as pathology, ecology, genetics, physiology, mass production, formulation and application strategies. The research, development and final commercialization of fungal biological control agents (BCAs) continue to confront a number of obstacles, ranging from elucidating important basic biological knowledge to socio-economic factors. Considerable advances have been made in separate areas but it is important to integrate and communicate these new findings.

In this chapter we present a brief overview of some of the social and economic reasons for developing fungal BCAs, highlighting the commercial perspective. We also outline the main steps in developing fungal BCAs and draw attention to the chapters that correspond with each step in the commercialization process.

There is considerable interest in the exploitation of naturally occurring organisms, such as bacteria, viruses and fungi, for the control of crop pests, weeds and diseases. Although this chapter focuses on fungal BCAs, many of the concepts apply to other beneficial organisms which offer environmentally friendly alternatives to chemical pesticides. Fungal BCAs could be used where chemical pesticides are banned (e.g. organochlorines) or being phased out (e.g. methyl bromide) or where pests have developed resistance to conventional pesticides (see Chapter 2). It is generally recognized that some chemical pesticides contaminate groundwater and enter food-chains that have an impact on a wide range of organisms. Furthermore, pesticides can pose hazards to animal health and to the user spraying the chemical. Consumer perceptions worldwide are that chemical usage in agricultural production needs to be significantly

Table 1.1. Fungi developed or being developed for the biological control of diseases (data from Burges, 1998; Butt and Copping, 2000).

Product	Fungus	Target	Producer
Mycoparasites			
Rotstop	*Phlebiopsis* (=*Peniophora*) *gigantea*	*Heterobasidium annosus*	Kemira Agro Oy, Finland
Primastop	*Gliocladium catenulatum*	Several plant diseases	Kemira, Agro Oy, Finland
SoilGard (= GlioGard)	*Gliocladium virens*	Several plant diseases Damping off and root pathogens	ThermoTrilogy, USA
Cotans WG	*Coniothyrium minitans*	*Sclerotinia* spp.	Prophyta, Germany; KONI, Germany
AQ10 Biofungicide	*Ampelomyces quisqualis*	Powdery mildews	Ecogen Inc., USA
YieldPlus	*Cryptococcus albidus*	*Botrytis* spp., *Penicillium* spp.	Anchor Yeast, South Africa
Aspire	*Candida oleophila*	*Botrytis* spp., *Penicillium* spp.	Ecogen Inc., USA
Endothia parasitica	*Endothia parasitica* (non-pathogenic strain)	*Endothia parasitica* (chestnut blight)	CNICM
Fusaclean	*Fusarium oxysporium*	*Fusarium oxysporium*	Natural Plant Protection, France
Biofox C	*F. oxysporium*	*F. oxysporium, Fusarium moniliforme*	SIAPA, Italy
Polygandron polyversum	*Pythium oligandrum*	*Pythium ultimum*	Plant Protection Institute, Slovak Republic
Trichoderma 2000	*Trichoderma harzianum*	*Rhizoctonia solani, Sclerotium rolfsii, Pythium*	Mycontrol (EfA1) Ltd, Israel
Trichopel	*T. harzianum*	Wide range of fungal diseases	Agrimm Technologies Ltd, New Zealand
T-22 and T-22HB Bio-Trek, RootShield	*T. harzianum*	*Pythium, Rhizoctonia, Fusarium, Sclerotinia*	BioWorks (= TGT Inc.), Geneva, USA
Supresivit	*T. harzianum*	Various fungi	Borregaard and Reitzel, Denmark; Fytovita, Czech Republic
Trichodowels, Trichoject, Trichoseal and others	*T. harzianum* and *Trichoderma viride*	*Chondrostereum purpureum* and other soil and foliar pathogens	Agrimms Biologicals, New Zealand, and others
Binab T	*T. harzianum, Trichoderma polysporum*	Fungi causing wilt, wood decay and take-all	Bio-Innovation, Sweden
Trichodex	*T. harzianum*	Fungal diseases, e.g. *Botrytis cinerea*	Makhteshim-Agan, Israel, several European companies, e.g. DeCeuster, Belgium

Table 1.2. Fungi developed or being developed for the biological control of pests (data from Burges, 1998; Butt and Copping, 2000).

Product	Fungus	Target	Producer
Entomogenous fungi			
Mycotal	*Verticillium lecanii*	Whitefly and thrips	Koppert, the Netherlands
Vertalec	*V. lecanii*	Aphids	Koppert, the Netherlands
Biogreen	*Metarhizium anisopliae*	Scarab larvae on pasture	Bio-care Technology, Australia
Metaquino	*M. anisopliae*	Spittlebugs	Brazil
Bio-Path	*M. anisopliae*	Cockroaches	EcoScience, USA
Bio-Blast	*M. anisopliae*	Termites	Ecoscience, USA
Cobican	*M. anisopliae*	Sugar-cane spittlebug	Probioagro, Venezuela
Conidia	*Beauveria bassiana*	Coffee-berry borer	Live Systems Technology, Colombia
Ostrinil	*B. bassiana*	Corn-borer	Natural Plant Protection (NPP), France
CornGuard	*B. bassiana*	European corn-borer	Mycotech, USA
Mycotrol GH	*B. bassiana*	Grasshoppers, locusts	Mycotech, USA
Mycotrol WP & BotaniGard	*B. bassiana*	Whitefly, aphids, thrips	Mycotech, USA
Naturalis-L	*B. bassiana*	Cotton pests including boll-worms	Troy Biosciences, USA
Proecol	*B. bassiana*	Army worm	Probioagro, Venezuela
Boverin	*B. bassiana*	Colorado beetle	Former USSR
Boverol	*B. bassiana*	Colorado beetle	Czechoslovakia
Boverosil	*B. bassiana*	Colorado beetle	Czechoslovakia
Engerlingspilz	*Beauveria brongniartii*	Cockchafers	Andermatt, Switzerland
Schweizer	*B. brongniartii* *Beauveria* spp.	Cockchafers	Eric Schweizer, Switzerland
Melocont	*B. brongniartii*	Cockchafers	Kwizda, Austria
Green Muscle	*Metarhizium flavoviride*	Locusts, grasshoppers	CABI BioScience, UK
PFR-97	*Paecilomyces fumosoroseus*	Whitefly	ECO-tek, USA
Pae-Sin	*P. fumosoroseus*	Whitefly	Agrobionsa, Mexico
Laginex	*Lagenidium giganteum*	Mosquito larvae	AgraQuest, USA
Nematophagous fungi			
DiTera	*Myrothecium verrucaria*	Plant-parasitic nematodes	Valent (Sumitomo), USA, Japan
Product under development	*Duddingtonia flagrans*	Animal-parasitic nematodes	Christian Hansen, Denmark
Product under development	*Verticillium chlamydosporium*	Plant-parasitic nematodes	DeCeuster, Belgium

reduced. In order to satisfy this demand, biological control strategies, especially for the growing organic market, are urgently required. Several members of the European Union (EU) (Sweden, Denmark, the Netherlands) decided in the mid–late 1980s to decrease the chemical input in agriculture by 50% within a 10-year period, but these countries may be unable to meet their goals unless a greater impetus is given to the development of new, environmentally friendly pest and disease management strategies

Table 1.3. Fungal agents being developed or commercially available for the biological control of weeds (data from Templeton and Heiny, 1989; Butt *et al*., 1999; Butt and Copping, 2000).

Product	Commercial name	Supplier or country where registered	Target weed
Acremonium diospyri		USA	Persimmon (*Diospyros virginiana*) in Oklahoma rangeland
Alternaria zinniae		Italy	Noogoora burr (*Xanthium occidentale*)
Alternaria eichhornia		India	Water hyacinth (*Eichhornia crassipes*)
Alternaria cassiae	Casst	USA	Sicklepod (*Cassia obtusifolia*) and coffee senna (*Cassia occidentalis*) in soybeans and groundnuts
Cercospora rodmanii	ABG 5003	Abbott Labs, USA	Water hyacinth (*Eichhornia crassipes*)
Colletotrichum coccodes	Velgo	USA, Canada	Velvet-leaf (*Abutilon theophrasti*) in maize and soybeans
Colletotrichum gloeosporioides f. sp. *cuscutae*	Luboa 2	PR China	*Cuscuta chinensis, Cuscuta australis* in soybeans
C. gloeosporioides f. sp. *malvae*	Biomal	Canada	Mallow (*Malva pusilla*) in wheat and lentils
C. gloeosporioides f. sp. *aeschynomene*	Collego	Encore Technologies, USA	Northern joint vetch (*Aeschynomene virginica*) in rice
Colletotrichum orbiculare		Australia	Spiny burr (*Xanthium spinosum*)
Chondrosterium purpureum	BioChon	Koppert, the Netherlands	Black cherry (*Prunus serotina*) in forestry in the Netherlands
Phytophthora palmivora	Devine	Sumitomo, Valent, USA	Milkweed vine (*Morrenia odorata*) in Florida citrus

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(Matteson, 1995). Plant protection is at present partly trapped between the increasing number of prohibited chemical compounds and the lack of safe, efficient alternatives.

Some Benefits and Problems in Developing Fungal BCAs

Natural methods of pest, weed and disease control may be more labour-intensive and less efficient than chemical pesticides but they can lead to:

- job and wealth creation because of the numerous niche markets they would have to satisfy if chemicals were phased out altogether;
- more sustainable methods of crop production;
- more income for the grower because of the premium on pesticide-free and organic produce.

De novo development and implementation of alternative crop protection programmes will take time and investment as well as re-education, particularly of growers and extension service workers (see Chapter 14). Maintaining biodiversity is of paramount importance from an amenity (i.e. public interest in natural systems) and from a medical (i.e. potential source of useful medicines) point of view. Development of natural agents could have many spin-offs, such as the development of pharmaceu-

Table 1.4. Bioactive compounds of pharmaceutical importance

Compound	Source	Function
Swainsinone	*Metarhizium anisopliae*	Inhibitor of α-mannosidase II, inhibits metastasis and tumour growth
Cytochalasin C	*M. anisopliae*	Inhibits cytokines
SN-C (protein-bound polysaccharide)	*Cordyceps* (teleomorph of many entomogenous fungi)	Antitumour activity
Bassiatin	*Beauveria bassiana*	Platelet aggregation inhibitor
Viridiofungins	*Trichoderma viride*	Inhibitor of squalene synthetase
Zearalenone	*Fusarium* spp.	Oestrogenic
Cyclosporin	*Tolypocladium* spp.	Immunosuppressant

tical drugs, research tools and safer agrochemicals. Table 1.4 lists some compounds of medical interest isolated from fungal BCAs.

Unfortunately, there is relatively little investment in the research and development of microorganisms compared with that spent on the discovery of chemical pesticides (Whipps and Lumsden, 1989). Two reasons for this are that BCAs usually have a narrow host range and often give inconsistent and poor control in field trials. Consequently, more attention is being given to the selection of broad-spectrum BCAs and improvements in the production, formulation and application technologies (Butt *et al.*, 1999). Efforts are also being made to optimize the impact of these agents by integrating them with other novel crop protection strategies (Pickett *et al.*, 1995; see also Chapter 3).

The Commercial Perspective

One major factor to consider is the market potential of BCAs. Currently, only specialized, niche markets exist. Their full potential has not been realized because of the following:

1. Absence of strong incentives to develop these agents and/or discourage chemical pesticides.
2. Availability of new, biodegradable chemical pesticides.
3. Absence or breakdown of the infrastructure, which facilitates transfer of new technologies and research knowledge to the end-user (i.e. grower).
4. Absence of a universally acceptable registration procedure.
5. Restrictions in the use of exotic BCAs.
6. Lack of robust and reliable field effects.
7. Very few growers or extension workers know how to use BCAs.

Progress is also slow because the main producers of BCAs are often small–medium-size enterprises (SMEs), which have limited resources for the effective development and marketing of products. According to Lisansky (1999), some of the characteristics of successful companies are:

- Low production costs. This remains the key to cost-effective products and yet it attracts neither research money nor speculative investment. Cost-competitive products will succeed, sometimes even where control is imperfect.

- Good market research. This is essential because markets for BCAs are smaller and generally require more input than markets for chemicals. Companies must take a very precise look at their markets and know who will buy and use their products. Experience in agrochemicals is not sufficient nor is simple awareness of socio-economic trends, e.g. expansion of the organic farming sector and public sensitivity to health risks and environmental pollution.
- Corporate commitment. Good companies commit funds to ensure that a good, cost-effective product will reach the market, i.e. they do not enter the market half-heartedly. The commitment is not limited to sale of products but includes the follow-through to ensure that end-users will be successful when using BCAs.
- Good management. This is important to ensure that the company remains focused and does not diffuse its resources (i.e. spread the risk).

According to Lisansky (1999), companies can make three fundamental mistakes in their approach to BCAs:

- They believe what is said about BCAs in print, namely that they are easy, quick and cheap to make and are in great, yet untapped, demand.
- They overestimate their own capabilities, believing that they, unlike nearly 200 of their predecessors, will avoid the pitfalls and pick the winners.
- They under-budget in time and resources and try to succeed on the cheap.

Several fungal BCAs have been or are being developed as commercial BCAs, often with global markets in mind (see Tables 1.1–1.3). In order to survive, many SMEs market products of other companies or produce BCAs under licence. Presumably, this mutualism will decline as the use of BCAs increases (i.e. the market expands) and it becomes more lucrative for individual companies to develop their own agents.

The Search for and Development of Commercially Viable Fungal BCAs

This usually entails several steps:

1. Isolation of BCAs from the environment. Some methods for the isolation of fungi are given in Butt and Goettel (2000).

2. Studies to generate knowledge of the ecology, physiology and taxonomy of potential fungal BCAs. It is important to identify organisms as some resemble less desirable organisms, e.g. the entomogenous fungus *Metarhizium anisopliae* can be mistaken for *Aspergillus* species (cause of aspergillosis). This information is also essential to meet the registration requirements. Knowledge of BCA ecology can contribute to a better understanding of the effect of environmental factors on the survival and distribution of BCAs. This in turn can enable scientists to predict when to apply inoculum and/or promote habitats that encourage amplification of natural inoculum and the induction of epidemics/epizootics (see Chapters 2–6). Biochemical and molecular markers help to monitor pathogens in the field (Chapter 7). Such markers can also help to distinguish between exotic and native isolates, as well helping to elucidate how epidemics/epizootics develop in the field.

3. For effective pest, weed and disease control, laboratory and field bioassays will help identify the most antagonistic/virulent, ecologically fit strains. Dose–mortality

studies determine the minimum amount of inoculum required to cause disease in pests/weeds or to suppress plant pathogens. Such studies also indicate the time it will take for BCAs to have an impact on target organisms. Overall, ecological fitness is a fundamental requirement because of the relatively narrow window of environmental parameters, particularly relative humidity, over which many fungal BCAs are able to grow effectively in the natural environment. This should not be confused with biological fitness, which refers to the ability of the organism to reproduce successfully. It is possible that during culture in artificial media fungi lose properties that facilitate survival and infection in the field. Genetic manipulation, specific production systems and formulations may help overcome some of these problems (see Chapters 8–10).

4. Economical, mass production of stable inoculum is vital for the successful development of fungal BCAs. With entomogenous fungi, attenuation of virulence/ antagonism is a poorly understood phenomenon and research is under way at a number of universities to elucidate the underlying mechanisms. SMEs are investigating methods for reducing their production costs and increasing both the inoculum yield and the shelf-life (Chapters 9 and 10).

5. Formulation can improve the field efficacy of the pathogen by protecting against desiccation and harmful ultraviolet (UV) radiation. Some formulations can enhance fungal virulence by improving spore attachment to the host surface, diluting the fungistatic compounds in the epicuticular waxes and stimulating germination. Progress in this area is reviewed in Chapter 10.

6. Application strategies can have a profound impact on the efficacy of fungal BCAs but this has often been a neglected area. This is because pest, weed and disease control is dose-related. Not all the inoculum will infect the host; some is removed due to natural causes (e.g. during preening by the insect, shed during ecdysis), destroyed by UV irradiation or washed off by rain. Some of the strategies and tools for delivering the inoculum are dealt with in Chapter 11.

7. One of the big economic hurdles in the commercialization of fungal BCAs is in risk assessment. Risk assessment trials are essential for registration purposes. Fungal BCAs must be shown to be safe both to humans and to other non-target organisms. Besides the high cost of conducting the trials, additional technical protocols are often required by the registration authorities. All this takes a lot of time and is expensive, sometimes prohibitively so for SMEs. The cost of delay in getting a product to the market is often higher than one might imagine – in fact, it is the current discounted cash value of every single year's sales coming 1 year later than expected. Some companies have gone bankrupt waiting for sales to start. The toxins and safety of fungal BCAs are reviewed in Chapters 12 and 13, respectively.

8. Last, but not least, are the training processes whereby fungal BCAs are integrated into a unified crop protection programme that is easy to manage by the grower or other end-user. Integrated, sustainable crop and animal protection management programmes often require imagination and daring – for example, the use of bees that not only pollinate flowers but concomitantly spread beneficial fungi for pest and disease control (Vanneste, 1996; Butt *et al.*, 1998). Many challenges still remain to be dealt with at the technical, agronomic, socio-economic and political levels; these are discussed in the final chapter of this book with recommendations on how we can proceed to accelerate commercialization and effectively deploy fungal BCAs.

References

Burges, H.D. (1998) *Formulation of Microbial Pesticides.* Kluwer Academic Publishers, Dordrecht, The Netherlands.

Butt, T.M. and Copping, L. (2000) Fungal biological control agents. *Pesticide Outlook* 11, 186–191.

Butt, T.M. and Goettel, M.S. (2000) Bioassays of entomogenous fungi. In: Navon, A. and Ascher, K.R.S. (eds) *Bioassays of Entomopathogenic Microbes and Nematodes*. CAB International, Wallingford, UK, pp. 141–195.

Butt, T.M., Carreck, N.L., Ibrahim, L. and Williams, I.H. (1998) Honey bee mediated infection of pollen beetle (*Meligethes* spp.) by the insect-pathogenic fungus, *Metarhizium anisopliae*. *Biocontrol Science and Technology* 8, 533–538.

Butt, T.M., Harris, J. and Powell, K. (1999) The European scene – opportunities for biopesticides. In: Menn, J. and Hall, F.R. (eds) *Biopesticides: Use and Delivery*, Vol. 5. Humana Press, Totowa, New Jersey, pp. 23–44.

Lisansky, S.G. (1999) *Biopesticides: Markets, Technology, Legislation and Companies.* CPL Scientific Information Services Limited, Newbury, UK.

Matteson, P.C. (1995) The '50% pesticide cuts' in Europe: a glimpse of our future? *American Entomologist* Winter, 210–220.

Pickett, J.A., Butt, T.M., Doughty, K.J., Wallsgrove, R.M. and Williams, I.H. (1995) Minimising pesticide input in oilseed rape by exploiting natural regulatory processes. Plenary lecture. In: *Proceedings of the GCIRC 9th International Rapeseed Congress, Cambridge, UK, 4–7 July 1995*, Vol. 2, pp. 565–571.

Templeton, G.E. and Heiny, D.K. (1989) Improvement of fungi to enhance mycoherbicide potential. In: Whipps, J.M. and Lumsden, R.D. (eds) *Biotechnology of Fungi for Improving Plant Growth.* Cambridge University Press, Cambridge, UK, pp. 127–151.

Vanneste, J.L. (1996) Honeybees and epiphytic bacteria to control fire blight, a bacterial disease of apple and pear. *Biocontrol News and Information* 17, 67N–78N.

Whipps, J.M. and Lumsden, R.D. (eds) (1989) *Biotechnology of Fungi for Improving Plant Growth.* Cambridge University Press, Cambridge, UK.

2 Commercial Use of Fungi as Plant Disease Biological Control Agents: Status and Prospects

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Introduction

Public concerns over the use of pesticides in agriculture and their effects on the environment are continuing to increase. These concerns, which are compounded by perceived risks associated with genetic modification of plants, have resulted in a desire for a more environmentally sustainable approach to agriculture, horticulture, forestry and related industries (including, for example, ornamental plants and turf grass). Globally, these views have resulted in greater restrictions on chemical pesticide use in the developed nations and a worldwide ban on the use of methyl bromide. Individual countries have been implementing their own initiatives in this area at a range of social levels. For example, in the Netherlands in 1991, the Dutch government instigated the Multiyear Crop Protection Plan, which aimed to reduce the use of pesticides by 50% by the year 2000. In the UK, major food retailers have introduced schemes for their contracted growers to minimize pesticide applications with the intention, in the long term, to dispense with their use altogether. Interestingly, these more 'natural' products are attracting a premium on price. In both the USA and the UK, there are organic farming certification standards, which severely restrict the use of chemicals of all forms (Lipson, 1997; Anon., 1999). As far as the control of plant disease is concerned, there are increasing trends for natural, non-chemical or organic approaches to disease control. This raises the question as to the role of disease biological control agents (BCAs) in modern agriculture and horticulture.

Some of the relative potential advantages and disadvantages for the development of BCAs over chemicals for control of plant diseases are listed in Table 2.1. What is clear is that the key determinants are influenced by public views and perceptions, scientific facts or observations, as well as commercial or financial considerations. Whether a property of a biocontrol agent is viewed as advantageous or not may be a matter of

Table 2.1. Perceived advantages and disadvantages of the commercial development of biological disease control agents relative to existing chemical control measures.

Advantages	Disadvantages
Environmentally compatible; naturally occurring	Inconsistent and often low levels of control
Positive, 'green image'	Subject to environmental influences
Not persistent; low environmental impact	May lack persistence to give long-term control
Broad or narrow targets depending on organism	Chance of mutation and variation
Can be site-specific	Too specific or slow-acting
Less prone to resistance	Inoculum not robust: may have poor shelf-life
Cost-effective for specialized applications or where no chemical controls exist	Not cost-effective for certain existing markets
Application methods easily adaptable	Expensive and more difficult to use
Low cost of development	Needs novel fermentation facilities
Rapid and cheaper registration[a]	Costs of registration and toxicity testing excessive for niche markets
Small markets viable	Not practical for large-acreage agronomic crops
Integrated control possible, reducing chemical use	May not be compatible with accepted practices

[a] The US Environmental Protection Agency (EPA) has a less expensive rapid registration process for biological in comparison with chemical fungicides. In the UK, fees charged by the Ministry of Agriculture, Fisheries and Food (MAFF) for assessment of a biopesticide package are only 25% of that charged for chemical pesticides.

opinion. On one hand, it may be considered that BCAs are ideal and environmentally acceptable for disease control and should be implemented irrespective of cost and relative efficacy, or on the other, BCAs may simply be considered unreliable, ineffective, or too costly. In practice, the truth probably lies somewhere between these extremes. This chapter considers the current and future status of commercial fungal BCAs with particular emphasis on products. Examples of specific products will be used to illustrate important relevant points rather than attempting an all-encompassing review.

Products Available

The number of fungal products on the market used to control plant diseases is increasing, and nearly 40 have been reported in recent sources (Cook *et al.*, 1996; Whipps, 1997a; Fravel *et al.*, 1998; US Department of Agriculture/Agricultural Research Service/BPDL (USDA/ARS/BPDL) Biocontrol of Plant Diseases Laboratory webSite http://www.barc.usda.gov/psi/bpdl/bioprod.htm). However, many of these materials are not registered as BCAs (also termed biopesticides); rather, they are sold as some form of 'plant growth promoter' or 'stimulant', 'soil conditioner', 'plant strengthener' or 'wound protectant'. By not claiming fungicidal activity, producers of these materials avoid the need for registration and costs for obtaining efficacy, toxicology and environmental fate data. Although this speeds up entry of the product to the market-place, it also introduces an element of potential environmental and health risks in those cases where extensive experimental background information has not been accumulated. Making pesticidal claims for a product without formal registration and permission can lead to a ban on sales and the imposition of penalties (Federal Insecticide, Fungicide and Rodenticide Act (FIFRA)).

Table 2.2. Fungi registered and commercially marketed as biological control agents (revised from Whipps, 1997a; Fravel *et al.*, 1998).

Antagonist	Target pathogen(s)/activity	Disease/host	Product name and source
Soil and root microbiomes			
Coniothyrium minitans	*Sclerotinia minor*; *Sclerotinia sclerotiorum*	Protected vegetable and field crops	Contans WG (Prophyta Biologischer Pfanzenschutz GmbH, Germany)
		Glasshouse crops and amenity areas	KONI (Bioved Ltd, Szigetszentmiklos, Hungary)
Gliocladium (*Trichoderma*) *virens*	*Pythium ultimum*; *Rhizoctonia solani*	Damping-off of bedding plants	SoilGard (GL-21), formerly GlioGard (Thermo Trilogy, Columbia, Maryland, USA)
Trichoderma harzianum	*Fusarium* spp; *P. ultimum*; *R. solani*; *Sclerotinia homeocarpa*	Range of crops, ornamentals and turf	T-22G, T-22 Planter Box, Bio-Trek and Root Shield (Bio-Works Inc., Geneva, New York, USA)
	Various fungi		Supresivit (Borregaard and Reitzel, Denmark, or Fytovita, Czech Republic)
T. harzianum + *Trichoderma polysporum*	Various root-infecting fungi	Glasshouse crops	BINAB-T WP(Bio-Innovation Eftr AB, Bredholmen, Sweden; or Svenska Predator AB, Sweden; or Bayer, Sweden)
Trichoderma viride	*Fusarium* sp.; *Pythium* sp.; *Rhizoctonia* sp.; *Macrophomina phaseolina*; *Phytophthora* sp.	Cotton, legumes, sunflower, tobacco and vegetables	Ecofit (Hoechst Schering AgrEvo Ltd, Chakala, India)
Aerial microbiomes			
Ampelomyces quisqualis	Powdery mildew	Curcubits, grapes, ornamentals, strawberries, tomatoes	AQ10 Biofungicide (Ecogen Inc., Langhorne, Pennsylvania, USA)
Peniophora (*Phlebiopsis*) *gigantea*	*Heterobasidion annosum*	Stem and root rot of pine	Pg suspension (Omex Environmental Ltd UK) and Rotstop (Kemira Agro Oy, Helsinki, Finland)
T. harzianum	*Botrytis cinerea* and other foliar pathogens	Cucumber, grape, nectarine, soybean, strawberry, sunflower, tomato	Trichodex (Makhteshim Chemical Works Ltd, Israel)
T. harzianum + *T. polysporum*	*B. cinerea*	Strawberry	BINAB-T WP (Bio-Innovation Eftr AB, Bredholmen, Sweden; or Svenska Predator AB, Sweden; or Bayer, Sweden)
	Chondrostereum purpureum; *Eutypa*	Silver-leaf disease and chlorotic leaf curl in stone-fruit and grapes	
T. harzianum + *T. viride* (combinations)	*C. purpureum*	Silver-leaf disease in pip- and stone-fruit trees	Trichodowels, Trichoject and Trichoseal (Agrimm Technologies Ltd, New Zealand)
Postharvest microbiomes			
Candida oleophila	*Botrytis* spp.; *Penicillium* spp.	Storage rots of pome fruit	Aspire (Ecogen Inc., Pennsylvania, USA)
Cryptococcus albidus	*B. cinerea*; *Penicillium expansum*	Storage rots of apple and pear	Yield*Plus* (Anchor Yeast, Cape Town, South Africa)

Surprisingly, only 20 products, made from nine species of fungi, are registered and sold commercially as BCAs or biopesticides (Table 2.2). Brief summaries of some of these products are given below. Where the information was provided by the manufacturers directly, they are not referenced further. Significantly, more than half of the products are *Trichoderma*- or *Gliocladium*-based preparations reflecting the widespread occurrence of these fungi, the relative ease of their production, their low toxicity and the huge volume of experimental data on these genera. Products are available for control of pathogens in soil and root, aerial and postharvest microbiomes (*sensu* Whipps *et al.*, 1988). These may be considered reasonably well-defined habitats that have distinct physicochemical properties containing characteristic microbial communities.

Soil and root microbiomes

Coniothyrium minitans

Coniothyrium minitans is a mycoparasite of sclerotia of *Sclerotinia sclerotiorum* and *Sclerotinia minor*. Two products containing this BCA are available: Contans WG, in Germany and Switzerland, and KONI, in Hungary. Both are granular formulations, but Contans WG is sprayed and incorporated into soil after dispersal in water whereas KONI is incorporated into soil directly. Application must be made several weeks prior to planting crops to allow time for the sclerotia to be destroyed. Currently, although use is restricted to glasshouses and polyethylene tunnels for a range of high-value crops, use on field crops and amenity areas is planned. *C. minitans* strain CON/M/91-08 in Contans WG is undergoing consideration for full European registration under the European Union (EU) Council Directive 91/414 and in spring 2001 received approval from the US-EPA and Austria.

Gliocladium virens *(=* Trichoderma virens*)*

The BCA *Gliocladium virens* has appeared on the market in two formulations, GlioGard™, an alginate prill formulation, and SoilGard™, a granular fluid-bed formulation (Lumsden *et al.*, 1996). These products target damping-off diseases of vegetable and ornamental plant seedlings caused by *Rhizoctonia solani* and *Pythium* spp. Application was confined to greenhouse or interior container use (Lumsden *et al.*, 1996). Only the product SoilGard is now produced and is marketed by Thermo Trilogy Corp., Columbia, Maryland, USA.

Trichoderma harzianum

A commercial formulation of *Trichoderma harzianum* strain 1295-22 (T-22) is manufactured by BioWorks, Inc., Geneva, New York, USA, and sold through several distributors as T-22 Planter Box™. This conidial formulation is designed for application to large-seeded crops such as maize, beans, cotton and soybeans, and in most cases can be applied to seeds already treated with fungicides (Harman and Björkman, 1998). The seed treatment delivers the *T. harzianum* inoculant to the growing seedling where it colonizes the spermosphere and also the developing root system, protecting crop plants from damping-off diseases.

Similar products using the same strain 1295-22 (T-22) include a granular formulation used as a greenhouse soil amendment, which is called RootShield™ and con-

tains the entire thallus of *T. harzianum* colonized on clay particles. Another product, RootShield drench, consists of conidia and inert ingredients for use as a water-suspensible drench. In either case, the product is thought to colonize the root system of the crop to be protected (Harman and Björkman, 1998). This product is claimed to control root diseases caused by *Fusarium, Rhizoctonia* and *Pythium* spp., but not *Phytophthora* spp.

Another *T. harzianum* product available in the Czech Republic and Denmark for glasshouse use is Supresivit. This dispersible powder containing conidia of strain PV5736-89 is applied to soil or potting mixes to control disease complexes causing damping-off or root rots of ornamentals and forest-tree seedlings, and as a pea seed treatment to control damping-off.

Trichoderma viride

Trichoderma viride is available as a BCA in India from Hoechst Schering AgrEvo Ltd in a product named Ecofit. It is a talc-based powder sold for the control of root rot, seedling rot, damping-off, collar rot and *Fusarium* wilt in cotton, chick-pea, pigeon-pea, Bengal gram, groundnut, sunflower, soybean, tobacco and vegetables. Depending on the plant and disease of interest, Ecofit can be applied before sowing as a dry powder or slurry seed treatment, before planting as a rhizome, tuber or set dip, or as a soil drench for soil incorporation following a preliminary scale-up procedure involving prior inoculation on to farmyard manure.

Aerial microbiomes

Ampelomyces quisqualis

Ampelomyces quisqualis, formulation AQ10, is the first biocontrol fungus developed specifically for controlling powdery mildew. AQ10 is water-dispersable and acts as a mycoparasite on powdery mildews affecting leaves, stems or fruits of plants. The range of plants protected includes strawberry, tomato, grape, tree fruit and ornamentals (Dik *et al.*, 1998). As with many other plant diseases, powdery mildews have developed resistance to commonly used chemical treatments, such as sulphur and demethylation-inhibiting fungicides. AQ10 is useful in powdery mildew management programmes to ward off resistance problems and can extend the usefulness of these chemical treatments for a reduced time and amount of application.

Phlebiopsis (Peniophora) gigantea

Phlebiopsis gigantea is a common wood-rotting saprotroph that is applied to freshly cut stumps of pine to prevent their colonization by the root-rotting fungus *Heterobasidion annosum*. It is not a biocide that kills the target organism but rather it competes for the food base that the pathogen would otherwise use. Commercial products containing oidia are available in the UK from Omex Environmental Ltd and in Finland from Kemira Agro Oy as PG Suspension and Rotstop, respectively. *P.* gigantea is also available in other Scandinavian countries and Poland. Significantly, after 30 years of field use, PG Suspension has become the first fungal disease BCA approved in the UK under the Control of Pesticides Regulation (COPR) 1986 (Pratt *et al.*, 1999).

Trichoderma harzianum

Strain T39 of *T. harzianum* has been used for greenhouse control of *Botrytis cinerea*. It is produced by Makhteshim Chemical Works and is marketed as Trichodex™ in Europe and Israel. The strategy for best control involves alternating chemical and biological control treatments (Elad *et al.*, 1994). This approach, as with *A. quisqualis*, reduced the use of chemicals and may also reduce the incidence of chemical resistance developed by *B. cinerea*.

Trichoderma harzianum + Trichoderma polysporum

A combination of strains IMI 206040 and IMI 206039 of *T. harzianum* and *T. polysporum*, respectively, sold as BINAB-T, is one of the oldest commercial biopesticide preparations still available. Produced by Bio-Innovation Eftr AB in Sweden it has been used for over 20 years. Because of the long period of safe use it will continue to receive exemptions from the new pesticide regulations in Sweden (Kemikalieinspektionen) until a decision is made concerning a current application for registration made under the new regulations. BINAB-T has been registered and used in the past for control of numerous diseases, but currently, in Sweden and Denmark, is used largely for the control of grey mould (*B. cinerea*) on strawberries, with some minor use in glasshouse crops to control soil-borne pathogens. The other main market is Chile, where it is used for the suppression of silver-leaf disease (*Chondrostereum purpureum*) and chlorotic leaf curl (*Eutypa*) in stone fruit and grapes, respectively.

Trichoderma harzianum + Trichoderma viride

Agrimm technologies in New Zealand have three registered products containing various combinations of *T. harzianum* and *T. viride* sold for the control of silver-leaf disease in pip- and stone-fruit trees. Trichodowels are small, *Trichoderma*-impregnated wooden dowels, inserted into the plant via a 6 mm hole drilled into the stem or trunk; Trichoject is a liquid preparation of *Trichoderma* injected into the stem or trunk; and Trichoseal is a wound paint for treating pruning wounds.

Postharvest microbiomes

Candida oleophila

Aspire™, a biocontrol product containing the yeast *Candida oleophila* as the BCA is registered in the USA and in Israel. The product is used to reduce rot diseases, green and blue mould, caused by *Penicillium digitatum* and *Penicillium italicum*, respectively. It is also efficacious against sour rot, caused by *Geotrichum candidum* (Wilson *et al.*, 1993; Droby *et al.*, 1998). Aspire works best in combination with reduced application rates of thiabendazole, and this treatment often reduces the incidence of decay as effectively as conventional, full-rate fungicide treatments (sodium O-phenyl phenate, thiabendazole, imazalil and metalaxyl). Aspire is marketed in the USA by Ecogen Corporation, Langhorn, Pennsylvania.

Cryptococcus albidus

This yeast was developed for use on pome fruits, especially apples and pears, against grey and blue mould caused by *B. cinerea* and *Penicillium expansum*, respectively. A product, Yield*Plus*, is produced commercially by Anchor Yeast, Capetown, South Africa (de Koch, 1998; C.L. Wilson, personal communication).

Long-term Developments

Both the commercial use and the acceptability of fungal BCAs are likely to depend on the perceived need for the products, the level of impetus for their development and the ability of the developers to overcome constraints or bottlenecks. Some of the relevant factors are discussed below.

Impetus for development of commercial disease biocontrol agents

Legislation reflecting the need to reduce the use of chemical pesticides in the environment and the levels of pesticide residues in food has become one of the major driving forces for the development of commercial BCAs, either as direct substitutes for chemicals or in integrated management systems, where rates of chemical usage are reduced. However, a reduction in the use of agrochemicals may also influence the development of alternatives, especially if the chemical withdrawn was very effective at controlling diseases. For example, the loss of methyl bromide for the control of numerous soil-borne pathogens of ornamental and vegetable crops and of storage rots of fruits and vegetables is likely to have a significant impact (Ristaino and Thomas, 1997) since for many of these diseases there is no other economically viable means of control. Under these circumstances, BCAs may thus provide an option where no other control measures exist. Indeed, this may already be the case for diseases such as take-all (caused by *Gaemannomyces graminis* var. *tritici*) on wheat, chestnut blight (caused by *Cryphonectria parasitica*) and club-root (caused by *Plasmodiophora brassicae*) on brassicas, where no resistant cultivars or varieties exist and no chemical control products are available or effective. Here, crops are not grown where the pathogen is well established or a continual loss due to disease is accepted as normal.

Opportunities for future development of fungal-based biocontrol products

Agricultural practice is changing as a result of demands to reduce the use of chemical pesticides, including fungicides, and to provide abundant feed, food and fibre using environmentally friendly, sustainable systems. Much of this change has been initiated and mandated or encouraged by national and international legislatures. The potential roles of biological methods in these evolving practices of pest control (in this sense including plant pathogens, weeds and insects as 'pests', as all are detrimental to agricultural production) are considered below.

Sustainable agriculture

According to the US government 1990 Farm Bill, sustainable agriculture is:

> an integrated system of plant and animal production practices having a site specific application that will, over the long-term, satisfy human food and fiber needs; enhance environmental quality and the natural resource base, upon which the agricultural economy depends; make efficient use of non-renewable resources and on-farm resources, and integrate, where appropriate, natural biological cycles and controls; sustain the economic viability of farm operations; and enhance the quality of life for farmers and society as a whole.

The same concept of sustainable agricultural systems has been expounded by the Canadian government to address environmental health, economic profitability and social and economic equity (Acton and Gregorich, 1995). Fungal BCAs have not been used to a large extent in the development of sustainable systems, but they have the potential to do so by providing alternatives to chemical pesticides.

Pesticide control actions

FIFRA has been a guiding force in the regulation of pesticides in the USA for many years. Similar guidelines are in place in the UK, such as COPR 1986, contained within the Food and Environment Protection Act (FEPA) 1985, and the Control of Substances Hazardous to Health (COSHH) Regulations 1988, made under the Health and Safety at Work Act 1974. Importantly, provisions of European legislation affecting all members of the European Community in the Plant Protection Products Directive 91/414/EEC are now being implemented in Britain by the Plant Protection Products Regulations (PPPR) 1995. In addition to the general regulations on the use of pesticides, several countries have implemented their own specific policies to reduce pesticide use. For example, Denmark, Sweden and the Netherlands have passed legislation requiring a reduction of 50% or more in the total use of agricultural pesticides by the year 2000 (Matteson, 1995). There are also similar US state and regional initiatives to reduce pesticide use (Matteson, 1995). Understandably, alternatives to chemical pesticides or products that allow reduced usage in terms of fewer or reduced rates of application are beginning to appear on the market in the form of fungal BCAs that can be used in integrated systems.

Integrated Pest Management (IPM)

IPM is defined as a systems approach to pest management that combines multiple crop production practices with careful monitoring of pests (including plant pathogens) and their natural enemies (such as fungal antagonists). In 1977, the US Federal budget funded the USDA's IPM initiative to help agricultural producers implement IPM practices on 75% of total crop acreage by the year 2000. IPM as it relates to plant pathology was recently reviewed (Jacobsen, 1997). The concept of IPM was first introduced in relation to insect pest control through integrating the use of pesticides and biological control organisms. In practice, entomological applications of IPM are most advanced, but strides are being made to couple biocontrol of plant diseases with other disease control strategies. This is particularly important as often the current need to resort to chemical treatment for disease control disrupts an otherwise successful biological programme of pest control. For example, one strategy of IPM is to develop

fungal BCAs with tolerance to fungicides or to incorporate fungicide resistance into antagonists (Locke *et al.*, 1985; Locke and Lumsden, 1989; O'Neill *et al.*, 1996). Combining resistant or tolerant fungal BCAs with fungicides can sometimes have twofold advantages in the treatment of seeds: a high level of seed protection is provided early on by the chemical component and then the biological component becomes active later in seedling development and can provide protection of root systems for improved plant health and function (Harman and Björkman, 1998). Integration of biocontrol may also be feasible in other systems, such as with disease forecasting for potato late blight control, in tandem with other strategies, or to substitute for the general biocidal properties of methyl bromide.

Methyl bromide replacement

In November 1992, the Montreal Protocol, an international environmental treaty, was amended to include the agricultural fumigant methyl bromide on its official list of substances believed to harm the earth's protective ozone layer. The US Environmental Protection Agency (EPA) classifies methyl bromide as an acute toxin. The 1990 US Clean Air Act, as well as actions taken at a 1995 meeting in Vienna, Austria, determined a phase-out of the use of methyl bromide worldwide, to begin in 2001 and to be completely banned by 2010. The effect of this ban on agriculture, especially in the USA, and on the options for the control of soil-borne plant pathogens is significant (Ristaino and Thomas, 1997). The role that biological control may have in replacing methyl bromide (a potent biocide that kills everything it contacts) is unclear. However, the proposal itself has stimulated research for replacement materials, and significant progress has been made. For example, saprophytic strains of *Fusarium oxysporum* have been discovered which are antagonistic to *F. oxysporum* f. sp. *lycopersici*, the cause of *Fusarium* wilt of tomato (Larkin and Fravel, 1998). This may bode well for states such as Florida where large quantities of methyl bromide are currently used for the control of *Fusarium* wilt and other soil pests. Legislation similar to that which resulted in the methyl bromide ban is now beginning to be established for uses of other synthetic chemicals for disease control, illustrating the growing concern over the use of pesticides in general.

Food Quality Protection Act (FQPA)

This act reforms US food safety laws and was made into law in 1996. It amends the two other laws involving pesticides, namely FIFRA (mentioned earlier) and the Federal Food, Drug and Cosmetic Act (FFDCA). FQPA potentially affects implementation of biocontrol in two ways. First, the law re-authorizes and increases user fees for the review of older pesticides to ensure they meet current standards. Many fungicides are likely to be dropped because of the review costs incurred, especially for those formulations involved in minor-use applications. The law affects all groups of pesticides that share a common mechanism of action, such as all organophosphates. Thus, when calculating the permitted amount of pesticide in the environment or as residues on food, all organophosphates used for both pathogen and pest control count together. This is expected to result in the loss of clearance for use on minor crops in order to get total exposure of all pesticides with the same mode of action under the tolerance set for that general class of pesticides. This is popularly called a 'risk cup'. The legislation may have the effect of opening up opportunities for fungal BCAs to be used in niche market

applications on minor crops. Similar reregistration requirements are active in the UK. The second opportunity for biologicals in the USA is the provision in the law for a quick review of reduced-risk pesticides (under which BCAs would be considered) to enable them to reach the market sooner in order to replace older, potentially more risky chemicals. This 'advantage' remains to be proved, but may stimulate registrations, similar to the UK reduced-cost incentives. Other incentives for the increased use of BCAs may develop through organic certification initiatives.

Organic farming and organic food certification

Countries around the world are developing national standards for organic farming and for the marketing of organic products. In Canada, the National Standard for Organic Agriculture operates with 47 self-administered organic certification bodies comprising the Canadian Organic Advisory Board (COAB). Israel has established the Israel Bio-organic Agricultural Association, which has defined all organic standards and regulations relating to growing, manufacturing and marketing bio-organic products. New Zealand has two certification systems – BIOGRO and Demeter – developed in the early 1980s, whose labels are issued by the Biodynamic Farming and Gardening Association and the NZ Biological Producers Council. In the UK, the Register of Organic Food Standards provides guidelines for certification for organic food production and maintains a register of approved producers. Chemical use is severely restricted but disease control using naturally occurring organisms against specific disease targets is permitted. Interest in organic farming is growing with the recent launch by the Ministry of Agriculture, Fisheries and Food (MAFF) of an organic farming initiative that includes research on plant disease control.

The US Organic Foods Production Act (OFPA) was enacted in 1990 as Title XXI of the Farm Bill. The USDA is currently developing standards for the use of the term 'organic' (Lipson, 1997). Agreement is now under negotiation to exclude such practices as utilizing municipal sludge compost and marketing produce derived from genetically modified plants. Significantly, biological control of weeds, insects and plant diseases is an integral component of the accepted practices for organic certification. However, distinct areas of uncertainty have developed in which, for example, a formulation process used in the manufacture of a BCA may not be compatible with perceived organic production standards. In addition, biologicals may not be acceptable if used routinely where the focus is on the inputs of biological pesticide and not on the understanding and management of agricultural ecological processes (Lipson, 1997: p. 63).

The intention of the OFPA is for the USDA to establish national standards for the production and handling of foods labelled 'organic'. Previously, private and state agencies have been certifying organic practices with no uniformity and therefore no guarantee from state to state or certifier to certifier. How differences in the development and interpretation of these standards are resolved remains to be seen.

All these legislative initiatives promote and encourage the use of fungal BCAs in agricultural production. However, the quantities that will be used in agriculture are unclear. The total number of applications available for immediate use are limited, and their future depends on the scientific community discovering and developing new BCAs, the industry adapting and marketing them, the end-users adopting biocontrol practices and the consumer accepting the technology. The future is bright but uncertain.

Constraints on the development of commercial disease biocontrol agents

One of the major limitations with biological disease control is the inconsistency in efficacy which is often observed when useful antagonists reach the stage of large-scale glasshouse or field testing, and can arise from a variety of causes reflecting the biological nature of the control microorganism. Essentially the organism must first survive application and then retain activity in the environment of use throughout the period when active control is required, which may be several months for some soilborne pathogens. During this time, it must survive fluctuations in the physical environment and the action of the indigenous and competitive microbiota. In many cases, potential BCAs have been selected and tested in artificial *in vitro* systems that bear little resemblance to the environment of use. Consequently, failure at the scale-up stage is always likely to be high. In response to this problem, it has been suggested that all selection, screening and development processes should adopt an ecological approach that takes into account the features of the environment of use and should improve the number of active BCAs reaching the market (Deacon, 1991; Whipps, 1997a, b, c). The use of appropriate inoculum production, formulation and application technologies together with quality control checks should also help in this process. Nevertheless, even if reliable BCAs can be produced, they must still be easy to use and cost-effective, or they will either never reach the market-place or not be used by growers. At the moment, many chemical fungicides are cheap and effective and will not be substituted for by BCAs unless they are withdrawn from use. If they are withdrawn, market-driven forces will then dictate whether BCAs become commercially viable for use on the same crop or whether an alternative crop is grown, thereby avoiding the need for BCAs. Nevertheless, disease can be expected to build up in alternative crops over time, regardless of rotation, and some control measures will eventually be required. Appropriate BCAs need to be developed in advance of this situation, so as to be ready for the time when they become cost-effective for use. The situation in the glasshouse is somewhat different, as good hygiene can prevent or control many diseases. However, applications of BCAs may be cost-effective in this more controlled environment, where, in general, reproducibility is easier to achieve and profit margins are higher in comparison with field crops.

Another constraint concerns registration. Currently there are no fungal biocontrol products registered and sold worldwide. Some, such as those based on *P. gigantea*, are available in several countries while others, such as AQ10 and Aspire, are sold in two countries (Israel and the USA). Most of the others appear to be sold only in the country of development. This reflects the problems associated with registration requirements in different countries, and includes concerns about releasing non-indigenous microorganisms. There has always been a requirement for a registration package, generally including toxicology and efficacy data for each individual organism and formulated product, in every intended country of use. The high costs associated with this process have consequently stifled commercial development of BCAs for what are often small niche markets. In turn, this has led to a large number of products appearing on the market which actually work by controlling plant pathogens but which purport to be plant growth promoters, soil conditioners, biofertilizers, biological activators or similar microorganism-based materials that require no registration. Unfortunately, without the rigours of a registration package involving toxicological and efficacy data, safe use cannot be assured and consistent beneficial effects on disease control and crop growth are not always seen (Cook *et al.*, 1996). Regulatory authorities are now aware

of this anomaly and are attempting to encourage legal registration and use in a variety of ways. For example, in the USA the EPA claims a more rapid and cheaper registration process for biological pesticides in comparison with chemical pesticides, and in the UK the fees charged by MAFF for assessment of a biopesticide package are only 25% of that charged for a chemical pesticide. Moreover, in the European Community, regulatory authorities in member countries are now beginning to implement the Plant Protection Product Directive 91/414/EEC, which paves the way for rapid pan-European registration once it is obtained in one of the member states (Klingauf, 1995). Authorities in Europe are also aware that legislation drafted essentially for chemical pesticides is not always applicable to biological pesticides, and the requirements for registration of biological pesticides are currently under discussion for appropriate review. The authorities in the USA have been aware of this situation for many years and use a realistic, case-by-case basis to interpret the existing legislation when considering BCAs. For discussion of this approach with regard to *G. virens* in GlioGard (now SoilGard), see Mintz and Walter (1993) and Lumsden and Walter (1995).

Conclusions

It is now clear that the stage is set for fungal biological disease control agents to play a greater part in agriculture and horticulture. The need for alternatives to chemical fungicides, when viewed against a groundswell of feeling by the public for more natural or organic food production systems, makes this a priority. What is required to make this a reality is a long-term commitment from those involved in food production and environmental protection to collaborate. This would include researchers and extension scientists, government, producers, grower organizations and levy boards, retailers and agrochemical companies. The MAFF Horticulture LINK Scheme in the UK has several excellent examples of such consortia acting together. From such consortia, sufficient funding would need to be forthcoming to allow realistic screening, selection and efficacy testing to obtain antagonists with proved activity against specific target pathogens. At this stage the decision as to whether further characterization and development into a commercial product should take place has to be made. Cost–benefit analyses, toxicology and registration must be carried out. Importantly, providing that an appropriate collaboration agreement and royalty rights are agreed at the outset of each stage requiring funding, all those members of the consortia involved with developing the BCA will eventually benefit financially. This approach would undoubtedly encourage environmentally desirable products that are wanted by the public to reach the market-place rapidly.

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Disclaimer

Mention of a trademark proprietary product does not constitute a guarantee or warranty by the USDA, MAFF or the BBSRC and does not imply approval by the exclusion of other products not mentioned that may also be suitable.

References

Acton, D.F. and Gregorich, L.J. (eds) (1995) *The Health of our Soils: Toward Sustainable Agriculture in Canada.* Agriculture and Agri-Food Canada, Centre for Land and Biological Resources Research, Publ. 1906-E, Ottawa, Ontario, 138 pp.

Anon. (1999) *United Kingdom Register of Organic Food Standards (UKROFS).* UKROFS, London.

Cook, R.J., Bruckart, W.L., Coulson, J.R., Goettel, M.S., Humber, R.A., Lumsden, R.D., Maddox, J.V., McManus, M.L., Moore, L., Meyer, S.F., Quimby, P.C., Stack, J.P. and Vaughn, J.L. (1996) Safety of microorganisms for pest and plant disease control: a framework for scientific evaluation. *Biological Control* 7, 333–351.

Deacon, J.W. (1991) Significance of ecology in the development of biocontrol agents against soil-borne plant pathogens. *Biocontrol Science and Technology* 1, 5–20.

Dik, A.J., Verhaar, M.A. and Bélanger, R.R. (1998) Comparison of three biological control agents against cucumber powdery mildew (*Sphaerotheca fuliginea*) in semi-commercial-scale glasshouse trials. *European Journal of Plant Pathology* 104, 413–423.

Droby, S., Cohen, L., Daus, A., Weiss, B., Horev, B., Chalutz, E., Katz, H., Keren-Tzur, M. and Shachnai, A. (1998) Commercial testing of Aspire™: a yeast preparation for the biological control of postharvest decay of citrus. *Biological Control* 12, 97–101.

Elad, Y., Shtienberg, D. and Niv, A. (1994) *Trichoderma harzianum* T39 integrated with fungicides: improved biocontrol of grey mold. In: *Proceedings of the Brighton Crop Protection Conference*. British Crop Protection Council, Farnham, Surrey, pp. 1109–1114.

Fravel, D.R., Connick, W.J. Jr and Lewis, J.A. (1998) Formulation of microorganisms to control plant diseases. In: Burges, H.D. (ed.) *Formulation of Microbial Biopesticides.* Kluwer Academic Publishers, Boston, Massachusetts, pp. 187–202.

Harman, G.E. and Björkman, T. (1998) Potential and existing uses of *Trichoderma* and *Gliocladium* for plant disease control and plant growth enhancement. In: Harman, G.E. and Kubicek, C.P. (eds) Trichoderma *and* Gliocladium: *Enzymes, Biological Control and Commercial Applications*, Vol. 2. Taylor & Francis, London, pp. 229–265.

Jacobsen, B.J. (1997) Role of plant pathology in integrated pest management. *Annual Review of Phytopathology* 35, 373–391.

Klingauf, F.A.J. (1995) Registration requirements of biological control agents in Germany and in the European Union. In: Hokkainen, H.M.T. and Lynch, J.M. (eds) *Biological Control: Benefits and Risks.* Cambridge University Press, Cambridge, pp. 283–290.

de Koch, S. (1998) Control of post-harvest decay of fruit by means of antagonistic microorganisms. PhD Dissertation, University of Stellenbosch, Capetown, South Africa.

Larkin, R.P. and Fravel, D.R. (1998) Efficacy of various fungal and bacterial biocontrol organisms for control of *Fusarium* wilt of tomato. *Plant Disease* 82, 1022–1028.

Lipson, M. (1997) *Searching for the 'O-Word'*. Organic Farming Research Foundation, Santa Cruz, California.

Locke, J.C., Marois, J.J. and Papavizas, G.C. (1985) Biological control of *Fusarium* wilt of greenhouse-grown chrysanthemums. *Plant Disease* 69, 167–169.

Locke, J.C. and Lumsden, R.D. (1989) Interaction of the biocontrol agent, *Gliocladium virens*, with several soil drench fungicides in a zinnia damping-off system, 1988. *Biological and Cultural Control Tests* 4, 85.

Lumsden, R.D. and Walter, J.F. (1995) Development of the biocontrol fungus *Gliocladium virens*: risk assessment and approval for horticulture use. In: Hokkainen, H.M.T. and Lynch, J.M. (eds) *Biological Control: Benefits and Risks.* Cambridge University Press, Cambridge, pp. 263–269.

Lumsden, R.D., Walter, J.F. and Baker, C.P. (1996) Development of *Gliocladium virens* for damping-off disease control. *Canadian Journal of Plant Pathology* 18, 463–468.

Matteson, P.C. (1995) The '50% pesticide cuts' in Europe: a glimpse of our future? *American Entomologist* Winter, 210–220.

Mintz, A.S. and Walter, J.F. (1993) A private industry approach: development of GlioGard™ for disease control in horticulture. In: Lumsden, R.D. and Vaughn, J.L. (eds) *Pest Management: Biologically Based Technologies.* American Chemical Society, Washington, DC, pp. 398–403.

O'Neill, T.M., Elad, Y., Shtienberg, D. and Cohen, A. (1996) Control of grapevine grey mould with *Trichoderma harzianum* T39. *Biocontrol Science and Technology* 6, 139–146.

Pratt, J.E., Gibbs, J.N. and Webber, J.F. (1999) Registration of *Phlebiopsis gigantea* as a forest biocontrol agent in Britain: recent experience. *Biocontrol Science and Technology* 9, 113–118.

Ristaino, J.B. and Thomas, W. (1997) Agriculture, methyl bromide, and the ozone hole: can we fill the gaps? *Plant Disease* 81, 964–977.

Whipps, J.M. (1997a) Developments in the biological control of soil-borne plant pathogens. *Advances in Botanical Research* 26, 1–134.

Whipps, J.M. (1997b) Ecological considerations involved in commercial development of biological control agents for soil-borne diseases. In: Van Elsas, J.D., Wellington E.M.H. and Trevors, J.T. (eds) *Modern Soil Microbiology.* Marcel Dekker, New York, pp. 525–546.

Whipps, J.M. (1997c) Interactions between fungi and plant pathogens in soil and the rhizosphere. In: Gange, A.C. and Brown, V.K. (eds) *Multitrophic Interactions in Terrestrial Systems.* Blackwell Science, Oxford, pp. 47–63.

Whipps, J.M., Lewis, K. and Cooke, R.C. (1988) Mycoparasitism and plant disease control. In: Burge, M.N. (ed.) *Fungi in Biological Control Systems.* Manchester University Press, Manchester, pp. 161–187.

Wilson, C.L., Wisniewski, M.E., Droby, S. and Chalutz, E. (1993) A selection strategy for microbial antagonists to control postharvest diseases of fruits and vegetables. *Scientia Horticulturae* 50, 105–112.

3 Use of Hyphomycetous Fungi for Managing Insect Pests

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Introduction

The development of resistance to chemical insecticides and concerns over the deleterious effects of chemicals on environmental and human safety have provided a strong impetus for the development of microbial control agents for use in integrated control of insect pests. A diverse assemblage of microorganisms are currently under consideration as control agents of insects, including viruses, bacteria, protozoa and fungi. Of the fungi, considerable effort has focused on the development and utilization of entomopathogenic Hyphomycetes (see next section). There are numerous examples of the efficacious suppression of pest insects with this group of microorganisms demonstrating their considerable potential as biological control agents (BCAs). However, their application has not always provided consistent suppression of insect pests. The factors responsible for the initiation and development of epizootics in insect populations are extremely complex, involving interactions among the pathogen(s), insect host, environment and time. An understanding of this dynamic interaction is important, and elucidation of the factors that limit disease initiation and development may allow us to overcome constraints and thereby achieve efficacious control of insect pests.

Reviews dealing with entomopathogenic Hyphomycetes and their development as microbial control agents include those of Ferron (1985), McCoy *et al.* (1988), Evans (1989), Ferron *et al.* (1991), Glare and Milner (1991), Roberts and Hajek (1992), Tanada and Kaya (1993), Hajek and St Leger (1994), Boucias and Pendland (1998) and Wraight and Carruthers (1999). For methods and techniques used to study Hyphomycetes, readers are referred to Goettel and Inglis (1997), Butt and Goettel (2000), Goettel *et al.* (2000a) and Lacey and Kaya (2000). In this chapter we provide

 Fungi as Biocontrol Agents (eds T.M. Butt, C. Jackson and N. Magan)

a broad overview of the major factors influencing the efficacy of hyphomycetous fungi and provide examples of successful use of entomopathogenic Hyphomycetes in pest control, emphasizing some of the strategies that can be used to optimize their impact on pest populations.

Entomopathogenic Hyphomycetes

Fungi are a phylogenetically diverse group of microorganisms that are all heterotrophic (absorptive nutrition) eukaryotes, unicellular (i.e. yeasts) or hyphal (i.e. filamentous), and reproduce by sexual and/or asexual spores. The true fungi (kingdom: *Mycota*) are divided into four divisions: the *Chytridiomycota, Zygomycota, Ascomycota* and *Basidiomycota* (Hawksworth *et al.*, 1995). Although a variety of criteria are used, the most common character used to assign fungi to natural groups (i.e. based on genetic relationships) relies on sexual fruiting structures. However, a large group of fungi, including many entomopathogenic taxa, have lost the ability to produce or rarely produce sexual spores. This group of fungi was traditionally placed in the formed division, *Deuteromycota*, within the artificial class, Hyphomycetes. The members of this class are characterized by mycelial forms that bear asexual spores, termed 'conidia', borne on specialized conidiogenous cells. Conidiogenous cells are often formed on simple or branched hyphae, termed 'conidiophores', or from aggregations of conidiophores, referred to as synnemata (a group of erect and sometimes fused conidiophores bearing conidia at the apex only or on both apex and sides) or sporodochia (a mass of short conidiophores produced in a cushion-like structure).

Members of the Hyphomycetes were traditionally distinguished from fungi that produced conidia in conidiomata (i.e. *Coelomycetes*). These fungi produced conidia in discrete, often flask-shaped, conidiomata, termed 'pycnidia', or in saucer-shaped conidiomata, termed 'acervuli'. Most mycologists no longer accept *Deuteromycota* and its subclasses as formal taxonomic assemblages, and these fungi are typically handled together as 'mitosporic fungi'. Although few taxa of entomopathogenic mitosporic fungi have been connected with a sexual state, most exhibit ascomycetous affinities, including septate hyphae possessing relatively simple septal pores and lamellate hyphal walls with a thin electron-dense outer layer and a relatively thick electron-transparent inner layer. Furthermore, many of these fungi have been correlated with members of the *Ascomycota* on the basis of DNA homology (i.e. 18S ribosomal DNA. When a mitosporic fungus has been connected with a sexual state, it is referred to as the 'anamorph' or 'anamorphic state'. The sexual or perfect state alone is termed the 'teleomorphic state', whereas the whole fungus and all its 'morphs' (i.e. the sexual and asexual states together) are referred to as the 'holomorph'.

The nomenclature of fungi is governed by the International Code (see also selected papers of the IXth International Congress of Mycology of the International Union of Microbiological Societies in 1999). The Code permits the different states of fungi to be given separate names. However, if a teleomorph is present, the name automatically refers to that morph even if the anamorph is also evident (i.e. the correct name of a holomorph is that of its teleomorph). For example, the entomopathogenic taxon, *Paecilomyces farinosus* is the name given to the anamorphic state of the teleomorph, *Cordyceps memorabilis* (Domsch *et al.*, 1980). Since this fungus has been correlated with a teleomorphic state, according to the rules of nomenclature it must be named *C. memorabilis*. For fungi not correlated with a teleomorphic state, the use of the gen-

eral term 'mitosporic fungi' has been advocated (Hawksworth *et al.*, 1995). However, the use of 'mitosporic fungi, is not without controversy, and numerous authors have argued that the anamorphic and teleomorphic phases of a fungus are determined not simply by the type of cellular processes (e.g. mitosis) that precede sporulation, but also by morphological features. Largely due to tradition, we have retained the use 'entomopathogenic Hyphomycetes' in this chapter. For a more comprehensive review on the nomenclature of ascomycetes the reader is referred to Seifert (2000).

We have already indicated that the asexual spores produced by mitosporic fungi are termed 'conidia' but some taxa can also produce asexual resting spores, termed 'chlamydospores'. Traditionally, the classification of Hyphomycetes to genera was primarily based on spore characteristics and on the degree of aggregation of conidiophores into more complex conidiomatal structures (e.g. synnematal or sporodochial conidiomata). Beginning with Hughes (1953), the study of conidium formation on conidiogenous cells (i.e. conidiogenesis) provided an additional tool for classifying the Hyphomycetes. The most common entomopathogenic genera of hyphomycetous fungi include *Aspergillus, Beauveria, Culicinomyces, Hirsutella, Metarhizium, Nomuraea, Paecilomyces, Tolypocladium* and *Verticillium.* Each of these taxa is defined by its characteristic conidiogenesis (e.g. Barron, 1968; Samson *et al.*, 1988; Humber, 1997). However, placement of hyphomycetous fungi within formed genera based on conidiogenesis does not necessarily reflect phylogenetic groupings, and the application of molecular methods is shedding new light on generic and species concepts within this group of entomopathogenic fungi. Many of the above taxa of entomopathogenic Hyphomycetes are believed to possess a *Cordyceps* teleomorphic state (Ascomycota: Hypocreales).

A few taxa of entomopathogenic fungi rarely or never produce exogenous conidia. Two examples of such fungi are *Aschersonia* and *Sorosporella*. Conidia of *Aschersonia* are formed from conidiogenous cells within flask-shaped structures (i.e. pycnidia embedded in light to brightly coloured, hemispherical or cushion-shaped stromata, somewhat shrunken, opening by wide pores or ruptures) and are traditionally handled within the 'artificial' class, *Coelomycetes*; *Aschersonia* has been correlated with a *Hypocrella* teleomorph (Ascomycota: Hypocreales). *Sorosporella* is another mitosporic taxon that is a pathogen of insects (e.g. acridids). While *Sorosporella* readily produces brick-red chlamydospores in insect cadavers, it does not usually produce conidia. However, germinating chlamydospores can produce mycelium from which conidia are formed. The conidial state of this fungus is *Syngliocladium* (Petch, 1942).

Pathogenesis and Epizootiology

Fungi are unique among the insect pathogens in that they infect their hosts primarily through the external cuticle, although a few taxa (e.g. *Culicinomyces*) are able to invade through the alimentary canal. Conidia of most taxa of entomopathogenic Hyphomycetes strongly adhere to insect cuticles, and their attachment to cuticles is thought to involve non-specific adhesion mechanisms mediated by the hydrophobicity of the conidial cell wall (Boucias *et al.*, 1988, 1991). Once a propagule comes in contact with an appropriate insect cuticle, it may germinate and it may produce penetration structures (e.g. germ-tube swelling, appressorium or extracellular sheath) from which penetration hyphae are formed. Even if germination does occur, the fungus may not be able to penetrate the cuticle, due to a number of factors, such as an

inappropriate environment (e.g. conditions of moisture) and/or the presence of inhibitory factors, such as fatty acids or melanin, within the cuticle. To penetrate the cuticle, the entomopathogenic Hyphomycetes utilize a combination of enzymatic and mechanical mechanisms. The production of exoproteases are important in penetration by *Metarhizium anisopliae* and other fungi, but other enzymes, such as endoproteases, esterases, lipases, chitinases and chitobiases, are involved as well (St Leger, 1993; Boucias and Pendland, 1998; Butt *et al.*, 1998a).

Once the fungus reaches the haemocoele, it grows as hyphal bodies, which are single- or multicelled structures that lack a formal cell wall but do contain a thin, fibrillar layer on the plasma membrane (some forms are often referred to as 'blastospores'). Before the fungus can proliferate in the haemocoele, it must often overcome the insect's defence response, and the production of toxins by the fungus can debilitate the efficacy of the defence response mounted by the insect (see Chapter 12). The insect may respond to fungal infection using humoral (e.g. phenoloxidase, lectins and/or host defence proteins and peptides) and/or cellular (e.g. phagocytosis and/or encapsulation) mechanisms (Bidochka *et al.*, 1997; Boucias and Pendland, 1998). However, hyphal bodies of some species (e.g. *Nomuraea rileyi*) do not appear to be phagocytosed by haemocytes, either because they lack specific surface residues and therefore are not recognized by humoral lectins (i.e. opsonization) or because they mimic surface epitopes on insect haemocytes (Boucias and Pendland, 1998). Insect death may result from a combination of actions, including depletion of nutrients, physical obstruction or invasion of organs and toxicosis. For example, *Beauveria bassiana* produces a number of toxic compounds, including beauvericin, bassianolide and oosporein (see Chapter 12). Perhaps the best-studied toxins produced by entomopathogenic Hyphomycetes are the destruxins produced by *M. anisopliae*. Several of these are known to induce tetanic paralysis (Dumas *et al.*, 1996), while others can be immunosuppressive (Cerenius *et al.*, 1990).

Following death, the fungus often grows saprotrophically within the host, and metabolites produced by the various taxa (e.g. oosporein produced by *B. bassiana* and *Beauveria brongniartii*) may be involved in the competitive exclusion of competing microorganisms from the cadaver. Soon after host death, and under favourable conditions, hyphae emerge from the cadaver; they produce conidiogenous cells, sporulation occurs on the host surface and the conidia are liberated. Conidial dispersal is passive, relying principally on wind but other factors, such as rain, can play a role in dissemination. As mentioned previously, many hyphomycetous fungi (e.g. *Beauveria, Metarhizium* and *Paecilomyces*) produce conidia possessing hydrophobic properties due to cysteine-rich proteins called hydrophobins within the rodlet layer of the cell wall. In contrast, *Verticillium lecanii* (syn. *Cephalosporidum lecanii*) possesses hydrophilic conidia. The hydrophobicity of the conidial cell wall influences the biology of these fungi and is an important factor in deployment against insect pests.

Epizootics of hyphomycetous fungi can occur in field populations of insects, and they can be important in the natural regulation of insect pests. An epizootic is the result of a complex interaction among the host, the pathogen and the environment over time (i.e. the disease tetrad), and relatively little is known about the factors responsible for the initiation and continuance of epizootics for most fungal species. A threshold of inoculum is required to induce disease, and many pest managers have simply focused on the deposition of large quantities of virulent propagules on to an insect host. Often this approach has resulted in inadequate suppression of insect pests, since the inoculum threshold is not static and it is influenced by all aspects of the disease

tetrad (e.g. host susceptibility and environment). Much research is needed before we shall be able to adequately predict the conditions under which hyphomycetous fungal epizootics are initiated and developed. Such an understanding is necessary if we are to use these pathogens successfully in integrated pest management (IPM) programmes.

Perhaps the best-studied entomopathogenic hyphomycetous fungus from an epizootiological perspective is *N. rileyi* (Carruthers and Soper, 1987). This fungus often causes natural epizootics in populations of noctuids, such as soybean loopers (*Pseudoplusia includens*) and velvet-bean caterpillars (*Anticarsia gemmatalis*) in soybean. Numerous factors, such as the timing of the initial infection of the host population, the developmental lag associated with the disease incubation period, which is related to the host instar, the rate of spread of the pathogen (i.e. transmission) and various environmental parameters, are all known to control the occurrence of epizootics (Ignoffo *et al.*, 1977a; Kish and Allen, 1978; Ignoffo, 1981). Primary inoculum (i.e. the inoculum that initiates disease within a population) is thought to originate from ovipositing moths (Kish, 1975) or by overwintering inoculum within the field (Ignoffo *et al.*, 1977a). Secondary inoculum (i.e. inoculum produced from diseased individuals within a population) in field environments depends on the movement of infected host larvae and on the dispersal of airborne conidia from cadavers (Kish and Allen, 1978). Initially, infected insects occur in limited loci, but, given appropriate conditions, disease can spread very rapidly throughout a field (Fuxa, 1984). Infected hosts generally exhibit a more highly aggregated spatial pattern, and this probably contributes to higher densities of secondary inoculum in the vicinities of initial loci. Kish and Allen (1978) developed one of the first quantitative epizootiological models, based on a series of stimulus–response experiments, and model predictions were found to accurately predict field mortality in ~50% of the field situations examined. Due to the complexity of the factors controlling epizootic development, the use of quantitative models can provide critical information on the factors influencing disease. Such information can subsequently be used to facilitate the efficacious suppression of insect pests using inundative deployment strategies.

Factors Influencing Efficacy

The pathogen

Pathogenicity is the qualitative ability of a pathogen to cause disease and it is determined by a variety of factors, including the physiology of the host (e.g. defence mechanisms), the physiology of the fungus (e.g. pathogenicity factors, such as the production of enzymes and toxins) and the environment. Fungi, as a group, have one of the widest host ranges among the pathogens of arthropods. However, host spectra vary widely, depending on fungal species. For instance, *Aschersonia aleyrodis* only infects whiteflies, and *N. rileyi* almost exclusively infects lepidopterans within the Noctuidae. In contrast, species such as *B. bassiana* and *M. anisopliae* have much wider host ranges, spanning numerous orders within the Arthropoda. It is now recognized that *B. bassiana* and *M. anisopliae* contain a diverse assemblage of genotypes and probably comprise 'species complexes'. Therefore, it is not surprising that within these taxa (grouped on morphological characters) individual isolates or pathotypes can exhibit a substantially restricted host range. Physiological host range is usually determined within controlled

environment settings, and it is a very flexible characteristic where a variety of factors will determine the outcome (e.g. dose or application method). While the ability of a pathogen to incite disease is essential, a complex array of additional factors determines its suitability as a prospective BCA.

An important consideration in selecting a strain is its virulence, which is the quantitative amount of disease that a pathogen can incite in a group of insects. In a field setting, propagule densities must be sufficiently high to ensure a good probability that an insect will come in contact with an adequate number of propagules to exceed the inoculum threshold. Since a highly virulent pathogen will require fewer propagules to incite disease, selection of virulent genotypes has obvious consequences for efficacious microbial control of insects. Unfortunately, much of the research quantifying the virulence of entomopathogenic Hyphomycetes isolates (usually expressed as a median lethal dose or LD_{50}) has been conducted under conditions that are not necessarily indicative of field environments (e.g. constant and moderate temperatures). However, a few studies have attempted to mimic conditions that may limit the efficacy of entomopathogens in field environments (e.g. environmental constraints) in an attempt to select genotypes able to incite disease under such conditions.

The ability of an entomopathogenic Hyphomycetes species to persist in an environment is another important attribute of a successful BCA. For propagules that exhibit good persistence, there will be a higher probability of an insect coming in contact with sufficient propagules to cause disease. As mentioned previously, inoculum thresholds are dynamic and the prolongation of propagule survival until conditions are conducive for disease development will result in more efficacious control. For example, during sunny periods grasshoppers can optimize their body temperatures by basking. Despite the deposition of large numbers of virulent conidia on to grasshoppers, disease progression is prevented or reversed under such conditions. However, during cool overcast periods, when grasshoppers are incapable of elevating their body temperature, Inglis *et al.* (1997a) hypothesized that the inoculum threshold may drop sufficiently to permit disease initiation. By comparing disease development in grasshoppers inoculated with *B. bassiana* and placed in cages that were either exposed to full sunlight or protected from ultraviolet-B (UVB) radiation, they observed substantially more disease in the UVB-protected cage environments, and the onset of disease occurred during a relatively cool overcast period. There was no difference in the ability of grasshoppers to elevate their body temperatures between cage environments, but conidial persistence was enhanced in the UVB-protected environment. Their observations supported the hypothesis that prolonging conidial persistence until conditions are conducive for disease to develop – in this case, the grasshoppers were unable to elevate their body temperatures during an overcast period – can substantially enhance the efficacy of entomopathogenic Hyphomycetes against insect pests. Further research on the interaction among persistence, inoculum thresholds and efficacy is warranted.

A number of entomopathogenic Hyphomycetes frequently cause natural epizootics, and the ability of a pathogen to cycle and disperse is an important factor in the development of these epizootics. As indicated previously, transmission of *N. rileyi* inoculum is an important factor in the development of an epizootic. Kish and Allen (1978) summarized the salient factors influencing dispersal of *N. rileyi* in velvetbean caterpillar populations in soybean, which are: (i) conidiogenesis on cadavers required periods of high humidity ($\geq$ 70%), but conidia will form on cadavers independently of fluctuations in relative humidity as long as the cadavers do not rapidly

desiccate; (ii) dry windy conditions promote conidial dispersal; (iii) dry windy conditions retard conidial germination and infection, but promote infection if they are followed by humid conditions, providing no excess of free water exists; (iv) rain and vegetative wetting promote conidiogenesis on cadavers; (v) conidia are washed to the ground by rain or heavy vegetative wetting by dew; (vi) an excess of free water during the height of an epizootic has little net effect on the course of the epizootic, but an excess of free water in the early stages of an epizootic (< 10% infection) may retard the spread of infection, if it follows conidiogenesis but precedes conidial dispersal; and (vii) an alternation of wet and dry conditions is necessary for spread of infection, and short periods of vegetative wetting and high humidity with longer periods of dry conditions with light winds favour the increase and spread of infections.

The insect host

A complex array of physiological and morphological factors influence the susceptibility of insect pests to entomopathogenic fungi. Examples include population density, behaviour, age, nutrition, genetics and exposure to injuries caused by mechanical, chemical or non-microbial agents (e.g. predators and parasites). A detailed review of the influence of host factors on disease development is beyond the scope of this chapter, and here we focus on important factors influencing the efficacy of entomopathogenic Hyphomycetes in managing insect pests.

One of the most important paradigms in microbial control is that 'stressed' animals are more susceptible to entomopathogens than non-stressed animals (e.g. Steinhaus, 1958a; Vago, 1963). While a variety of factors are thought to stress insects and predispose them to entomopathogens (e.g. crowding, nutrition, exposures to chemical stressors, environment), the physiological mechanisms (i.e. depressed immune response) of stress and the influence of many environmental parameters on the physiological predisposition of insects to entomopathogens are relatively poorly understood. Insect nutrition is a very important factor regulating the susceptibility of insects to entomopathogens, and it is an often overlooked factor in disease development. Inadequate nutrition often leads to increased susceptibility to entomopathogens, and the utilization of resistant plant genotypes to induce nutritional stress can substantially enhance the efficacy of entomopathogens. Conversely, diet can also decrease the susceptibility of insect pests to entomopathogenic Hyphomycetes. For example, Ekesi *et al.* (2000) found that thrips (*Megalurothrips sjostedti*) were less susceptible to *M. anisopliae* on certain cow-pea cultivars because of plant-derived fungistatic compounds. The concentration of secondary metabolites in plants is said to be higher in young leaves than in older leaves, but older leaves contain fewer nutrients (i.e. nitrogen and water) (Fenny 1992).

Declining nutrient and water content in the mature foliage of perennial plants was reported to reduce the growth rates of lepidopteran larvae compared with those of closely related species feeding on younger leaves or on the foliage of herbaceous plants (Krischik and Denno, 1983). It has also been suggested that high protein concentrations in an insect's diet can counterbalance the toxic effect of secondary metabolites, such as alkaloids (Costa and Gaugler, 1989). It is now recognized that insects may sequester antifungal compounds from their diets as a defence against entomopathogens, and increasing attention is now focusing on the impact of insect

nutrition on entomopathogen success. The inclusion of physiological stressors in the formulation of entomopathogenic Hyphomycetes is another obvious strategy that can be applied to enhance the efficacy of entomopathogens, and this strategy will also be discussed later in the chapter.

Another host factor that has been demonstrated to play an important role in the success of entomopathogens is the development stage of an insect. Not all stages in an insect's life cycle are equally susceptible to infection by entomopathogenic Hyphomycetes. In some situations, immature insects are more susceptible to infection than mature insects. For example, young larvae of the European corn-borer (*Ostrinia nubilalis*) are more susceptible to *B. bassiana* than older larvae (Feng *et al.*, 1985). In contrast, adult western flower thrips (*Frankliniella occidentalis*) were more susceptible to *V. lecanii* than larvae (Vestergaard *et al.*, 1995). Most host factors, such as insect developmental rates, cannot be considered independent of environment (e.g. temperature). High temperatures accelerate insect development and will reduce the time between molts, which can subsequently reduce the prevalence of infection due to loss of inoculum on exuviae.

Insect density is of particular importance in the epizootiology of disease. As the density of insects increases, there is a higher probability of an insect coming into contact with a pathogen (i.e. with infected individuals or with the pathogen directly). This is particularly true of viral epizootics, but limited research has focused on epizootics caused by entomopathogenic Hyphomycetes. Crowding has also been shown to stress individuals within a population, and subsequently predispose them to infection by non-fungal entomopathogens (Steinhaus, 1958b). However, the impact of crowding on the predisposition of insects to infection by entomopathogenic Hyphomycetes has not received much attention.

The behaviour of insects can influence epizootic development, and can affect the dispersal of an entomopathogen. For example, insects infected with entomophthoralean fungi often climb to the tops of plants just prior to death (i.e. summit disease syndrome), where they die firmly clasping the plant (e.g. grasshoppers infected with *Entomophaga grylli*). Such adaptations help ensure that spores contact potential hosts within and beneath the plant canopy, although such behaviour has not been reported for insects infected with hyphomycetous fungi. Another behavioural trait that can influence transmission of a pathogen is grooming. Conidia of *M. anisopliae* are spread among individual termites by grooming (Kramm *et al.*, 1982). While grooming can contribute to disease severity, the sanitation behaviour exhibited by many social insects can also limit the spread of an entomopathogen. For example, the termite *Reticulitermes flavipes* is very resistant to entomopathogenic Hyphomycetes (e.g. *B. bassiana*) not because of any endogenous defence mechanisms, but as a result of complex social behaviours, including the removal of infected individuals from the colony (Boucias *et al.*, 1996).

The environment

A variety of environmental factors have been shown to have dramatic effects on the efficacy of entomopathogens against insect pests. Salient parameters influencing the success of entomopathogenic Hyphomycetes against insects are solar radiation, temperature, water availability, precipitation and wind. Although we most often focus on a particular variable, environmental parameters interact with each other in their impact

on entomopathogens and, where possible, these factors should be addressed interactively.

Solar radiation

One of the most important parameters affecting propagule persistence in epigeal habitats is deactivation by solar radiation. Conidia, hyphal bodies and hyphae of all taxa of hyphomycetous fungi are highly susceptible to damage by solar radiation, and in particular the UVB portion of the solar spectrum (285–315 nm). However, significant differences in susceptibility to irradiation among taxa and strains within species have been observed. For example, Fargues *et al.* (1996) observed that conidia of *M. anisopliae* var. *acridum* (syn. *Metarhizium flavoviride*) were generally the most resistant to artificial sunlight (295–1100 nm at a UVB irradiance of 0.3 W m^{-2}) followed by conidia of *B. bassiana, M. anisopliae* and *Paecilomyces fumosoroseus*. While the mechanisms of resistance to solar radiation within the entomopathogenic fungi have not been extensively studied, resistance to UV damage should be considered in selecting genotypes of fungi for use in IPM programmes.

The microhabitat in which fungi are deployed is another important factor influencing their persistence. The survival of conidia deposited on substrates exposed to direct solar radiation is substantially reduced relative to propagules in protected locations, such as within plant canopies (Inglis *et al.*, 1993). Since many insect pests feed or exist for periods of time within the plant canopy, the influence of microclimate should not be overlooked. Another strategy is to apply conidia on the abaxial surface of leaves (e.g. Sopp *et al.*, 1990) to enhance persistence, but this can be a difficult task (Wraight and Carruthers, 1999). The degree to which the targeting of abaxial leaf surfaces will be efficacious will also depend on the behaviour of the target insect pest. Some insects naturally feed on the undersides of leaves (e.g. whiteflies), but it may be necessary to stimulate other pest insects to move to the abaxial surface of leaves. For example, Amiri *et al.* (1999) demonstrated that crude preparations of destruxins from *M. anisopliae* applied to the abaxial leaf surface caused diamondback moth (*Plutella xylostella*) and mustard beetle (*Phaedon cochleariae*) larvae to move to the adaxial leaf surface.

Even within shaded areas, propagules will eventually be killed due to indirect irradiance (Smits *et al.*, 1996a, b), but conidial survival may be sufficiently prolonged to enhance the efficacy of control. While the UVB component of the solar spectrum is detrimental to fungal propagules, irradiation at higher wavelengths may be beneficial by stimulating photoreactivation counteracting the harmful effects of UV radiation. Although we are not aware of a photoreactivation phenomenon specifically within insect-pathogenic fungi, it has been demonstrated in other fungi (Leach, 1971) and consequently should not be overlooked.

The rapid inactivation of infectious propagules by solar radiation is considered by many to be a major impediment to the successful commercialization of entomopathogens against insect pests of field crops, and considerable effort has focused on the protection of entomopathogens. The most popular approach has involved the incorporation of solar blockers and/or UVB-absorbing chemicals (i.e. sunscreens) in formulations of propagules. A diverse array of sunscreens have been tested with entomopathogenic viruses and *Bacillus thuringiensis*, and to a lesser extent with fungi. A summary of sunscreens used to protect microorganisms is provided by Bernhard *et al.* (1998) and Burges and Jones (1998). While a number of sunscreens have

demonstrated excellent protection of fungal propagules in controlled environment studies, reports of enhanced persistence or efficacy in field settings are less common. For example, several oil-soluble sunscreens significantly increased the survival of *B. bassiana* and *M. anisopliae* var. *acridum* conidia exposed to artificial radiation (Moore *et al.*, 1993; Inglis *et al.*, 1995a), but they did not enhance survival in field settings (Inglis *et al.*, 1995a; Shah *et al.*, 1998). In contrast, a solar blocker (clay) and a UV-absorbing optical brightener (Tinopal) did increase the field persistence of *B. bassiana* conidia on grass leaves exposed to sunlight (Inglis *et al.*, 1995a). It has long been recognized that pigmented propagules (e.g. melanin) of the dematiaceous Hyphomycetes are substantially more resistant to solar inactivation than non-melanized fungi. Formulation of *B. thuringiensis* in melanin provided excellent photoprotection (Liu *et al.*, 1993), and the formulation of melanin and other polyphenolics (e.g. lignin) may increase the persistence of entomopathogenic Hyphomycetes.

While research to date has focused on survival of propagules on plant tissues and/or on inanimate objects, very little is known about the persistence of propagules on insects. Many researchers consider this to be unimportant because the amount of time a fungus spends on the outside of the insect is limited (i.e. germination and penetration occur within 24 h). However, some evidence indicates that conidia on insects are as susceptible to solar deactivation as they are on inanimate objects or leaf surfaces (Inglis *et al.*, 1997a), and that prolonging the survival of fungal propagules on the surface of insects until conditions are favourable for disease development may enhance efficacy in field environments (e.g. Inglis *et al.*, 1997a). The adverse effects of solar radiation on entomopathogens are often influenced by other variables as well, and often these act as confounding effects. For example, both the direct (e.g. germicidal) and indirect (e.g. host behaviour) effects of solar radiation influence the efficacy of *B. bassiana* against grasshoppers (Inglis *et al.*, 1996a, 1997a). Research that determines whether prolonging the survival of fungal propagules will subsequently translate to increased field efficacy and the impact of other variables on this relationship is warranted.

Temperature

Temperature is one of the principal factors influencing entomopathogen efficacy. It is well documented that ambient temperature influences the rate of infection and time to death of insects treated with entomopathogenic hyphomycetous fungi. Given that a window of opportunity often exists for the pathogen to infect its host (e.g. conditions of humidity or solar radiation may be suitable for short periods of time), the influence of temperature on the infection process is very important. For example, the optimum temperature for *M. anisopliae* infecting adult thrips is ~23°C (Vestergaard *et al.*, 1995), and a decrease in temperature of 3–5°C increases the time to death by ~1 day. A delay in death can be critical to a pest manager, especially in the management of pests with explosive population growth rates and/or pests of high-value crops (e.g. within greenhouses). Ideally, the thermal characteristics of isolates should be matched to the microhabitats in which they will be deployed for optimal performance.

The optimum temperature for most entomopathogenic Hyphomycetes is between 20 and 25°C, but infection and disease can occur at temperatures ranging between 15 and 30°C. Above 30°C, the vegetative growth of most taxa is inhibited and growth usually ceases at ~37°C. The inability of an entomopathogenic fungus to grow at mammalian body temperature is an important consideration in registration (see Chapter

13). Similarly to variation in solar deactivation, considerable variability exists among genotypes in their thermal characteristics. For instance, Fargues *et al.* (1997b) found that four isolates of *M. anisopliae* var. *acridum* equally induced 98–100% mortality in the desert locust at 25 and 30°C, and none at 40°C, but there were significant differences among the isolates at 35°C, with mortalities ranging from 40 to 100%. A number of researchers have investigated the possibility of selecting genotypes possessing specific thermal characteristics (e.g. higher optimal and cardinal temperatures) from specific geographical locations (e.g. if an isolate comes from a warm country it may tolerate high temperatures, or conversely if it comes from cold climates it will perform better at low temperatures). Some studies have reported no or weak correlations between the geographical origin and thermal characteristic (McCammon and Rath, 1994; Fargues *et al.*, 1997a; Ouedraogo *et al.*, 1997). Others have observed a stronger relationship between temperature characteristics *in vitro* and place of origin. For example, Vidal *et al.* (1997a) measured the effects of temperature on the growth rates of various isolates of *P. fumosoroseus* originating from various insect hosts (primarily *Bemisia tabaci*, and some species of Lepidoptera) from the southern USA, Europe, Pakistan, Nepal and India. Similarly to other studies, they observed considerable variability among isolates in their ability to grow at various temperatures. They also reported that most isolates originating from Europe exhibited growth at 8–30°C with optimal growth rates at 20°C, 20–25°C or 25°C. The temperature range for the isolates from the southern USA (both humid and dry subtropical climates) and from west Asia (humid tropical climate) was broader (8–35°C) with optimal growth at 25°C, 25–28°C or 28°C. Indian isolates possessed the greatest tolerance to high temperatures (32°C and 35°C). The thermal biology of insects in field settings is extremely complex, and it still remains to be determined whether isolates possessing slightly higher optimal growth rates *in vitro* will translate into increased efficacy in field conditions.

Within field settings, daily temperatures can fluctuate substantially. For example, during May–July, when control of grasshoppers is usually implemented in the northern great plains of North America, night-time temperatures of less than 5°C and daytime temperatures of 25–35°C are common. Fluctuations in temperature have been shown to significantly influence the *in vitro* growth of a number of hyphomycetous fungi. However, the influences of fluctuating temperatures *in vivo* are less common. Inglis *et al.* (1999) measured the effects of oscillating temperature on the competitive infection and colonization of grasshoppers (*Melanoplus sanguinipes*) by *B. bassiana* and *M. anisopliae* var. *acridum* applied alone and in combination. Nymphal mortality and proliferation of the fungi in the haemocoele were measured in four environments with the same mean daily temperature (25°C) but differing in the degree to which they oscillated daily (constant 25°C, 20 to 30°C, 15 to 35°C or 10 to 40°C). In general, as the amplitude of temperature increased, growth rates for both fungi decreased. A similar trend was observed for nymphal mortality.

While the relationship between fungal proliferation in the haemocoele and disease development is poorly understood, Inglis *et al.* (1999) showed that both *B. bassiana* and *M. anisopliae* var. *acridum* proliferated in the haemocoele of inoculated grasshoppers, and that the size of fungal populations within haemocoeles was significantly correlated with mortality. Although the same temperature regime was repeated on a daily basis, in nature temperature oscillations are variable and the influence of irregular oscillations on entomopathogens may further influence efficacy. The identification of temperature thresholds and the effect of cumulative exposures to high and low temperatures on efficacy of entomopathogenic Hyphomycetes, and the application of mathematical

models will facilitate our understanding of the effects of temperature on insect–fungal interactions. This will lead to the identification and implementation of strategies to overcome constraints of temperature on efficacy in entomopathogens.

The thermal constraints on entomopathogenic Hyphomycetes are not only the result of ambient temperatures but may also be exacerbated by host behaviour (i.e. thermoregulation). For example, many insects elevate their body temperatures higher than ambient by either directly or indirectly intercepting solar radiation (e.g. basking) (Heinrich, 1993, 1996). The influences of thermoregulation on disease development are less well documented, but basking behaviour in a number of insects, including houseflies (Watson *et al.,* 1993) and various acridids (e.g. Inglis *et al.*, 1996a), has been shown to reduce the severity of disease. Many acridids possess an optimal temperature of ~40°C, and optimization of body temperature via basking has been shown to adversely affect *B. bassiana* and to a lesser extent *M. anisopliae* var. *acridum* (Inglis *et al.*, 1996a, 1997b, 1999). The cardinal temperature for *B. bassiana* conidial germination and hyphal development is ~35°C, and the prevalence of mycosis decreased by ~45% and 90% in nymphs allowed to optimize their body temperature by basking for only 1 and 2 h day^{-1}, respectively (Inglis *et al.*, 1996a). While most insects possess lower temperature optima than acridids, these insects may exhibit a 'behavioural fever' response to infection. Behavioural fever is defined here as the elevation of body temperatures higher than normal levels using behavioural means. Most research on behavioural fever has utilized thermal gradients in which the body temperature of infected and non-infected insects is compared. For example, Inglis *et al.* (1996a) observed that a significantly higher prevalence of *B. bassiana*-infected nymphs selected hotter positions than non-infected nymphs on a heat gradient.

Field observations of behavioural fever are less common. Applying a grab-and-stab method at various times after application of *M. anisopliae* var. *acridum* against the Senegalese grasshopper (*Oedaleus senegalensis*), Blanford *et al.* (1998) observed that the body temperatures of grasshoppers sprayed with the fungus were 3°C higher on average than those of control grasshoppers, but that the fungus still provided good suppression, indicating that the behavioural fever response to infection did not confer resistance to disease in the treated population. However, behavioural fever in acridids infected with *M. anisopliae* var. *acridum* appears to increase the time to death and may also raise the inoculum threshold required for successful disease initiation and progression (Blanford *et al.*, 1998). The degree to which other insects exhibit a behaviour fever response and the implications of fever on the successful use of entomopathogens to manage insect pests in laboratory and field conditions is currently uncertain.

In insects possessing optimal temperatures that inhibit disease or that exhibit a behavioural fever response to infection, a number of strategies to overcome this constraint have been proposed. As mentioned previously, the selection of genotypes with the ability to grow and incite disease at higher temperatures is one possibility. Another strategy that is currently being pursued involves the use of behavioural modifiers and/or physiological stressors (e.g. Goettel *et al.*, 2000b). For example, sublethal doses of some insecticides have been observed to alter the behaviour of insects (e.g. following application insects become cryptic). Recently, renewed interest has focused on the use of insecticides (e.g. imidacloprid and diflubenzuron) in combination with entomopathogens. How these compounds interact with the pathogen and the insect host has not been well documented. Two other strategies that might be used to overcome the effects of high temperature include the use of novel targeting strategies (e.g. insect

stages at times of the year when behavioural optimization of body temperature is unlikely) and the development of predictive models that will facilitate the deployment of entomopathogens under suitable conditions (i.e. windows of opportunity).

Relative humidity

Relative humidity can influence fungal efficacy in several ways. Humidity, in combination with temperature, influences evaporation of spray droplets, which can result in the loss of small particles and thereby adversely affect targeting. Furthermore, moisture can also have very significant effects on the persistence of fungal inocula. For the most part, fungal conidia usually exhibit greatest stability under cool and dry conditions (Roberts and Campbell, 1977; Daoust and Roberts, 1983a; Hedgecock *et al.*, 1995; Hong *et al.*, 1997). In contrast, conidia of other fungi (e.g. *M. anisopliae*) survive better at moderate temperatures when relative humidity is high (e.g. Daoust and Roberts, 1983b). Not only is water essential for propagule germination, but it also regulates conidiogenesis on cadavers that have died from mycosis. In all instances, conidiogenesis on insect surfaces requires high moisture, and the production of conidia may influence horizontal transmission of the pathogen. For example, Fargues and Luz (2000) observed that a relative humidity of at least 97% was required for conidial production of *B. bassiana* on mummified cadavers of *Rhodnius prolixus* (vectors of *Trypanosoma*, the causal agent of Chagas' disease), and suggested that the occurrence of high humidity is the most crucial climatic constraint limiting the recycling ability of *B. bassiana* against *R. prolixus*. While high ambient humidity is a prerequisite for external conidiogenesis, evidence indicates that some taxa (e.g. *B. bassiana*) can form conidia within the haemocoel of cadavers under conditions of low humidity (Fernandes *et al.*, 1989).

The prerequisite for water during germination has led to the general belief that moist conditions are essential for effective use of fungi in microbial control against insects. This may hold true for some fungal taxa against some insect pests, but it is unlikely to hold true for all fungi in all circumstances. A number of studies indicate that dry conditions during or immediately following application of fungal propagules are less detrimental than previously thought. For example, ambient humidity does not influence infectivity of insects with *B. bassiana* and *M. anisopliae* var. *acridum* (e.g. Ramoska, 1984; Marcandier and Khachatourians, 1987; Fargues *et al.*, 1997b; Ferron, 1997). The ability of these fungi to germinate and infect the host under conditions of low ambient humidity is attributed to sufficient moisture within microhabitats. For example, a boundary layer surrounds plant vegetation, and a microclimate of high humidity probably also surrounds the integument of insects (e.g. due to water loss). Cuticular folds in insect exoskeletons may also be sites of high moisture. Although few studies have attempted to enhance infectivity of insects under conditions of low humidity, Bateman *et al.* (1993) reported that infection of desert locusts was enhanced for *M. anisopliae* var. *acridum* conidia applied in oil. On the basis of this report, many researchers now conclude that oil enhances infectivity at low humidities. However, Fargues *et al.* (1997b) found that *M. anisopliae* var. *acridum* conidia in oil did not enhance infectivity of desert locusts relative to an aqueous formulation. Evidence now indicates that there are complex interactions between inoculation method, carrier and humidity and this must be taken into consideration. Reports of spreading of propagules applied in oil over the surface of the integument (e.g. Inglis *et al.*, 1996b) and the influence that this has on infectivity (e.g. impact of microclimate) should be

addressed along with the impact of spatial distributions of propagules on the integument. Invert oils (water in oil emulsion) have been shown to enhance germination and penetration of plants by phytopathogenic fungi under low-moisture conditions, and the use of invert oils to enhance the infectivity of insects may prove to be beneficial.

Rainfall

In addition to increasing humidity, rainfall can serve to dislodge and disperse conidia from substrates as well as aid in the dispersion of propagules. The effects of precipitation and/or dew on the foliar persistence of entomopathogenic viruses and *B. thuringiensis* have been relatively extensively studied. In contrast, little is known about the persistence of entomopathogenic fungal propagules on insects and on foliage. Conidia of hyphomycetous fungi strongly adhere to insect cuticles (e.g. Boucias *et al.*, 1988, 1991). Anecdotal evidence, primarily based on poor recovery of conidia from insects following vigorous washing, has led to the opinion that rain does not 'wash off' significant numbers of conidia from the integument of insects (Burges, 1998). However, recent evidence indicates that rain does cause significant removal of *B. bassiana* and *M. anisopliae* conidia from foliage of monocotyledonous and dicotyledonous plants (Inglis *et al.*, 1995b, 2000; Inyang *et al.*, 2000) and the integument of Colorado potato beetle larvae (*Leptinotarsa decemlineata*) (Inglis *et al.*, 2000). Raintower experiments indicated that *M. anisopliae* conidia are readily dispersed by rain splash, but very little inoculum was found in the soil outside sprayed plots in a subsequent field experiment, raising questions as to the importance of rain in the redistribution of inoculum (Inyang *et al.*, 2000). While rain has been shown to remove fungal conidia from leaves and insect cuticles, the influence of a number of confounding variables, such as canopy density and architecture and insect behaviour, on conidial persistence remains to be determined.

Stickers are frequently incorporated into viral and *B. thuringiensis* formulations to increase the retention of spray deposits on foliage, and a diverse array of commercial stickers have been used to enhance persistence of these entomopathogens (Bernhard *et al.*, 1998). Very limited research has focused on the utilization of sticker formulations to enhance the persistence of fungal propagules. The formulation of *B. bassiana* conidia in vegetable and mineral oils only marginally improved the retention of conidia on potato leaves and not on Colorado potato beetle larvae (Inglis *et al.*, 2000). Additional research on the influence of 'stickers' on persistence of entomopathogens applied in oil-based formulations is necessary, but caution must be exercised in choosing stickers. Stickers may prevent the transfer of conidia from sprayed leaf surfaces to insects, but those that remain liquid and/or can be subsequently transferred to the passing insect may prove useful.

Soil factors

Many hyphomycetous fungal entomopathogens are considered to be soil-borne microorganisms and have demonstrated considerable potential against soil pests (Keller and Zimmermann, 1989). However, soil is an extremely complex milieu, and a number of factors, including soil type (i.e. texture, cation exchange capacity, organic matter content, pH, etc.), moisture (i.e. water availability) and the presence of a viable soil microflora, can influence the persistence and/or efficacy of entomopathogenic hyphomycetous fungi. Furthermore, a variety of strategies (e.g. formulation and appli-

cation methods, such as tillage) have been tested in an attempt to enhance the success of entomopathogenic Hyphomycetes in soil.

ABIOTIC FACTORS. Many entomopathogenic fungi are able to withstand high and variable temperatures, as well as conditions of high moisture and drought stress, in soils (Roberts and Campbell, 1977), and a number of studies have demonstrated that conidia applied directly on soil surfaces or incorporated into soil following application exhibit considerable persistence in temperate climates (Muller-Kogler and Zimmermann, 1986; Gaugler *et al.*, 1989; Storey *et al.*, 1989; Inglis *et al.*, 1997c). For example, *M. anisopliae* was found to persist for at least 7 years in pasture soils at levels equivalent to the applied dose (Rath *et al.*, 1997).

The influence of various soil factors on the occurrence or persistence of entomopathogenic Hyphomycetes has been addressed in a number of survey studies. In these studies, researchers have attempted to correlate the abundance of fungi with various soil parameters, but, due to the complexity of these factors, it is often not possible to draw conclusions about the effects of factors on either survival or efficacy. While the results of controlled experiments are less numerous than survey data, several studies have addressed the influence of various soil factors on persistence and efficacy of entomopathogenic Hyphomycetes. In a variety of experimental settings, soil moisture has been shown to adversely influence the persistence of *B. bassiana, B. brongniartii, M. anisopliae* and *V. lecanii* and others in soil. In most circumstances, limited vertical movement of conidia occurs in soils. However, in other situations, physical loss of inoculum may occur (Storey and Gardner, 1987, 1988). For example, Storey and Gardner (1988) observed that the vertical movement of *B. bassiana* conidia was correlated with the water infiltration value of soils, with most loss occurring in sandy relative to finer-textured soils. However, even in the sandy soil (87% sand), greater than 85% of the conidia applied to the soil remained at the soil surface. Soil texture and organic matter appear to be the most important factors determining vertical movement of fungal propagules in water, and sandy-textured and soils low in organic matter tend to retain fewer propagules than clay-textured and organic soils (Ignoffo *et al.*, 1977b; Storey and Gardner, 1988; Keller and Zimmermann, 1989; Fornallaz, 1992). The mechanisms responsible for the high retention of conidia in such soils are unknown, but may relate to their high cation exchange capacity and/or to their reduced pore sizes. Furthermore, the vertical movement of the propagules is not only dependent on the type of soil, but also on properties of the propagules themselves. For example, conidia of *B. brongniartii* are relatively large (~8 μm in length) and are less prone to be vertically displaced relative to smaller propagules.

The influence of other parameters, such as pH and ionic conductivity, are less well understood. A number of studies have demonstrated no or minimal effects of soil pH on the distribution of entomopathogenic Hyphomycetes (e.g. Rath *et al.*, 1995). In contrast, Groden and Lockwood (1991) observed that fungistasis against *B. bassiana* was correlated with pH but not with other soil characteristics, including texture and organic matter content. In a later study, Groden and Dunn (1996) found that soil pH and nitrogen fertilizers had an impact on the germination of *B. bassiana* conidia, but not on the infection of the Colorado potato beetle. Fungistatic effects on soil-borne phytopathogens are often expressed most strongly in neutral or slightly acidic soils (Lockwood, 1977), and the release of plant-derived phenolic compounds can inhibit entomopathogenic fungi in soils (Lopez-Llorca and Olivares-Bernabeu, 1997).

A variety of strategies have been applied in an attempt to increase the persistence

of entomopathogenic Hyphomycetes in soil. Incorporation of conidia into soils is thought to increase their survival by protecting propagules from solar radiation and buffering them from extremes of temperature and moisture (Gaugler *et al.*, 1989), and number of application strategies have been developed to facilitate efficient application (e.g. injection with a seed drill). However, conditions of temperature in the top few centimetres of soil may range over 40°C between daylight and night hours, and temperatures in excess of 50°C can occur (Carruthers and Soper, 1987). Therefore, incorporation of propagules into soil to enhance persistence will depend not only on climatic factors (e.g. ambient temperatures and vegetation), but also on other factors, such as shading or the depth to which propagules are incorporated. In addition to injections, the encapsulation of propagules is another formulation strategy that may facilitate penetration and persistence in soil, but this has not been extensively tested with entomopathogenic Hyphomycetes.

A number of formulations have been shown to influence the persistence of propagules in soil. The application of conidia in oil or oil-emulsion sprays on to the surfaces of soil have generally not substantially enhanced their persistence relative to aqueous preparations of entomopathogenic fungal conidia (e.g. Inglis *et al.*, 1997c). In contrast, other formulations have been shown to increase the survival of propagules in soil. For example, coating *B. bassiana* conidia in clay increased their persistence in various soils and with various water activities under controlled environment conditions (Studdert *et al.*, 1990), but it did not affect mortality against beet army worms (*Spodoptera exigua*) (Studdert and Kaya, 1990a). Considerable interest has focused on the use of granular formulations of entomopathogenic Hyphomycetes. A variety of different materials have been used to prepare granules (e.g. alginate), and conidia or mycelia are incorporated into the granule matrix. The ability of the fungus to grow and sporulate in and/or on the granule is usually desirable, and a number of materials that facilitate growth and sporulation have been tested. Increased persistence and efficacy of fungi applied in granules in soils have been observed (e.g. Storey *et al.*, 1989; Krueger *et al.*, 1992), and propagules applied in granule formulations have provided satisfactory field activity against a number of economically important soil pests. Several of these products have been commercialized (Shah and Goettel, 1999; see also next section – 'Mycoinsecticides in IPM').

The most extensively studied parameters affecting the efficacy of entomopathogenic Hyphomycetes are soil texture and moisture (e.g. water availability). However, other factors, such as temperature, pH and the organic matter content of soil, may also have an impact on the efficacy of hyphomycetous fungi against insect pests. Soil moisture has been shown to have a significant impact on the efficacy. For example, Krueger *et al.* (1991) demonstrated that a higher number of chinch-bugs (*Blissus leucopterus leucopterus*) died from infection by *B. bassiana* under conditions of low relative to high soil moisture. Similarly, Studdert and Kaya (1990a) observed that emergence of beet army-worm adults was substantially reduced in drier ($\leq$ 37 bars) than wetter soils ($\geq$ 15 bars) inoculated with *B. bassiana* conidia, and a substantially higher density of conidia was required to reduce adult emergence in the wetter soils. Management practices, such as the degree of tillage, can also have an impact on entomopathogen efficacy. Grivanov (1940) reported that deep ploughing of cereal stubble increased the efficacy of *B. bassiana* against overwintering thrips, but the reason for the increased efficacy is uncertain. Much more research is needed on how agromanagement practices can be altered to increase the efficacy of entomopathogenic Hyphomycetes against soil-dwelling insects.

BIOTIC FACTORS. Although many entomopathogenic Hyphomycetes are cosmopolitan in soil, very little is known about the saprotrophic ability of most taxa. Much circumstantial evidence suggests that many entomopathogenic Hyphomycetes (e.g. *B. bassiana* and *M. anisopliae*) are relatively weak competitors in soil, and it is common to observe relatively restricted vegetative growth emanating from insect cadavers that have died from mycosis in soil (e.g. Gottwald and Tedders, 1984). Supporting this supposition are observations of reduced vegetative growth in soils containing high to moderate levels of organic matter (Studdert and Kaya, 1990b) and in non-sterilized versus sterilized soils (e.g. Pereira *et al.*, 1993).

Studies attempting to elucidate the influence of the soil microflora on the persistence and efficacy of entomopathogenic Hyphomycetes are based on either anecdotal evidence or experimentation in which the microflora is eliminated (e.g. by sterilization) or modified (e.g. by organic amendments). Experimentation is often plagued by the extreme complexity of soils and by a variety of potentially confounding variables. For example, a number of researchers have compared persistence and/or efficacy of entomopathogenic Hyphomycetes in sterilized relative to unsterilized soils. The survival of *B. bassiana* conidia in non-sterile soil amended with carbon and/or nitrogen sources was found to be greatly decreased (Lingg and Donaldson, 1981). In contrast, populations of *B. bassiana* showed dramatic increases in sterile soil treated in the same manner, and Lingg and Donaldson (1981) postulated that the observed fungistasis in the non-sterile soils may have been due to the prevalence of the soil fungus *Penicillium urticae*, which produced a water-soluble inhibitor of *B. bassiana in vitro*. In another study, Inglis *et al.* (1998) observed that grasshoppers ovipositing into soils containing conidia of *B. bassiana* were highly susceptible to the fungus. While there was no effect of soil texture on mortality, on the number of eggs laid, on positioning of egg pods in the soil profile or on populations of *B. bassiana* recovered from female abdomens, a higher prevalence of mortality was observed for females ovipositing into sterilized than non-sterilized sandy-loam and clay-loam soils. All sterilization methods cause changes to the physical and/or chemical properties of soil, and extreme care must be taken in the interpretation of these types of studies.

Interactions among environmental variables

As indicated previously, the epizootics involve a dynamic interaction among the pathogen, host and environment in time. While the environment component of the disease tetrad is crucial to the success of a BCA, it is often overlooked. Furthermore, the interaction is extremely complex, and environmental parameters are rarely, if ever, static in time. Very few studies have attempted to empirically elucidate the importance of fluctuating climatic factors on the efficacy of entomopathogenic Hyphomycetes. A recent example by Fargues and Luz (2000) investigated the influence of fluctuating moisture and temperature regimes on sporulation of *B. bassiana* on *R. prolixus* cadavers. They found that high humidity was the most crucial parameter, but the intensity of conidial production was enhanced when diurnal temperatures were high (28 and 35°C). These types of mechanistic studies, while complex, are of paramount importance, and the integration of empirical studies with epizootiological modelling will enhance our understanding of epizootic initiation and development and facilitate the efficacious use of entomopathogenic Hyphomycetes.

Mycoinsecticides in IPM

There are three primary application strategies utilized against insects: (i) classical; (ii) inoculative (i.e. augmentative); and (iii) inundative approaches. In epigeal environments, entomopathogenic Hyphomycetes have primarily been applied utilizing an inundative application or microbial pesticide strategy. In soil environments, both inundative and inoculative strategies have been employed.

Entomopathogenic Hyphomycetes applied against insect pests using the inundative deployment strategy are frequently developed and assessed based on a chemical pesticide model and, in most instances, entomopathogens fit this model poorly. Here, we define a chemical pesticide model as the application of a specific quantity of an active ingredient to an insect population with the goal of inciting catastrophic and rapid mortality, thereby quelling an outbreak. Entomopathogenic Hyphomycetes are often relatively slow-acting or their effects may be sublethal. Furthermore, their efficacy is often assessed independent of environmental conditions. It is clear that new paradigms must be formulated and implemented within a biologically based framework if entomopathogens are to further contribute to the integrated management of insect pests.

Under optimal conditions, the death of an insect usually takes between 3 and 5 days from the time of application. In field environments, death can take substantially longer. This is usually due to conditions of suboptimal environment (see 'Factors Influencing Efficacy – The Environment'), which can prolong disease initiation and progress. For example, Sahelian grasshoppers treated with *M. anisopliae* var. *acridum* died more slowly when allowed to thermoregulate than those that were incubated in the shade (Langewald *et al.*, 1999). Disease progression by *B. bassiana* was arrested in Mormon crickets incubated at 37°C, but resumed when the crickets were transferred to 25°C, suggesting that mycosis could be typically delayed in the field by high temperatures and thermoregulation (J.H. Turnbow, personal communication).

Despite the delayed onset of mortality in field environments, the feeding behaviour of the insect pest can be affected during the period between infection and death. Although chemical pesticide models rarely take crop consumption into consideration in assessing efficacy, reduced feeding following the application of entomopathogenic Hyphomycetes can have a significant impact on crop protection. For instance, two examples in which reduced feeding was observed following infection are the Colorado potato beetle infected with *B. bassiana* and grasshoppers infected with *M. anisopliae* var. *acridum*. Fargues *et al.* (1994) observed that, 2 days following application of *B. bassiana*, substantial reductions (~57%) in consumption of foliage were observed in Colorado potato beetle larvae. Using faecal production as an indicator of food consumption, Thomas *et al.* (1997) also showed considerable reductions in feeding by grasshoppers (*Zonocerus variegatus*) infected with *M. anisopliae* var. *acridum* prior to death (< 7 days). A similar effect of *M. anisopliae* var. *acridum* on the acridid *Rhammatocerus schistocercoides* was observed in Brazil (Faria *et al.*, 1999).

An aspect of entomopathogen efficacy that is almost always neglected is the sublethal effects of infection. Sublethal infections occur when an insect does not die but various aspects of its biology are affected none the less. Reductions in feeding and fecundity and slowed developmental rates have been well documented for several viral and protozoan entomopathogens. In contrast, relatively little research has focused on the sublethal effects of entomopathogenic Hyphomycetes on insect pests. Fargues *et al.* (1991) demonstrated that the reproductive potential of Colorado potato beetle

females surviving infection by *B. bassiana* was reduced at 22°C but not at 25°C. Adults of the hymenopteran parasitoid of Russian wheat aphid (*Aphelinus asychis*) treated with *P. fumosoroseus* and incubated at high humidity were significantly less active (e.g. percentage of time walking, walking speed and distance covered) than untreated insects (Lacey *et al.*, 1997). Development times were prolonged and the predation efficacy of the coccinellid predator, *Serangium parcesetosum*, was similarly reduced in individuals sprayed with *B. bassiana* (Poprawski *et al.*, 1998). Authurs and Thomas (1999) observed that brown locusts (*Locusta pardalina*) infected by *M. anisopliae* var. *acridum* were more susceptible to predation. Similar effects have been shown for Sahelian grasshoppers (Thomas *et al.*, 1998). These recent reports demonstrate that sublethal effects occur in insect pests infected with entomopathogenic Hyphomycetes, and this should be considered in the evaluation of entomopathogenic fungi against insects.

Examples of hyphomycetous fungi used to manage insect pests

Entomopathogenic Hyphomycetes have been employed against a variety of different insect pests in a variety of different agroecosystems and they have demonstrated varying degrees of success. It is not possible to present a comprehensive review of the literature in this chapter, and we attempt to present examples in which efficacious management of insect pests has been achieved and/or novel approaches have been employed.

Glasshouses

Perhaps the greatest potential for managing pests with entomopathogenic Hyphomycetes is in glasshouses, where environment can be manipulated in favour of the pathogen. Several taxa have demonstrated excellent suppression of insect pests in greenhouses, including *Aschersonia* spp., *B. bassiana, M. anisopliae, P. fumosoroseus* and *V. lecanii*. Considerable research has focused on the development of *V. lecanii* against a variety of insect pests of glasshouse crops, including chrysanthemums (Hall and Burges, 1979; Hall, 1981). Aphids are economically important pests of chrysanthemums in glasshouses throughout the world, and the application of *V. lecanii* has demonstrated good efficacy against a variety of aphid species (Rombach and Gillespie, 1988). In addition, *V. lecanii* has shown considerable potential for managing whiteflies and thrips in greenhouse crops. Infection and penetration of aphids by *V. lecanii* can occur under conditions of reduced humidity (Hsiao *et al.*, 1992), but sporulation and transmission require high (~100%) humidity (Milner and Lutton, 1986).

Chrysanthemums are a short-day plant, and the normal procedure of covering plants with polyethylene sheeting from mid-afternoon until morning the following day during summer months creates a suitable environment (i.e. high humidity) for disease to occur. A single application of *V. lecanii* conidia just before plants were covered with the polyethylene was observed to provide satisfactory control of the important aphid pest, *Myzus persicae*, but less so of two minor aphid pests (Hall and Burges, 1979). It is also possible to easily and safely create high-humidity environments by fogging water over greenhouse crops at night. Helyer *et al.* (1992) observed that four consecutive nights of high humidity per week or a cycle of two nights of high humidity and two nights of ambient humidity resulted in excellent control of aphids, thrips and whiteflies by *V. lecanii* with no adverse impacts on the crop. The efficacy of *V.*

lecanii for controlling pests other than aphids, thrips and whiteflies (e.g. mites, nematodes and rusts) has also been demonstrated in a number of glasshouse crops (Verhaar *et al.*, 1996). The ability of *V. lecanii* to infect other fungi (i.e. mycoparasites) appears to be unique among the entomopathogenic Hyphomycetes (Askary *et al.*, 1998).

P. fumosoroseus (PRF-97®) and *B. bassiana* (Botaniguard®) are two other fungi that have recently been registered against an array of greenhouse pests, including aphids, thrips, whiteflies and spider mites (Shah and Goettel, 1999). See section on whiteflies below for examples of the use of fungi for control of whiteflies in field crops.

Field crops

COLORADO POTATO BEETLE. The Colorado potato beetle is an economically important pest of potatoes and other solanaceous crops throughout the world. The development of resistance to insecticides has prompted considerable research into alternative methods, including the application of *B. bassiana*, for managing this pest. Some studies have shown no or limited efficacy, while others have observed significant suppression of Colorado potato beetle (Campbell *et al.*, 1985; Hajek *et al.*, 1987; Anderson *et al.*, 1988; Jaques and Laing, 1988; Drummond and Groden, 1996; Poprawski *et al.*, 1997; Lacey *et al.*, 1999a). *B. bassiana* (e.g. Boverin) was used extensively (> 70 types of crops) in the former Soviet Union and satellite countries against a variety of insect pests. Against Colorado potato beetle, Boverin was applied alone or in combination with sublethal doses of dichlorodiphenyltrichloroethane (DDT) (Ferron, 1978). Several insecticides have been tested in combination with *B. bassiana* against Colorado potato beetle in North America, and some results have demonstrated an additive effect (e.g. Anderson *et al.*, 1989).

A number of studies have observed that foliar applications of *B. bassiana* to potatoes in North America provided satisfactory suppression of Colorado potato beetle populations. Foliar application of *B. bassiana* was used effectively in conjunction with the bacterium *B. thuringiensis*, and densities of beetles in the fungus–bacterium-treated plots declined yearly relative to other treatments (Drummond and Groden, 1996). Poprawski *et al.* (1997) observed that applications of *B. bassiana* conidia at 3–4-day intervals early in the season effectively reduced densities of older larvae and provided substantial foliar protection; larval densities were 10, 21 and 41 larvae per plant for the *B. bassiana*, insecticide (i.e. esfenvalerate, piperonyl butoxide, oxamyl and carbofuran) and control treatments, respectively. Lacey *et al.* (1999a) also observed significant effects of *B. bassiana* against Colorado potato beetle. Five weekly applications of conidia were made, but poor control of beetles was observed prior to row closure, after which point fair to good control was observed. Yields of tubers were rated as fair in plots treated with *B. bassiana*, but lower numbers of overwintering adult Colorado potato beetle were observed in these plots relative to the chemical control. Furthermore, the fungus enabled good survival in predatory Heteroptera and other non-target insects relative to the insecticide treatment. Reasons for the differential success among experiments in managing Colorado potato beetle with *B. bassiana* are currently unknown. While a number of reasons have been suggested for the poor efficacy of *B. bassiana* in some field trials, relatively limited research has specifically focused on the elucidation of constraints on disease development. The requirement for adequate canopy development (e.g. Lacey *et al.*, 1999a) suggests that microclimate is an important factor for efficacy. A commonality in most successful trials is that multiple applications of

B. bassiana have been utilized, which may ensure the presence of adequate inoculum until conditions are adequate for disease development.

In addition to foliar applications of *B. bassiana* against larvae and adults, the amendment of soils with conidia against overwintering adults has also been tested. Overwintering adults are often killed by indigenous *B. bassiana* in soil (e.g. Weber and Ferro, 1993), and applications of the fungus on to foliage or directly on to the soil surface have significantly reduced populations of emerging adults (Watt and LeBrun, 1984; Cantwell *et al.*, 1986; Anderson *et al.*, 1988).

EUROPEAN CORN-BORER. *B. bassiana* applied to maize foliage can effectively control European corn-borers (Hsiu *et al.*, 1973; Lewis and Bing, 1991; Lewis *et al.*, 1996). Recently, it was realized that *B. bassiana* forms an endophytic relationship with maize which has exciting possibilities for the management of insect pests. Initially, Bing and Lewis (1991) observed that most of the maize plants treated at a whorl stage of development with liquid or granular formulations of *B. bassiana* conidia became internally colonized by the fungus. Subsequent studies confirmed that *B. bassiana* entered maize tissues and, once in the plant, the fungus remained there throughout the growing season and provided season-long suppression of European corn-borer larvae (Bing and Lewis, 1991, 1992a, b, 1993; Lewis and Bing, 1991).

The timing of *B. bassiana* application was shown to influence the efficacy of endophytic *B. bassiana*. For example, maize plants inoculated with *B. bassiana* early in the season (e.g. at the whorl and late-whorl stage of development) had significantly more larval tunnelling than plants infested later in the season (i.e. at the pre-tassel stage) relative to their respective control treatments (Bing and Lewis, 1992a). This was attributed to more extensive colonization of plants by *B. bassiana* at later stages of development. Within maize plants, *B. bassiana* is commonly isolated within the pith of the plant during plant growth and senescence (Bing and Lewis, 1992b).

The adhesion, subsequent growth and penetration of maize leaves are very similar morphologically to the germination and penetration of insect cuticles by *B. bassiana* (Wagner and Lewis, 2000). On leaves, conidia germinate regardless of topographical signals, and hyphae from germinating conidia penetrate the cuticle directly; microscopic observations suggest both an enzymatic and a mechanical mechanism of penetration. Once in the plant, hyphae grow within the leaf apoplast and, in some cases, within the xylem elements. The ability to grow within xylem tissues may explain the systemic nature of the fungus within maize plants, which is important for its efficacy against European corn-borers. *B. bassiana* occurs naturally as an endophyte of maize in several tillage systems, and endophytism has been shown to be compatible with a number of chemical and microbial insecticides (e.g. Lewis *et al.*, 1996).

In the USA, substantial attention is now focusing on the use of *B. thuringiensis*-transgenic maize to control European corn-borers, and Lewis *et al.* (2000) observed no difference in rates of endophytism by *B. bassiana* between *B. thuringiensis*-transgenic and near-isogenic lines of maize. Studies to determine the impact of *B. thuringiensis*-transgenic maize and endophytic *B. bassiana* are currently in progress (L.C. Lewis, personal communication). The impact of endophytic *B. bassiana* on other insect pests of maize and whether *B. bassiana* exists as an endophyte in other plants are currently uncertain. Preliminary results from West Africa indicate that local strains of *B. bassiana* can exist endophytically within maize and provide some action against the corn-borer *Sesamia calamistis* (Cherry *et al.*, 1999).

LEPIDOPTEROUS (NOCTUIDAE) PESTS OF SOYBEAN. As indicated previously, *N. rileyi* frequently causes natural epizootics in noctuid populations. Unfortunately, naturally occurring epizootics often occur too late in the growing season to prevent economic crop losses (Ignoffo *et al.*, 1976; Kish and Allen, 1978; Boucias *et al.*, 1984; Fuxa, 1984), and a number of researchers have attempted to induce epizootics earlier in the growing season in an attempt to reduce losses. Most attempts to induce epizootics have involved the application of conidia as sprays or dusts, which have often resulted in high levels of disease (Getzin, 1961; Ignoffo *et al.*, 1976, 1978; Mohamed *et al.*, 1978; Ignoffo, 1981). However, the prolonged lag period between infection and disease development in older larvae allows them to continue feeding, which can result in unacceptable levels of damage (Carruthers and Soper, 1987). In an attempt to enhance crop protection, Ignoffo *et al.* (1976) demonstrated that early-season applications against young larvae could provide acceptable suppression of populations if the environmental conditions were adequate to allow epizootic development. Other researchers have attempted to increase early-season inoculum densities by distributing cadavers throughout the field (Sprenkel and Brooks, 1975), manipulating conditions of moisture through the application of irrigation (Kish and Allen, 1978) and manipulation of cultural practices, such as using early planting dates (Sprenkel *et al.*, 1979). Research has continued to focus on foliar applications of *N. rileyi*, but some evidence indicates that incorporation of conidia into soil may be an efficacious method of targeting some noctuids (Devi, 1995).

WHITEFLIES. The whiteflies *Bemisia argentifolii* and *B. tabaci* are two of the most important agricultural pests, affecting numerous crops worldwide. Damage occurs through direct feeding, vectoring of viruses, honeydew secretion and associated sooty-mould development. *P. fumosoroseus* is one of the most common fungal pathogens found attacking whiteflies and is responsible for epizootics that can significantly affect both greenhouse and field populations of the pests (Lacey *et al.*, 1996). However, natural epizootics are sporadic and cannot be depended upon for management of these pests.

P. fumosoroseus causes rapid infection and death of all whitefly stages; under optimal conditions, hyphae are present within the haemocoele within 24 h of inoculation, death occurs between 24 and 48 h, hyphae emerge and conidiogenesis occurs on the surface of the cadaver within 72 h (Osborne *et al.*, 1990). Optimal growth rates are between 20 and 30°C, with optima related to the microclimate of the fungal isolate's biotope (Vidal *et al.*, 1997a). Highly virulent isolates of *P. fumosoroseus* with considerable control potential against whiteflies are widespread and numerous (Lacey *et al.*, 1996; Vidal *et al.*, 1997b; Wraight *et al.*, 1998). Wraight *et al.* (2000) demonstrated that infection can take place at ambient relative humidities as low as 25%. Hyphal bodies are more virulent than conidia (Lacey *et al.*, 1999b) and can be rapidly produced in liquid culture, remaining viable and virulent following drying (Jackson *et al.*, 1997). *P. fumosoroseus* demonstrated limited lethal and sub-lethal effects on *S. parcesetosum*, an important coccinelid predator of whiteflies, suggesting that the integration of these two control agents in IPM may be possible (Poprawski *et al.*, 1998).

Inundative application of *P. fumosoroseus* conidia or hyphal bodies has been used successfully to manage whiteflies in field crops. In small-scale field trials using portable air-assist sprayers, multiple applications of *P. fumosoroseus* at 4–7-day intervals provided > 90% mortality of late-instar whiteflies on cucumber, cantaloupe melons and zucchini

squash (Wraight *et al.*, 2000). Although effects on nymphs were highly significant, the effects on adult whiteflies were minimal. Commercial products based on this fungus are now available for whitefly control (Shah and Goettel, 1999; Wraight *et al.*, 2000).

Although only occasionally found as a naturally occurring pathogen of whiteflies (Lacey *et al.*, 1996), *B. bassiana* has demonstrated comparable efficacy to *P. fumosoroseus* against whiteflies, and several isolates have been commercially developed for their control (Shah and Goettel, 1999; Wraight *et al.*, 2000). Mycotech Corporation, Butte, Montana, has developed an emulsifiable oil formulation, which can be readily targeted and applied with low-volume air-assist sprayers or moderate- to high-volume hydraulic sprayers (Wraight and Carruthers, 1999). Because whiteflies primarily inhabit the undersides of leaves, a special effort has to be made to adequately target this area. This can be accomplished by spraying upward from below the canopy level, using nozzles mounted on swivels on vertical tubes. For crops with low canopies (e.g. cucurbits), high-pressure hydraulic sprayers, fitted with drop nozzles carried at or slightly above canopy levels are effective. Application of a wettable powder formulation of *B. bassiana* (Mycotrol) at 560 g ha^{-1} at 2–4-weekly applications in cucumbers and five to seven applications in cantaloupe melons consistently provided 65–75% control of first-generation whitefly larvae (Wraight *et al.*, 2000).

Pasture and grassland

COMMON COCKCHAFER. Adults of the common or European cockchafer (*Melolontha melolontha*) feed on forest and fruit-tree leaves, in particular oaks, beech, maple, sweet chestnut, walnut and plum. Larvae are polyphagous, and they attack the roots of various crops including fruit or forest trees, cereals, red beet, potato and grasses. A host-specific strain of *B. brongniartii* has been tested in large-scale field tests in grassland regions in Austria, Italy and Switzerland over the last two decades. A number of formulations have been tested (Keller *et al.*, 1997; Strasser, 1999). In an attempt to use egg-laying females as vectors of the fungus, hyphal bodies were applied to woodland borders in 1985 and 1988 and, at two-thirds of the 15 sites, 50–80% reductions in cockchafer populations were observed (Keller *et al.*, 1997). From these trials, the authors concluded that spraying the adults with hyphal bodies to control cockchafer larvae has both advantages and disadvantages. The great advantage is that only a small area must be treated because the egg-laying females disseminate the fungus to an area 50 times larger than the treated one. However, the quantity of hyphal bodies needed can only be produced in large fermenters on a commercial scale, and they cannot be stored for more than 4 weeks without loss of efficacy (Keller *et al.*, 1997).

Cockchafer flight depends strongly on weather conditions and the spraying of the adults can only be done during a short period (i.e. when females reach the swarming sites). Therefore, efficacious application often necessitates the use of helicopters, which is less cost-effective. To overcome the disadvantages of applying hyphal bodies, an alternative method of applying *B. brongniartii* in and/or on barley kernels was developed (Aregger, 1992; Zelger, 1993; Strasser, 1999). Based on the results of field experiments by Fornallaz (1992), colonized barley was applied with a slit seeder at various times of the year to pastures, grasslands, sports fields, orchards, forests, tree nurseries and vineyards. The incorporation of inoculum into soil to a depth of 5–10 cm was found to increase efficacy by placing the fungus in close contact with the pest. A threshold of inoculum in soil is required to ensure epidemic levels in the pastures ($> 2 \times 10^4$

spores g^{-1} dry weight of soil (Ferron, 1979), and the results of field trials conducted between 1995 and 1999 with the barley inoculum indicated that population sizes of *B. brongniartii* increased continuously through five applications conducted in autumn 1994 and spring and autumn of 1995 until 1997 (Strasser, 1999). With the exception of the site with sandy-textured soil, fungal populations within soils persisted at above threshold levels. The application of *B. brongniartti* using barley kernels provided sufficient suppression of cockchafer populations after only 2 years of application (> 20% prevalence of mycosis), and populations were reduced from > 70 larvae m^{-2} to less than 22 larvae m^{-2} after 5 years. Since the spring of 1998, no relevant damage by *M. melolontha* has been reported at treated sites (Strasser, 1999).

RED-HEADED PASTURE COCKCHAFER. The subterranean scarab, *Adoryphorus couloni*, is an important root-feeding pest of pasture and field crops, and the application of *M. anisopliae* to pasture soils has shown considerable potential for the control of cockchafers in Australia. Conidia can be applied directly to soil or the fungus can be applied on/in grains by direct drilling at the time of pasture renovation. Application of *M. anisopliae* to pasture soil in the winter caused substantial mortality of larvae and pupae by 27 weeks, and decline in populations continued in subsequent years (Rath *et al.*, 1995). Reductions in larval numbers led to greater retention of pasture grasses, reduced weed invasion and an increase in pasture productivity. Throughout the 4-year duration of the study, *M. anisopliae* remained at levels equal to or above the applied concentrations. While autumn applications may not be sufficient to control pest populations initially, the excellent persistence of *M. anisopliae* in soil and effects on subsequent populations demonstrate the potential for long-term management of cockchafers in pastures. In some instances, it is not necessary to re-inoculate soil for a 5–10-year period. A commercial product of *M. anisopliae* (i.e. BioGreen®) is currently being marketed for use against red-headed pasture cockchafers in Australia (Shah and Goettel, 1999).

GRASSHOPPERS AND LOCUSTS. Acridids have a tremendous impact on agricultural production throughout the world. They are capable of decimating cultivated crops, pastures and rangeland, and historically have been indirectly responsible for death by starvation or death by diseases associated with starvation of untold thousands of people annually. *M. anisopliae* var. *acridum* is being developed as a BCA of acridids in various regions of the world, including Africa (e.g. Lomer *et al.*, 1997a), Australia (e.g. Milner, 1997) and Brazil (e.g. Magalhaes *et al.*, 2000). In a large research programme conducted in Africa, conidia are produced on rice (Jenkins *et al.*, 1998) and they are dried and stored for various periods of time at low (>18 months) and/or ambient (~12 months) temperatures (Lomer *et al.*, 1997a). Conidia are formulated in oils (primarily paraffinic oils used alone or as blends with botanical oils) and applied at ultra-low volumes, using hand-held Micro-Ulva applicators.

Trials conducted at a variety of locations in Africa have demonstrated satisfactory suppression of field populations of grasshoppers (e.g. *Z. variegatus, Hieroglyphus daganensis* and *O. senegalensis*) by *M. anisopliae* var. *acridum* in a number of instances (Lomer *et al.*, 1997a); in some cases, reductions in field populations of ≥ 70% have been observed (e.g. Kooyman *et al.*, 1997; Lomer *et al.*, 1997a,b). Field testing of the fungus against locusts has been problematic, primarily because of the high mobility of locust adults and nymphs. Efficacy has been demonstrated in captured locusts that were maintained in cages following the application of conidia (Lomer *et al.*, 1997a).

However, efficacy in cages does not necessarily correlate with reductions in field populations of acridids, due to the substantially different microclimatic conditions between the two environments (Inglis *et al.*, 1997a), and extreme care must be taken in extrapolating efficacy from cage assessments. The efficacy of *M. anisopliae* var. *acridum* is probably enhanced by secondary spore pick-up; there are observations that acridids coming in contact with vegetation infested with conidia pick up inoculum and often become infected and die by mycosis (Bateman *et al.*, 1998; Thomas *et al.*, 1998). Furthermore, the fungus can persist in fragments of infected grasshopper cadavers and survive adverse environmental conditions (e.g. the Sahelian dry season), which potentially serves as a source of secondary inoculum (i.e. horizontal transmission), enhancing the efficacy of *M. anisopliae* var. *acridum* against acridids (Thomas *et al.*, 1996). However, the biotic and abiotic factors regulating horizontal transmission in field settings are still poorly understood.

Tree crops

The coconut-palm rhinoceros beetle, *Oryctes rhinoceros*, is a major pest of Asian- and Pacific-grown coconut- and oil-palms. The adults feed on palm fronds, boring into the axils and destroying plant tissues, and the immature stages are found in soil and litter (e.g. Bedford, 1980). Larvae are naturally infected by *M. anisopliae*, and the fungus is considered an important natural mortality factor (Carruthers and Soper, 1987). A control programme, involving the release of *M. anisopliae* and a baculovirus (*Rhabdionvirus oryctes*), was initiated in Tonga to manage rhinoceros beetles. The virus was found to induce epizootics, but a low prevalence of infections caused by *M. anisopliae* was observed 15 months after release (Young, 1974). Two years after release, *M. anisopliae* was barely detectible in natural breeding populations. The beetles are highly susceptible to *M. anisopliae*, and reasons for the poor efficacy in the field are speculative. Anecdotal evidence suggests that limited dispersal and longevity of inoculum are the most likely reasons for the poor efficacy observed (Latch and Falloon, 1976; Carruthers and Soper, 1987). More recent research using *M. anisopliae* has focused on the inundative application of conidia against the beetles, and the use of the fungus in IPM programmes with the baculovirus (Young, 1986).

Forests

While a number of entomopathogenic Hyphomycetes have demonstrated considerable potential as BCAs against a diverse array of insect pests of forest trees in controlled environments or small-scale field tests, there are relatively few examples of large-scale applications of entomopathogenic Hyphomycetes against forest pests. However, a substantial effort in China has gone into the widespread application of entomopathogenic Hyphomycetes against forest insect pests. For example, the annual production of *B. bassiana* conidia in China in the late 1980s was ~10,000 t which allowed the treatment of 0.8–1.3 million ha (Feng *et al.*, 1994). A major forest pest in China is the pine caterpillar (*Dendrolimus* spp.), and large-scale applications of *B. bassiana* in oil or oil–emulsion formulations have provided excellent suppression of caterpillars; more than 300,000 ha of forest were spayed by aeroplane over a 5-year period and mortalities ranging from 43 to 93% were recorded during this period (Pan and Zheng, 1988). In some instances, repeated applications of *B. bassiana* in a given year resulted in better control (Lü and Zhao, 1988). The cost of managing pine caterpillars with *B. bassiana*

was low (≤ US$3 ha^{-1}) regardless of the application method, and has been shown to be a cost-effective management practice (Feng *et al.*, 1994).

Urban pests

TERMITES. Termites cause considerable damage to wood structures throughout the world. A number of entomopathogenic Hyphomycetes, including *M. anisopliae* and *B. bassiana*, have demonstrated considerable potential for controlling termites. These entomopathogens are commonly isolated from termite colonies (e.g. Milner *et al.*, 1998a). In Australia, the application of *M. anisopliae* conidia to mound- and tree-nesting termites has been shown to incite substantial mortality, but indirect treatment using feeding sites and baits was not successful (Milner and Staples, 1996; Milner *et al.*, 1998b). In Brazil, a high prevalence of termite mortality (~100%) was observed in 19 of 20 nests treated with *M. anisopliae*, and the remaining nest exhibited 70% mortality (Alves *et al.*, 1995).

The efficacy of entomopathogenic Hyphomycetes against termites is facilitated by the horizontal transmission of conidia between individuals (Kramm *et al.*, 1982; Rosengaus and Traniello, 1997), and one of the major factors limiting the efficacy involves the behavioural response of healthy termites to conidia and diseased termites. The frequency of grooming increases during and after exposure to *M. anisopliae* conidia, and this increases the survival of termites (Rosengaus *et al.*, 1998a). Termites in direct contact with high concentrations of conidia can also exhibit a striking vibratory display, which appears to convey information to unexposed nest-mates, which are stimulated to stay clear of the infested individual (Rosengaus *et al.*, 1999).

Microorganisms associated with soil, the termite gut and gallery systems can be inhibitory to the fungus (Boucias *et al.*, 1996; Rosengaus *et al.*, 1998b; Ramakrishnan *et al.*, 1999). While the repellence of viable conidia to termites may represent an obstacle in the development of effective bait formulations, this characteristic has been used to provide a barrier treatment in soil (Milner and Staples, 1996). Evidence now indicates that it may be possible to overcome the repellency of conidia, and Rath and Tidbury (1996) observed that *M. anisopliae* conidia formulated in attapulgite clay and surfactant were attractive to termites. Almeida *et al.* (1998) reported that the formulation of *B. bassiana* conidia in a bait with insecticides provided good suppression of termites in sugar cane in Brazil. Since infection of termites primarily occurs through the external integument, the efficacy of bait formulations will be dependent on the degree to which individuals become surface-contaminated during handling and ingestion of the bait. Therefore, development of bait formulations should focus on baits that promote tactile contact by termites, thereby enhancing transfer of conidia to the integument surface.

As indicated earlier, sublethal doses of chemicals can adversely affect the behaviour of termites. Most of the work on behavioural modifiers has focused on the insecticide imidacloprid. Imidacloprid is a systemic, chloronicotinyl insecticide that causes a blockage in a type of neuronal pathway (nicotinergic), which leads to the accumulation of acetylcholine, resulting in insect paralysis and, at high enough doses, death (Kidd and James, 1991). A number of researchers have observed that combining entomopathogenic Hyphomycetes with sublethal doses of imidacloprid substantially increases efficacy of fungi against termites (Boucias *et al.*, 1996; Almeida *et al.*, 1998; Ramakrishnan *et al.*, 1999). This is attributed to the interference with normal tunnelling and termite grooming and associated trophallaxis activities, factors that play a

major role in conferring resistance to entomopathogenic Hyphomycetes. At present, a commercial formulation of *M. anisopliae* (i.e. BioBlast®) has demonstrated substantial control of termites on pine boards in the USA, but was more effective when used with a chemical barrier (Quarles, 1999).

COCKROACHES. The Bio-Path® Cockroach Control Chamber is the first microbial agent registered for control of cockroaches in the USA. Designed like a traditional bait station, cockroaches entering the station become infested with conidia of *M. anisopliae*. In the Bio-Path system, *M. anisopliae* is present on an agar medium, and the cockroaches entering the bait station come in contact with conidia. Horizontal transmission is important for the efficacy of entomopathogenic Hyphomycetes against cockroaches (Kaakeh *et al.*, 1996). Once cockroaches have left the station they may infest other individuals with inoculum from the trap, or they become infected and eventually die, and conidia subsequently sporulating on the cadaver serve as a source of secondary inoculum (Fehrenbach, 1993; Andis, 1994; Milner, 1994). The fungus has been marketed as being safe for use in hotels, restaurants and museums. Additional research has focused on the incorporation of entomopathogenic Hyphomycetes into bait formulations in which cockroaches become surface-infested during ingestion of the bait substrate (e.g. Mohan *et al.*, 1999). Dead infected cockroach nymphs are not cannibalized, suggesting an avoidance behaviour by healthy nymphs (Kaakeh *et al.*, 1996). The incorporation of imidacloprid into baits has been shown to increase the efficacy of *M. anisopliae* against cockroaches (Kaakeh *et al.*, 1997), but the mechanism of action is uncertain at present.

Compatibility with agrochemicals

Entomopathogenic Hyphomycetes will not supplant the need for chemical pesticides in all commercial production systems. For example, insecticides may be needed to suppress a rapidly expanding pest population or to control pests not targeted by fungi. Fungicides will often be required to control plant diseases, but many fungicides possess broad spectra of activity and they can adversely affect the efficacy of entomopathogenic fungi. Herbicides and plant growth regulators are also used extensively in most agroecosystems, and their compatibility with fungi is often uncertain. While it is not possible to draw specific conclusions on the compatibility of all agrochemicals with entomopathogenic Hyphomycetes, it is crucial that the pest manager be cognizant of the adverse impacts that these chemicals can have on entomopathogen efficacy. Furthermore, it is often necessary to obtain information on the compatibility of specific fungal strains with the agrochemicals used in a particular agroecosystem. This will allow growers to select appropriate compounds and schedule fungal or chemical treatments accordingly to minimize any deleterious effects on pathogen efficacy (i.e. compartmentalization).

Considerable research has focused on the influence of various agrochemicals on the germination and growth of entomopathogenic fungi *in vitro*. Here we attempt to summarize the salient findings of this research. All classes of agrochemicals are potentially inhibitory to entomopathogenic Hyphomycetes, including herbicides, insecticides and fungicides. There is a tremendous array of agrochemicals currently in use throughout the world, and their inhibitory properties will vary, both between and within chemical classes. In some instances, carriers and/or adjuvants associated with the active

ingredient are responsible for the fungistatic properties of a chemical pesticide, and the compositions of the formulations also vary tremendously.

The inhibitory effects of agrochemicals on the germination and growth of entomopathogenic Hyphomycetes often vary among taxa and strains (e.g. Vanninen and Hokkanen, 1988; Anderson *et al.*, 1989; Majchrocwicz and Poprawski, 1993; Li and Holdom, 1994). Therefore, it may be possible to select genotypes which are naturally less susceptible. Another approach is to 'genetically engineer' resistance to pesticides. In this strategy, a gene conferring resistance to a specific pesticide is introduced into a susceptible entomopathogen. For example, *M. anisopliae* was transformed with a gene conferring resistance to the fungicide benomyl (Benlate®) (Goettel *et al.*, 1990a), and *P. fumosoroseus* was transformed with a gene conferring resistance to the herbicide gluphosinate (Cantone and Vandenberg, 1999). However, pesticides that are inhibitory in the laboratory do not always exhibit the same action in field environments. This can be a function of pesticide concentration or due to compartmentalization (i.e. the entomopathogen may never come in contact with the pesticide). For example, Keller *et al.* (1993) observed that one-third of the fungicides tested *in vitro* completely inhibited mycelial growth of *B. brongniartii* and the others either prevented or reduced conidial germination. Despite the highly inhibitory nature of the fungicides *in vitro*, the growth of the fungus on barley kernels on soils sprayed with fungicides was substantially less inhibited, and the authors concluded that most of the fungicides tested would not have an adverse effect on *B. brongniartii* applied to soils. In another study, Jaros-Su *et al.* (1999) demonstrated that the timing of fungicide application was not detrimental to *B. bassiana* applied against the Colorado potato beetle if applications were made asynchronously. These examples emphasize the potential inaccuracy of extrapolating field inhibition from *in vitro* results. There are several examples where fungicides that are highly inhibitory *in vitro* are successfully used in conjunction with entomopathogenic fungi to manage insect pests. For instance, in chrysanthemum and cucumber production, *V. lecanii* and benomyl are commonly used together (Gardner *et al.*, 1984; van der Schaaf *et al.*, 1991).

While the potential inhibitory effects of pesticides on entomopathogenic Hyphomycetes cannot be ignored, there are numerous examples where the application of chemical pesticides have enhanced the efficacy of entomopathogens against insect pests. As indicated previously, sublethal doses of chemical insecticides can act as physiological stressors and/or behavioural modifiers and thereby predispose insects to disease. There are now a number of examples where the application of entomopathogenic Hyphomycetes with sublethal doses of insecticides has substantially enhanced the efficacy of pathogens. An early example of this phenomenon was reported by Easwaramoorthy *et al.* (1978), where two insecticides that inhibited *V. lecanii in vitro* enhanced the efficacy of the fungus when they were applied in combination against the coffee green scale. Anderson *et al.* (1989) detected higher insect mortality when *B. bassiana* and sublethal concentrations of insecticides were applied to control Colorado potato beetle, attributing the higher rates to synergism between the two agents. A recent example of enhanced efficacy for combination treatments is for *B. bassiana* and *M. anisopliae* applied with imidacloprid against the sugar-cane rootstock borer weevil (*Diaprepes abbreviatus*) (Quintela and McCoy, 1998). As indicated previously, imidacloprid is a neural toxin that can substantially affect the behaviour of various insects, such as termites (Boucias *et al.*, 1996). The enhanced efficacy of entomopathogenic Hyphomycetes applied in combination with imidacloprid against the sugar-cane rootstock borer weevils was attributed to reduced mechanical removal of

conidia from the surfaces of larval cuticles; imidacloprid severely impaired larval movement in soil (Quintela and McCoy, 1998). However, in contrast, only additive effects were obtained when *B. bassiana* was applied with diflubenzuron against acridids in Mali (Delgado *et al.*, 1999). Diflubenzuron, a member of a larger group of highly selective insecticides known as benzoylphenylureas, works by inhibiting chitin synthesis, and the cuticle of insects treated with diflubenzuron provides less resistance to penetration by hyphae of entomopathogenic Hyphomycetes (Hassan and Charnley, 1989). This would suggest that increased efficacy of an entomopathogenic fungus would occur due to enhanced penetration, but, if this occurred, it did not translate into increased mortality in the trial against field populations of acridids in Mali.

The beneficial effects of insecticides may also potentially expand the pest host range of fungal agents. For example, *P. fumosoroseus* was not effective against greenhouse infestations of the aphids *Aphis gossypii* and *Macrosiphoniella sanborni*, but when applied with azadirachtin (Margosan-O®) efficacy was enhanced and good control of these aphids was attained, thereby increasing the cost-effectiveness of the chemical control strategy (Lindquist, 1993). If greater efficiency can be obtained, fewer applications will be required to achieve the desired degree of pest control. Many researchers are now taking a mechanistic approach to such research, which will facilitate the efficacious use of entomopathogen combinations.

As modern agriculture moves to adopt more 'environmentally friendly' practices, there is increasing interest in the use of 'biorational' pesticides, such as insect growth regulators, insecticidal soaps and horticultural oils; these materials have minimal effects on beneficial insects, low mammalian toxicity and reduced residual activity. Some of these products are effective against insect pests, and they have excellent potential for inclusion in IPM strategies (Allen *et al.*, 1993). However, there are few documented studies on interactions between biorational pesticides and entomopathogenic fungi. As the mode of action of most biorational pesticides relies on disruption of an insect-specific process, they should be relatively innocuous to fungi. While the specific mechanisms of activity differ among the products, they all affect the insect cuticle – its formation, deposition, structure or integrity – to varying degrees. Insect growth regulators and botanicals, such as neem (azadirachtin) directly interfere with insect development and cuticle formation or the moulting process (Staal, 1987; Rembold, 1989). If deposition, hardening and tanning of the new cuticle are affected, its ability to serve as an effective barrier to fungal infection is compromised and the chances of a lethal mycosis developing may be enhanced (Zimmermann, 1994). Synergism between *Metarhizium* spp. and benzoylphenylurea insecticides (e.g. diflubenzuron and teflubenzuron), which interfere with chitin synthesis, have been demonstrated for a number of insects (Hassan and Charnley, 1983; Joshi *et al.*, 1992). Alternatively, if the mode of action of the biorational pesticide can induce morphogenetic damage, preventing moulting and maturation, then successful fungal invasion is more likely to occur. Insecticidal soaps affect the waxy layers of the insect cuticle. These layers prevent desiccation and are important in the insect defence system; their disruption could predispose insects to fungal infection. Horticultural oils kill insects by asphyxiation, and insecticidal oils have been used in the formulation of several fungal species, enhancing efficacy and application against the target host insect (see Chapters 10 and 11).

Compatibility with other biological control agents

The contribution of predators, parasitoids and pathogens is an important aspect of IPM. Consequently, the use of entomopathogenic Hyphomycetes in any IPM programme must take into consideration their possible deleterious effects on other components, especially other biological agents. On the other hand, many Hyphomycetes may act additively or synergistically with other biological agents, especially other entomopathogens.

Predators and parasitoids

Safety to non-target organisms and more specifically to predators and parasites is of primary concern when registering a microbial control agent (see Chapter 13). While many entomopathogenic Hyphomycetes have wide physiological host ranges, their pathogenicity and virulence vary by species and strain and by insect host (Goettel *et al.*, 1990b; see also Chapter 13). Entomopathogenic Hyphomycetes have been shown to be compatible with a number of beneficial insects (Bethke and Parrella, 1989; Goettel *et al.*, 1990b; James and Lighthart, 1994). Although laboratory tests are often a prerequisite in first establishing the susceptibility of non-targets to candidate fungal strains, there are often poor correlations between these and field test data (see Chapter 13). In laboratory tests, higher doses of inoculum are generally used against stressed hosts, which are held under conditions favouring infection and disease development. Under field conditions, the likelihood of high infection rates occurring in non-targets is greatly reduced. Even if some agents are incompatible with certain predators or parasites, consideration must be given to the usually devastating effects to most predators and parasites when chemical pesticides are used instead.

Entomopathogens

Surprisingly little attention has focused on the possibility of co-applying entomopathogens, such as viruses, protozoa and some bacteria, with Hyphomycetes fungi to enhance efficacy. The high infectivity and often debilitating effects on insects and the substantially different modes of action relative to fungi, which may reduce interspecies antagonism, would indicate that there is great potential in this approach. There are only a few reports where entomopathogenic Hyphomycetes have been combined with either viruses or protozoa (e.g. Lecuona and Alves, 1988). A number of researchers have tested combinations of bacteria and entomopathogenic Hyphomycetes. In some instances, increased efficacy was observed. For example, Glare (1994) observed that the co-application of *M. anisopliae* and *Serratia entomophila* provided synergistic mortality of early- but not later-instar grass grub (*Costelytra zealandica*) larvae. Lewis *et al.* (1996) observed that the application of *B. thuringiensis* to whorl-stage and pollen-shedding maize enhanced the suppression of European corn-borers by endophytic *B. bassiana*. In other instances, there was no apparent advantage of the co-treatment (e.g. Lewis and Bing, 1991). Understanding how the two pathogens interact during pathogenesis may facilitate efficacious use of pathogen combinations.

Nematodes are increasingly being considered for control of a range of insects (e.g. Tomalek, 1994; Ehlers, 1998). The work of Barbercheck and Kaya (1990, 1991) indicated that co-infections by nematodes and *B. bassiana* could speed up the lethal infection rate and cause higher mortality in a treated pest population. Antagonistic

interactions between *B. bassiana* and the nematodes in dual-infected hosts, however, could adversely affect pathogen or progeny development. This could affect the spread of the fungus or the nematode within the pest population, but, if inundative releases of both organisms were contemplated in a control strategy, this would not necessarily prevent their combined use.

Very few studies have reported the combined application of entomopathogenic Hyphomycetes with the aim of increasing efficacy. As indicated previously, the detrimental effects of high temperatures, exacerbated by the ability of acridids to elevate their body temperatures, is now recognized as an important constraint on the use of *B. bassiana* against grasshoppers. Substantial fluctuations in diurnal temperatures occur in the northern plains of North America, and Inglis *et al.* (1997b, 1999) tested the hypothesis that the co-application of *B. bassiana* and *M. anisopliae* var. *acridum* could be used to increase the temperature range over which the individual fungi alone would incite mortality in acridids. In controlled environment studies, they observed that the final mortality was greater for the combination treatment than for *M. anisopliae* var. *acridum* alone in a simulated hot-temperature environment, and equal to *B. bassiana* alone in a simulated cool-temperature environment. While these were relatively simple simulations, they demonstrate the potential value of utilizing fungal 'cocktails' to overcome the constraints of temperature on entomopathogenic hyphomycetous fungi against acridids.

Some innovative strategies

Development of IPM often requires innovative strategies. Hyphomycetous fungi offer unique opportunities and strategies for use in IPM programmes. For example, recent studies by Butt *et al.* (1998b) showed that honey-bees could be used to disseminate fungal inoculum and the improved targeting resulted in better control of pollen beetles (*Meligethes aeneus*) in oil-seed rape (= canola) than conventional spraying methods. This method has also been shown to control seed weevil (*Ceutorhynchus assimilis*) and has the potential to control most floral pests, including thrips (T.M. Butt, unpublished observations). Many entomopathogenic fungi fail to kill bees, partly because of the elevated temperatures in the beehive (Butt *et al.*, 1994). The 'push–pull' strategy is another novel pest control method, which, briefly, entails insect pests being driven out of the main crop with feeding deterrents and drawn into a trap crop, where they could be controlled by inundation with benign pathogens, such as *M. anisopliae*. To encourage pests into the trap crop, lures such as favoured plant varieties (i.e. those more attractive than the crop) and chemical attractants may be used.

Semiochemicals

When evaluating *V. lecanii* for control of *A. gossypii*, Hockland *et al.* (1986) observed an increased infection rate among aphids in plots sprayed with the aphid alarm pheromone, (E)-β-farnesene. In response to the pheromone, the aphids became agitated and increased their movement over the treated leaf surface, and in so doing, acquired more infective conidia, resulting in higher rates of infection. Could a similar approach be considered for other insect pests? Teerling *et al.* (1993) identified an alarm pheromone in droplets of anal fluid produced by western flower thrips. Both nymphs and adults responded to the pheromone by walking away from the source of

application. The thrips are highly thigmotactic and often abundant inside flowers, where they are difficult to reach with contact insecticides, including fungi. If the alarm pheromone could stimulate insects to vacate these refugia and increase their exposure to conidia deposited on leaves or petals, better control might be achieved.

Host-plant resistance

As discussed earlier, the host plant can influence the susceptibility of insects to fungal infection. In addition to considerations of resistance to insect pests and phytopathogens, it may also be beneficial to consider the efficacy of entomopathogens in selecting plant germ-plasm. Insects tend to be more stressed on resistant plants, and perhaps more prone to infection. Furthermore, if the developmental rate is affected, then the intermoult period is extended, providing a longer period of susceptibility to infection. Differences in insect susceptibility have been observed, depending on the type of host plant on which insects have been reared (Hare and Andreadis, 1983; Ramoska and Todd, 1985). While traits conferring resistance to insects have been bred into many agricultural crops, most plant breeding programmes, particularly in ornamentals, have focused on the development of resistance to phytopathogens. Although this in itself may not directly affect insect pests, the use of disease-resistant cultivars will decrease or eliminate the application of fungicides, etc., thereby reducing harmful effects on entomopathogenic fungi used against insect pests. Furthermore, the development of resistance to plant viruses transmitted by insect vectors (e.g. thrips, whitefly and aphids) would reduce the need to maintain plants virtually insect-free, allowing for greater flexibility in the type of control tactics that can be incorporated into an insect pest control programme.

Trap crops

Trap crops are crops that are grown to attract insects pests and thereby protect primary crops from pest attack. Once the insects are attracted to the trap crop, they are usually managed there, thus decreasing or eliminating the need to target the entire field. While trap crops that are preferred hosts for the insect pest are commonly used, a number of other strategies may be used, such as the 'push–pull method'. Limited work has focused on the use of entomopathogens to manage insect pests in trap crops, but this may represent an excellent strategy for deploying entomopathogenic Hyphomycetes. Since mortality is partly dependent upon the application rate of the fungus and host density, this strategy may significantly improve the effectiveness of fungal BCAs on pest populations. It may also be possible to modify environmental conditions within the trap crops to favour infection and disease development within pest insects. For example, Amiri *et al.* (1999) showed that forcing adults and larvae of *P. cochleariae* to the underside of Chinese cabbage leaves with a feeding deterrent increased their susceptibility to *M. anisopliae*. The enhanced efficacy may have resulted from higher humidity, which can be important in the infection process and on sporulation on cadavers, and increased conidial persistence, because they are less exposed to harmful UV radiation and are less likely to be dislodged by rain (Inglis *et al.*, 1993; Inyang *et al.*, 2000).

Conclusions

Fungi will not be cure-alls for pest problems on all crops and in all agricultural settings, and it is unlikely that they will ever totally supplant the management of insect pests with chemical insecticides. Nevertheless, they represent a valuable management resource to be utilized within an IPM framework, and will contribute significantly to reductions in chemical pesticide use (Lacey and Goettel, 1995). Research to identify constraints on efficacy and the utilization of biologically based strategies to overcome these constraints will continue to increase the consistency of insect suppression with entomopathogenic Hyphomycetes. Furthermore, the integration of this group of fungi with other management practices will require detailed compatibility studies and the development of effective guidelines for their use. For example, in a typical agroecosystem, a range of pest species, including phytopathogens and insects, are encountered and have to be combated simultaneously. Thus the role of mycoinsecticides in pest control must be viewed within the context of a total crop management programme. Such integration will be more complex than the use of traditional chemical control tactics, and will rely upon efficient monitoring to optimize the levels of control obtained from each application. Recent developments portend well for the future use of entomopathogenic Hyphomycetes in pest management programmes.

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References

Allen, W.R., Tehrani, B. and Luft, R. (1993) Effect of horticultural oil, insecticidal soap, and film-forming products on the western flower thrips and the tomato spotted wilt virus. *Plant Disease* 77, 915–918.

Almeida, J.E.M., Alves, S.B., Moino, A. and Lopes, R.B. (1998) Control of the termite *Heterotermes tenuis* (Hagen) using termitrap baits with insecticides associated with the entomopathogenic fungus *Beauveria bassiana* (Bals.) Vuill. *Anais da Sociedade Entomologica do Brasil* 27, 639–644.

Alves, S.B., Almeida, J.E.M., Moino, A., Stimac, J.L. and Pereira, R.M. (1995) Use of *Metarhizium anisopliae* and *Beauveria bassiana* for control of *Cornitermes cumulans* (Kollar, 1832) in pastures. *Ecossistema* 20, 50–57.

Amiri, B., Ibrahim, L. and Butt, T.M. (1999) Antifeedent properties of destruxins and their potential use with the entomogenous fungus *Metarhizium anisopliae* for improved control of crucifer pests. *Biocontrol Science and Technology* 9, 487–498.

Anderson, T.E., Roberts, D.W. and Soper, R.S. (1988) Use of *Beauveria bassiana* for suppression of Colorado potato beetle populations in New York State (Coleoptera: Chrysomelidae). *Environmental Entomology* 17, 140–145.

Anderson, T.E., Hajek, A.E., Roberts, D.W., Preisler, H.K. and Robertson, J.L. (1989) Colorado potato beetle (Coleoptera: Chrysomelidae): effects of combinations of *Beauveria bassiana* with insecticides. *Journal of Economic Entomology* 82, 83–89.

Andis, M. (1994) The Bio-Path Cockroach Control Chamber uses nature to control nature's pests. *Pest Control* 62, 44.

Aregger, E. (1992) Conidia production of the fungus *Beauveria brongniartii* on barley and quality evaluation during storage at 2°C. *Journal of Invertebrate Pathology* 59, 2–10.

Askary, H., Carriere, Y., Belanger, R.R. and Brodeur, J. (1998) Pathogenicity of the fungus *Verticillium lecanii* to aphids and powdery mildew. *Biocontrol Science and Technology* 8, 23–32.

Authurs, S.P. and Thomas, M.B. (1999) Factors affecting horizontal transmission of entomopathogenic fungi in locusts and grasshoppers. In: Thomas, M.B. and Kedwards, T. (eds) *Challenges in Applied Population Biology. Aspects of Applied Biology* 53, 89–97.

Barbercheck, M.E. and Kaya, H.K. (1990) Interactions between *Beauveria bassiana* and the entomogenous nematodes, *Steinernema feltiae* and *Heterorhabditis heliothidis. Journal of Invertebrate Pathology* 55, 225–234.

Barbercheck, M.E. and Kaya, H.K. (1991) Competitive interaction between entomopathogenic nematodes and *Beauveria bassiana* (Deuteromycotina: Hyphomycetes) in soilborne larvae of *Spodoptera exigua* (Lepidoptera: Noctuidae). *Environmental Entomology* 20, 707–712.

Barron, G.L. (1968) *The Genera of Hyphomycetes from Soil.* Robert E. Krieger Publishing Company, Malabar, Florida.

Bateman, R.P., Carey, M., Moore, D. and Prior, C. (1993) The enhanced infectivity of *Metarhizium flavoviride* in oil formulations to desert locusts at low humidities. *Annals of Applied Biology* 122, 145–152.

Bateman, R.P., Douro-Kpindou, O.K., Kooyman, C., Lomer, C. and Ouambama, Z. (1998) Some observations on the dose transfer of mycoinsecticide sprays to desert locusts. *Crop Protection* 17, 151–158.

Bedford, G.O. (1980) Biology, ecology and control of palm rhinoceros beetles. *Annual Review of Entomology* 25, 309–339.

Bernhard, K., Holloway, P.J. and Burges, H.D. (1998) Appendix I: A catalogue of formulation additives: function, nomenclature, properties and suppliers. In: Burges, H.D. (ed.) *Formulation of Microbial Biopesticides*, Kluwer Academic Publishers, Dordrecht, the Netherlands, pp. 333–365.

Bethke, J.A. and Parrella, M.P. (1989) Compatibility of the aphid fungus *Cephalosporium lecanii* with the leafminer parasite, *Diglyphus beninii* (Hymenoptera: Eulophidae). *Pan Pacific Entomologist* 65, 385–396.

Bidochka, M.J., St Leger, R.J. and Roberts, D.W. (1997) Mechanisms of deuteromycete fungal infections in grasshoppers and locusts: an overview. In: Goettel, M.S. and Johnson, D.L. (eds) *Microbial Control of Grasshoppers and Locusts. Memoirs of the Entomological Society of Canada*, 171, 213–224.

Bing, L.A. and Lewis, L.C. (1991) Suppression of the European corn borer, *Ostrinia nubilalis* (Hübner) (Lepidoptera: Pyralidae), by endophytic *Beauveria bassiana* (Balsamo) Vuillemin. *Environmental Entomology* 20, 1207–1211.

Bing, L.A. and Lewis, L.C. (1992a) Temporal relationships between *Zea mays, O. nubilalis* (Lepidoptera: Pyralidae) and endophytic *Beauveria bassiana. Entomophaga* 37, 525–536.

Bing, L.A. and Lewis, L.C. (1992b) Endophytic *Beauveria bassiana* (Balsamo) Vuillemin in corn: the influence of the plant growth stage and *Ostrinia nubilalis* (Hübner). *Biocontrol Science and Technology* 2, 39–47.

Bing, L.A. and Lewis, L.C. (1993) Occurrence of the entomopathogen, *Beauveria bassiana* (Balsamo) Vuillemin in different tillage regimens and in *Zea mays* L. and virulence towards *Ostrinia nubilalis* (Hübner) (Lepidoptera: Pyralidae). *Agricutural Ecosystems and Environment* 45, 147–156.

Blanford, S., Thomas, M.B. and Langewald, J. (1998) Behavioural fever in the Senegalese grasshopper *Oedaleus senegalensis* and its implications for biological control. *Ecological Entomology* 23, 9–14.

Boucias, D.G. and Pendland, J.C. (1998) *Principles of Insect Pathology*. Kluwer Academic Publishers, Boston, Massachusetts.

Boucias, D.G., Bradford, D.L. and Barfield, C.S. (1984) Susceptibility of the velvetbean caterpillar and soybean looper to *Nomuraea rileyi*: effects of pathotype, dosage, temperature and host age. *Journal of Economic Entomology* 77, 247–253.

Boucias, D.G., Pendland, J.C. and Latge, J.P. (1988) Nonspecific factors involved in attachment of entomopathogenic Deuteromycetes to host insect cuticle. *Applied and Environmental Microbiology* 54, 1795–1805.

Boucias, D.G., Pendland, J.C. and Latge, J.P. (1991) Attachment of mycopathogens to cuticle: the initial event of mycoses in arthropod hosts. In: Cole, G.T. and Hoch, H.C. (eds) *The Fungal Spore and Disease Initiation in Plants and Animals*. Plenum Press, New York, pp. 101–128.

Boucias, D.G., Stokes, C., Storey, G. and Pendland, J.C. (1996) The effects of imidacloprid on the termite *Reticulitermes flavipes* and its interaction with the mycopathogen *Beauveria bassiana. Pflanzenschutz-Nachrichten Bayer* 49, 103–144.

Burges, H.D. (1998) Formulation of mycoinsecticides. In: Burges, H.D. (ed.) *Formulation of Microbial Biopesticides*. Kluwer Academic Publishers, Dordrecht, the Netherlands, pp. 131–185.

Burges, H.D. and Jones, K.A. (1998) Formulation of bacteria, viruses and protozoa to control insects. In: Burges, H.D. (ed.) *Formulation of Microbial Biopesticides*. Kluwer Academic Publishers, Dordrecht, the Netherlands, pp. 33–127.

Butt, T.M. and Goettel, M.S. (2000) Bioassays of entomogenous fungi. In: Navon, A. and Ascher, K.R.S. (eds) *Bioassays of Entomopathogenic Microbes and Nematodes*. CAB International, Wallingford, UK, pp. 141–195.

Butt, T.M., Ibrahim, L., Ball, B.V. and Clark, S.J. (1994) Pathogenicity of the entomogenous fungi *Metarhizium anisopliae* and *Beauveria bassiana* against crucifer pests and the honey bee. *Biocontrol Science and Technology* 4, 207–214.

Butt, T.M., Segers, R.J., Leal, S.C.M. and Kerry, B.R. (1998a) Variation in the subtilisins of fungal pathogens of insects and nematodes. In: Bridge, P., Couteaudier, Y. and Clarkson, J. (eds) *Molecular Variability of Fungal Pathogens.* CAB International, Wallingford, UK, pp. 149–169.

Butt, T.M., Carreck, N.L., Ibrahim, L. and Williams, I.H. (1998b) Honey bee mediated infection of pollen beetle (*Meligethes* spp.) by the insect-pathogenic fungus, *Metarhizium anisopliae. Biocontrol Science and Technology* 8, 533–538.

Campbell, R.K., Anderson, T.E., Semel, M. and Roberts, D.W. (1985) Management of Colorado potato beetle using the entomogenous fungus *Beauveria bassiana. American Potato Journal* 62, 29–37.

Cantone, F.A. and Vandenberg, J.D. (1999) Genetic transformation and mutagenesis of the entomopathogenic fungus *Paecilomyces fumosoroseus. Journal of Invertebrate Pathology* 74, 281–288.

Cantwell, G.E., Cantelo, W.W. and Schroder, R.F.W. (1986) Effect of *Beauveria bassiana* on underground stages of Colorado potato beetle, *Leptinotarsa decemlineata* (Coleoptera: Chrysomelidae). *Great Lakes Entomologist* 19, 81–84.

Carruthers, R.I. and Soper, R.S. (1987) Fungal diseases. In: Fuxa, J.R. and Tanada, Y. (eds) *Epizootiology of Insect Diseases*. John Wiley & Sons, New York, pp. 357–416.

Cerenius, L., Thornqvist, P.-O., Vey, A., Johansson, M.W. and Soderhall, K. (1990) The effects of the fungal toxin destruxin E on isolated crayfish haemocytes. *Journal of Insect Physiology* 6, 785–789.

Cherry, A.J., Lomer, C.J., Djegui, D. and Schulthess, F. (1999) Pathogen incidence and their potential as microbial control agents in IPM of maize stem borers in West Africa. *Biocontrol* 44, 301–327.

Costa, S.D. and Gaugler, R. (1989) Influence of *Solanum* host plants on Colorado potato beetle

(Coleoptera: Chrysomelidae) susceptibility to the entomopathogen *Beauveria bassiana*. *Environmental Entomology* 18, 531–536.

Daoust, R.A. and Roberts, D.W. (1983a) Studies on the prolonged storage of *Metarhizium anisopliae* conidia: effect of temperature and relative humidity on conidial viability and virulence against mosquitoes. *Journal of Invertebrate Pathology* 41, 143–150.

Daoust, R.A. and Roberts, D.W. (1983b) Studies on the prolonged storage of *Metarhizium anisopliae* conidia: effect of growth substrate on conidial survival and virulence against mosquitoes. *Journal of Invertebrate Pathology* 41, 161–170.

Delgado, F.X., Britton, J.H., Onsager, J.A. and Swearingen, W. (1999) Field assessment of *Beauveria bassiana* (Balsamo) Vuillemin and potential synergism with diflubenzuron for control of savanna grasshopper complex (Orthoptera) in Mali. *Journal of Invertebrate Pathology* 73, 34–39.

Devi, P.S.V. (1995) Soil treatment with *Nomuraea rileyi*: a promising technique for control of *Spodoptera litura* on groundnut. *Biocontrol Science and Technology* 5, 361–364.

Domsch, K.H., Gams, W. and Anderson, T.H. (1980) *Paecilomyces farinosus.* In: Domsch, K.H., Gams, W. and Anderson, T.H. (eds) *Compendium of Soil Fungi.* Academic Press, London, pp. 527–528.

Drummond, F.C. and Groden, E. (1996) Insect pests and natural enemies. In: *The Ecology, Economics, and Management of Potato Cropping Systems: A Report of the First Four Years of the Maine Potato Ecosystem Project*. Maine Agricultural and Forest Experiment Station Bulletin 843, Orono, Maine, pp. 80–118.

Dumas, C., Matha, V., Quiot, J.-M. and Vey, A. (1996) Effects of destruxins, cyclic depsipeptide mycotoxins, on calcium balance and phosphorylation on intracellular proteins in lepidopteran cell lines. *Comparative Biochemistry and Physiology* 114C, 213–219.

Easwaramoorthy, S., Regupathy, A., Santharam, G. and Jayaraj, S. (1978) The effect of subnormal concentration of insecticides in combination with the fungal pathogen, *Cephalosporium lecanii* Zimm. in the control of coffee green scale, *Coccus viridis* Green. *Zeitschrift für Angewandte Entomologie* 86, 161–166.

Ehlers, R.U. (1998) Entomopathogenic nematodes – save biocontrol agents for sustainable ecosystems. *Phytoprotection* 79 (suppl.), 94–102.

Ekesi, S., Maniania, N.K. and Lwande, W. (2000) Susceptibility of the legume flower thrips to *Metarhizium anisopliae* on different varieties of cowpea. *Biocontrol* 45, 79–95.

Evans, H.C. (1989) Mycopathogens of insects of epigeal and aerial habitats. In: Wilding, N., Collins, N.M., Hammond, P.M. and Webber, J.F. (eds) *Insect–Fungus Interactions*. Academic Press, London, pp. 205–238.

Fargues, J. and Luz, C. (2000) Effects of fluctuating moisture and temperature regimes on sporulation of *Beauveria bassiana* on cadavers of *Rhodnius prolixus*. *Biocontrol Science and Technology* 8, 323–334.

Fargues, J., Delmas, J.C., Augé, J. and Lebrun, R.A. (1991) Fecundity and egg fertility in the adult Colorado beetle (*Leptinotarsa decemlineata*) surviving larval infection by the fungus *Beauveria bassiana. Entomologica Experimentalis et Applicata* 61, 45–51.

Fargues, J., Delmas, J.C. and LeBrun, R.A. (1994) Leaf consumption by larvae of the Colorado potato beetle (Coleoptera: Chrysomelidae) infected with the entomopathogen, *Beauveria bassiana. Journal of Economic Entomology* 87, 67–71.

Fargues, J., Goettel, M.S., Smits, N., Ouedraogo, A., Vidal, C., Lacey, L.A., Lomer, C.J. and Rougier, M. (1996) Variability in susceptibility to simulated sunlight of conidia among isolates of entomopathogenic Hyphomycetes. *Mycopathologia* 135, 171–181.

Fargues, J., Goettel, M.S., Smits, N., Ouedraogo, A. and Rougier, M. (1997a) Effect of temperature on vegetative growth of *Beauveria bassiana* isolates from different origins. *Mycologia* 89, 383–392.

Fargues, J., Ouedraogo, A., Goettel, M.S. and Lomer, C.J. (1997b) Effects of temperature, humidity and inoculation method on susceptibility of *Schistocerca gregaria* to *Metarhizium flavoviride. Biocontrol Science and Technology* 7, 345–356.

Faria, M.R., Almeida, D., Magalhaes, B.P., de Faria, M.R. and de-O-Almeida. (1999) Food consumption of *Rhammatocerus schistocercoides* Rehn (Orthoptera: Acrididae) infected by the fungus *Metarhizium flavoviride* Gams & Rozsypal. *Anais da Sociedade Entomologica do Brasil* 28, 91–99.

Fehrenbach, P. (1993) Biological warfare. *Pest Control Technology* 21, 42–44.

Feng, M.G., Poprawski, T.J. and Khachatourians, G.G. (1994) Production, formulation and application of the entomopathogenic fungus *Beauveria bassiana* for insect control: current status. *Biocontrol Science and Technology* 4, 3–34.

Feng, Z., Carruthers, R.I., Roberts, D.W. and Robson, D.S. (1985) Age-specific dose–mortality effects of *Beauveria bassiana* on the European corn borer, *Ostrinia nubilalis. Journal of Invertebrate Pathology* 46, 259–264.

Fenny, P. (1992) The evolution of chemical ecology: contributions from the study of herbivorous insects. In: Rosenthal, G.A. and Berenbaum, M.R. (eds) *Herbivores: their Interaction with Secondary Plant Metabolites 2(2): Ecological and Evolutionary Process*. Academic Press, San Diego, California, pp. 1–44.

Fernandes, P.M., Magalhaes, B.P., Alves, S.B. and Roberts, D.W. (1989) Effects of physical and biological factors on the conidiogenesis and survival of *Beauveria bassiana* (Bals.) Vuill. inside cadavers of *Cerotoma arcuata* (Oliver, 1791) (Coleoptera: Chrysomelidae). *Anais da Sociedade Entomologica do Brasil* 18, 147–156.

Ferron, P. (1978) Biological control of insect pests by entomogenous fungi. *Annual Review of Entomology* 23, 409–442.

Ferron, P. (1979) Study of the virulence of some mutants of *Beauveria brongniartii* (*Beauveria tenella*) fungi infecting *Melolontha melolontha. Journal of Invertebrate Pathology* 34, 71–77.

Ferron, P. (1985) Fungal control. In: Kerkut, G.A. and Gilbert, L.I. (eds) *Comprehensive Insect Physiology, Biochemistry and Pharmacology.* Pergamon Press, Oxford, UK, pp. 313–346.

Ferron, P. (1997) Influence of relative humidity on the development of fungal infection caused by *Beauveria bassiana* (Fungi Imperfecti: Moniliales) in imagines of *Acanthoscelides obtectus* (Coleoptera: Bruchidae). *Entomophaga* 22, 393–396.

Ferron, P., Fargues, J. and Riba, G. (1991) Fungi as microbial insecticides against pests. In: Arora, D.K., Ajello, L. and Mukerji, K.G. (eds) *Handbook of Applied Mycology*. Marcel Dekker, New York, pp. 665–706.

Fornallaz, C. (1992) Demoekologische Grundlagen und praktische Durchfuehrung der mikrobiellen Bekaempfung des Maikaefers *Melolontha melolontha* L. mit dem Pilz *Beauveria brongniartii* (Sacc). Thesis, Petch. ETH-Zürich, pp. 1–127.

Fuxa, J.R. (1984) Dispersion and spread of the entomopathogenic fungus, *Nomuraea rileyi* in a soybean field. *Environmental Entomology* 13, 252–258.

Gardner, W.A., Oetting, R.D. and Storey, G.K. (1984) Scheduling of *Verticillium lecanii* and benomyl applications to maintain aphid (Homoptera: Aphidae) control on chrysanthemums in greenhouses [*Chrysanthemum morifolium, Myrus persicae, Macrosiphoniella sanborni*]. *Journal of Economic Entomology* 77, 514–518.

Gaugler, R., Costa, S.D. and Lashomb, J. (1989) Stability and efficacy of *Beauveria bassiana* soil inoculations. *Environmental Entomology* 18, 412–417.

Getzin, L.W. (1961) *Spicaria rileyi* and entomogenous fungus of *Trichoplusia ni. Journal of Invertebrate Pathology* 3, 2–10.

Glare, T.R. (1994) Stage-dependent synergism using *Metarhizium anisopliae* and *Serratia entomophila* against *Costelytra zealandica. Biocontrol Science and Technology* 4, 321–329.

Glare, T.R. and Milner, R.J. (1991) Ecology of entomopathogenic fungi. In: Arora, D.K., Ajello, L. and Mukerji, L.G. (eds) *Handbook of Applied Mycology*, Vol. 2: Humans, Animals, and Insects. Marcel Dekker, New York, pp. 547–612.

Goettel, M.S. and Inglis, G.D. (1997) Fungi: Hyphomycetes. In: Lacey, L.A. (ed.) *Manual of Techniques in Insect Pathology*. Academic Press, London, pp. 213–249.

Goettel, M.S., St Leger, R.J., Bhairi, S., Jung, M.K., Oakley, B.R., Roberts, D.W. and Staples,

R.C. (1990a) Pathogenicity and growth of *Metarhizium anisopliae* stably transformed to benomyl resistance. *Current Genetics* 17, 129–132.

Goettel, M.S., Poprawski, T.J., Vandenberg, J.D., Li, Z. and Roberts, D.W. (1990b) Safety to nontarget invertebrates of fungal biocontrol agents. In: Laird, M., Lacey, L.A. and Davidson, E.W. (eds) *Safety of Microbial Insecticides*. CRC Press, Boca Raton, Florida, pp. 209–232.

Goettel, M.S., Inglis, G.D. and Wraight, S.P. (2000a) Fungi. In: Lacey, L.A. and Kaya, H.K. (eds) *Field Manual of Techniques in Invertebrate Pathology*. Kluwer Academic Press, Dordrecht, the Netherlands, pp. 255–282.

Goettel, M.S., Duke, G.M. and Inglis, G.D. (2000b) Lack of effects of sublethal doses of the chemical pesticides imidacloprid, diflurobenzuron and buprofezin on the susceptibility of thermoregulating grasshoppers to the fungus *Beauveria bassiana.* In: *Program and Abstracts, Society for Invertebrate Pathology 33rd Annual Meeting, Guanajuato, Mexico, 13–18 August*, p. 44.

Gottwald, T.R. and Tedders, W.L. (1984) Colonization, transmission, and longevity of *Beauveria bassiana* and *Metarhizium anisopliae* (Deuteromycotina: Hyphomycetes) on pecan weevil larvae (Coleoptera: Curculionidae) in the soil. *Environmental Entomology* 13, 557–560.

Grivanov, K.P. (1940) Deep ploughing instead of burning the stubble for the control of the wheat thrips *Haplothirps tritici. Review of Applied Entomology* A 28, 69–70.

Groden, E. and Dunn, T. (1996) Germination, host infection, and survival of *Beauveria bassiana* conidia in natural soils. In: Jackson, T.A. and Glare, T.R. (eds) *Microbial Control of Soil Dwelling Pests*. The Microbial Control Group, AgResearch, Lincoln, New Zealand, pp. 137–145.

Groden, E. and Lockwood, J.L. (1991) Effects of soil fungistasis on *Beauveria bassiana* and its relationship to disease incidence in the Colorado potato beetle, *Leptinotarsa decemlineata*, in Michigan and Rhode Island soils. *Journal of Invertebrate Pathology* 57, 7–16.

Hajek, A.E. and St Leger, R.J. (1994) Interactions between fungal pathogens and insect host. *Annual Review of Entomology* 39, 293–322.

Hajek, A.E., Soper, R.S., Roberts, D.W., Anderson, T.E., Biever, K.D., Ferro, D.N., LeBrun, R.A. and Storch, R.H. (1987) Foliar applications of *Beauveria bassiana* (Balsamo) Vuillemin for control of Colorado potato beetle, *Leptinotarsa decemlineata* (Say) (Coleoptera: Chrysomelidae): an overview of pilot test results from northern United States. *Canadian Entomologist* 119, 959–974.

Hall, R.A. (1981) The fungus *Verticillium lecanii* as a microbial insecticide against aphids and scales. In: Burges, H.D. (ed.) *Microbial Control of Pests and Plant Diseases 1970–1980*. Academic Press, New York, pp. 483–498.

Hall, R.A. and Burges, H.D. (1979) Control of aphids in glasshouses with the fungus *Verticillium lecanii. Annals of Applied Biology* 93, 235–246.

Hare, J.D. and Andreadis, T.G. (1983) Variation in the susceptibility of *Leptinotarsa decemlineata* (Coleoptera: Chrysomelidae) when reared on different host plants to the fungal pathogen, *Beauveria bassiana* in the field and laboratory. *Environmental Entomology* 12, 1871–1896.

Hassan, A.E.M. and Charnley, A.K. (1983) Combined effects of diflubenzuron and the entomopathogenic fungus *Metarhizium anisopliae* on the tobacco hornworm, *Manduca sexta.* In: *Tenth International Congress of Plant Protection 1983*, Vol. 2. Brighton, UK, p. 790.

Hassan, A.E.M. and Charnley, A.K. (1989) Ultrastructural study of the penetration by *Metarhizium anisopliae* through Dimilin-affected cuticle of *Manduca sexta. Journal of Invertebrate Pathology* 54, 117–124.

Hawksworth, D.L., Kirk, P.M., Sutton, B.C. and Pegler, D.N. (eds) (1995) *Ainsworth and Bisby's Dictionary of the Fungi*. CAB International, Wallingford, UK.

Hedgecock, S., Moore, D., Higgins, P.M. and Prior, C. (1995) Influence of moisture content on temperature tolerance and storage of *Metarhizium flavoviride* conidia in an oil formulation. *Biocontrol Science and Technology* 5, 371–377.

Heinrich, B. (1993) *The Hot-blooded Insects*. Harvard University Press, Cambridge, Massachusetts.

Heinrich, B. (1996) *The Thermal Warriers: Strategies for Survival*. Harvard University Press, Cambridge, Massachusetts.

Helyer, N., Gill, G., Bywater, A. and Chambers, R. (1992) Elevated humidities for control of chrysanthemum pests with *Verticillium lecanii. Pesticide Science* 36, 373–378.

Hockland, S.H., Dawson, G.W., Griffiths, D.C., Marples, B., Pickett, J.A. and Woodcock, C.M. (1986) The use of aphid alarm pheromone ((E)-β-farnesene) to increase effectiveness of the entomophilic fungus *Verticillium lecanii* in controlling aphids on chrysanthemums under glass. In: Samson, R.A., Vlak, J.M. and Peters, D. (eds) *Fundamental and Applied Aspects of Invertebrate Pathology*. Foundation of the Fourth International Colloquium in Invertebrate Pathology, Wageningen, the Netherlands, p. 252.

Hong, T.D., Ellis, R.H. and Moore, D. (1997) Development of a model to predict the effect of temperature and moisture on fungal spore longevity. *Annals of Botany* 79, 121–128.

Hsiao, W.F., Bidochka, M.J. and Khachatourians, G.G. (1992) Effect of temperature and relative humidity on the virulence of the entomopathogenic fungus, *Verticillium lecanii*, towards the oat-bird berry aphid, *Rhopalosiphum padi* (Hom., Aphidae). *Journal of Applied Entomology* 114, 484–490.

Hsiu, C.F., Chang, Y., Kwei, C.M., Han, Y.M., Wang, H.H., Wang, L. and Liu, H.P. (1973) Field application of *Beauveria bassiana* for controlling the European corn borer. *Acta Entomolologica Sinica* 16, 203–206.

Hughes, S.J. (1953) Conidiophores, conidia and classification. *Canadian Journal of Botany* 31, 577–659.

Humber, R.A. (1997) Fungi: identification. In: Lacey, L.A. (ed.) *Manual of Techniques in Insect Pathology*. Academic Press, London, pp.153–185.

Ignoffo, C.M., Puttler, B., Hostetter, D.L. and Dickerson, W.A. (1976) Susceptibility of the cabbage looper, *Trichoplusia ni* and the velvet bean caterpillar, *Anticarsia gemmatalis*, to several isolates of the entomopathogenic fungus *Nomuraea rileyi. Journal of Invertebrate Pathology* 28, 259–262.

Ignoffo, C.M., Garcia, C., Hostetter, D.L. and Pinnel, R.E. (1977a) Laboratory studies of the entomopathogenic fungus, *Nomuraea rileyi*: soil-borne contamination of soybean seedlings and dispersal of diseased larvae of *Trichoplusia ni. Journal of Invertebrate Pathology* 29, 147–152.

Ignoffo, C.M., Garcia, C., Hostetter, D.L. and Pinnel, R.E. (1977b) Vertical movement of conidia of *Nomuraea rileyi* through sand and loam soils. *Journal of Economic Entomology* 70, 163–164.

Ignoffo, C.M., Garcia, C., Hostetter, D.L. and Pinnel, R.E. (1978) Stability of conidia of an entomopathogenic fungus, *Nomuraea rileyi*, in and on soils. *Environmental Entomology* 7, 724–727.

Ignoffo, C.M. (1981) The fungus *Nomuraea rileyi* as a microbial insecticide. In: Burges, H.D. (ed.) *Microbial Control of Pests and Plant Diseases 1970–80*. Academic Press, London, pp. 513–538.

Inglis, G.D., Goettel, M.S. and Johnson, D.L. (1993) Persistence of the entomopathogenic fungus, *Beauveria bassiana*, on phylloplanes of crested wheatgrass and alfalfa. *Biological Control* 3, 258–270.

Inglis, G.D., Goettel, M.S. and Johnson, D.L. (1995a) Influence of ultraviolet light protectants on persistence of the entomopathogenic fungus, *Beauveria bassiana. Biological Control* 5, 581–590.

Inglis, G.D., Johnson, D.L. and Goettel, M.S. (1995b) Effects of simulated rain on the persistence of *Beauveria bassiana* conidia on leaves of alfalfa and wheat. *Biocontrol Science and Technology* 5, 365–370.

Inglis, G.D., Johnson, D.L. and Goettel, M.S. (1996a) Effects of temperature and thermoregulation on mycosis by *Beauveria bassiana. Biological Control* 7, 131–139.

Inglis, G.D., Johnson, D.L. and Goettel, M.S. (1996b) Effect of bait substrate and formulation on infection of grasshopper nymphs by *Beauveria bassiana. Biocontrol Science and Technology* 6, 35–50.

Inglis, G.D., Johnson, D.L. and Goettel, M.S. (1997a) Effects of temperature and sunlight on mycosis (*Beauveria bassiana*) (Hyphomycetes: Sympodulosporae) of grasshoppers under field conditions. *Environmental Entomology* 26, 400–409.

Inglis, G.D., Johnson, D.L., Cheng, K.-J. and Goettel, M.S. (1997b) Use of pathogen combinations to overcome constraints of temperature on entomopathogenic Hyphomycetes against grasshoppers. *Biological Control* 8, 143–152.

Inglis, G.D., Duke, G.M., Kanagaratnam, P., Johnson, D.L. and Goettel, M.S. (1997c) Persistence of *Beauveria bassiana* in soil following application of conidia through crop canopies. In: Goettel, M.S. and Johnson, D.L. (eds) *Microbial Control of Grasshoppers and Locusts*. *Memoirs of the Entomological Society of Canada* 171, 253–263.

Inglis, G.D., Johnson, D.L., Kawchuk, L.M. and Goettel, M.S. (1998) Effect of soil texture and soil sterilization on the susceptibility of ovipositing grasshoppers to *Beauveria bassiana*. *Journal of Invertebrate Pathology* 71, 73–81.

Inglis, G.D., Duke, G.M., Kawchuk, L.M. and Goettel, M.S. (1999) Influence of oscillating temperatures on the competitive infection and colonization of the migratory grasshopper by *Beauveria bassiana* and *Metarhizium flavoviride*. *Biological Control* 14, 111–120.

Inglis, G.D., Ivie, T.J., Duke, G.M. and Goettel, M.S. (2000) Influence of formulations on rainfastness of *Beauveria bassiana* conidia on potato leaves and Colorado potato beetle larvae. *Biological Control* 18, 55–64.

Inyang, E., Butt, T.M., McCartney, A., Oyejola, B., Ibrahim, L., Pye, B.J. and Archer, S. (2000) Effect of formulation, application and rain on the persistence of the entomogenous fungus *Metarhizium anisopliae* on oilseed rape. *Mycological Research* 104, 653–661.

Jackson, M.A., McGuire, M.R., Lacey, L.A. and Wraight, S.P. (1997) Liquid culture production of desiccation tolerant blastospores of the bioinsecticidal fungus *Paecilomyces fumosoroseus*. *Mycological Research* 101, 35–41.

James, R.R. and Lighthart, B. (1994) Susceptibility of the convergent lady beetle (Coleoptera: Coccinellidae) to four entomogenous fungi. *Environmental Entomology* 23, 190–192.

Jaques, R.P. and Laing, D.R. (1988) Effectiveness of microbial and chemical insecticides in control of the Colorado potato beetle (Coleoptera: Chrysomelidae) on potatoes and tomatoes. *Canadian Entomologist* 120, 1123–1131.

Jaros-Su, J., Groden, E. and Zhang, J. (1999) Effects of selected fungicides and the timing of fungicide application in *Beauveria bassiana*-induced mortality of the Colorado potato beetle (Coleoptera: Chrysomelidae). *Biological Control* 15, 259–269.

Jenkins, N.E., Heviefo, G., Langewald, J., Cherry, A.J. and Lomer, C. (1998) Development of mass production technology for aerial conidia for use as mycopesticides. *Biocontrol News and Information* 19, 21N–31N.

Joshi, L., Charnley, A.K., Arnold, G., Brian, P. and Bateman, R.P. (1992) Synergism between entomopathogenic fungi, *Metarhizium* spp., and the benzoylphenyl urea insecticide, teflubenzuron, against the desert locust, *Schistocerca gregaria*. In: *Proceedings of the Brighton Crop Protection Conference, Pests and Diseases*. Brighton, UK, pp. 23–26.

Kaakeh, W., Reid, B.L. and Bennett, G.W. (1996) Horizontal transmission of the entomopathogenic fungus *Metarhizium anisopliae* (Imperfect fungi: Hyphomycetes) and hydramethylnon among German cockroaches (Dictyoptera: Blattellidae). *Journal of Entomological Science* 31, 378–390.

Kaakeh, W., Reid, B.L., Bohnert, T.J. and Bennet, G.W. (1997) Toxicity of imidacloprid in the German cockroach (Dictyoptera: Blattellidae), and the synergism between imidacloprid and *Metarhizium anisopliae* (Imperfect Fungi: Hyphomycetes). *Journal of Economic Entomology* 90, 473–482.

Keller, S. and Zimmermann, G. (1989) Mycopathogens of soil insects. In: Wilding, N., Collins, N.M., Hammond, P.M. and Webber, J.F. (eds) *Insect–Fungus Interactions*. Academic Press, London, pp. 239–270.

Keller, S., Pärli, B., Lujan, M. and Schweizer, C. (1993) Der Einfluß von Fungiziden auf den

insektenpathogenen Pilz *Beauveria brongniartii* (Sacc.) Petch. *Anzeiger für Schädlingskunde Pflanzenschutz und Umweltschutz* 66, 108–114.

Keller, S., Schweizer C., Keller, E. and Brenner, H. (1997) Control of white grubs (*Melolontha melolontha* L.) by treating adults with the fungus *Beauveria brongniartii. Biocontrol Science and Technology* 7, 105–116.

Kidd, H. and James, D.R. (eds) (1991) *The Agrochemicals Handbook*, 3rd edn. Royal Society of Chemistry Information Services, Cambridge, UK.

Kish, L.P. (1975) The Biology and Ecology of *Nomuraea rileyi*. PhD dissertation, University of Florida, Gainesville, Florida.

Kish, L.P. and Allen, G.E. (1978) *The Biology and Ecology of* Nomuraea rileyi *and a Program for Predicting its Incidence on* Anticarsia gemmatalis *in Soybean.* Florida Agricultural Experimental Station Bulletin 795, University of Florida, Gainesville, Florida.

Kooyman, C., Bateman, R.P., Langewald, J., Lomer, C.J., Ouambama, Z. and Thomas, M.B. (1997) Operational-scale application of entomopathogenic fungi for control of Sahelian grasshoppers. *Proceedings of the Royal Society of London* B 264, 541–546.

Kramm, K.R., West, D.R. and Rockenbach, P.G. (1982) Termite pathogens: transfer of the entomopathogen *Metarhizium anisopliae* between *Reticulitermis* sp. termites. *Journal of Invertebrate Pathology* 40, 1–6.

Krischik, V.A. and Denno, R.F. (1983) Individual, population, and geographical patterns in plant defense herbivorous insects, weed control. In: Denno, R.F. and McClure, M.S. (eds) *Variable Plants and Herbivores in Natural and Managed Systems*. Academic Press, New York, pp. 463–512.

Krueger, S.R., Nechols, J.R. and Ramoska, W.A. (1991) Infection of Chinch Bug, *Blissus leucopterus leucopterus* (Hemiptera: Lygaediae), adults from *Beauveria bassiana* (Deuteromycotina: Hyphomycetes) conidia in soil under controlled temperature and moisture conditions. *Journal of Invertebrate Pathology* 58, 19–26.

Krueger, S.R., Villani, M.G., Martins, A.S. and Roberts, D.W. (1992) Efficacy of soil applications of *Metarhizium anisopliae* (Metsch.) Sorokin conidia, and standard and lyophilized mycelial particles against scarab grubs. *Journal of Invertebrate Pathology* 59, 54–60.

Lacey, L.A. and Goettel, M.S. (1995) Current developments in microbial control of insect pests and prospects for the early 21st century. *Entomophaga* 40, 1–25.

Lacey, L.A. and Kaya, H.K. (eds) (2000) *Field Manual of Techniques in Invertebrate Pathology*. Kluwer Academic Press, Dordrecht, the Netherlands.

Lacey, L.A., Fransen, J.J. and Carruthers, R. (1996) Global distribution of naturally occurring fungi of *Bemisia*, their biologies and use as biological control agents. In: Gerling, D. and Mayer, R.T. (eds) *Bemisia 1995: Taxonomy, Biology, Damage, Control and Management*. Intercept Limited, Andover, UK, pp. 401–433.

Lacey, L.A., Mesquita, A.L.M., Mercadier, G., Debire, R., Kazmer, D.J. and Leclant, F. (1997) Acute and sublethal activity of the entomopathogenic fungus *Paecilomyces fumosoroseus* (Deuteromycotina: Hyphomycetes) on adult *Aphelinus asychis* (Hymenoptera: Aphelinidae). *Environmental Entomology* 26, 1452–1460.

Lacey, L.A., Horton, D.R., Chauvin, R.L. and Stocker, J.M. (1999a) Comparative efficacy of *Beauveria bassiana, Bacillus thuringiensis*, and aldicarb for control of Colorado potato beetle in an irrigated dessert agroecosystem and their effects on biodiversity. *Entomologica Experimentalis et Applicata* 93, 189–200.

Lacey, L.A., Kirk, A.A., Millar, L., Mercadier, G. and Vidal, C. (1999b) Ovicidal and larvicidal activity of conidia and blastospores of *Paecilomyces fumosoroseus* (Deuteromycotina: Hyphomycetes) against *Bemesia argentifolii* (Homoptera: Aleyrodidae) with a description of a bioassay system allowing prolonged survival of control insects. *Biocontrol Science and Technology* 9, 9–18.

Langewald, J., Ouambama, J., Mamadou, A., Peveling, R., Stolz, I., Bateman, R., Attignon, S., Blanford, S., Arthurs, S. and Lomer, C. (1999) Comparison of an organophosphate

insecticide with a mycoinsecticide for control of *Oedaleus senegalensis* (Orthoptera: Acrididae) and other Sahelian grasshoppers at an operational level. *Biocontrol Science and Technology* 9, 199–214.

Latch, G.C.M. and Falloon, R.E. (1976) Studies on the use of *Metarhizium anisopliae* to control *Oryctes rhinoceros*. *Entomophaga* 21, 39–48.

Leach, C.M. (1971) A practical guide to the effects of visible and ultra-violet light on fungi. In: Booth, C. (ed.) *Methods in Microbiology*, Vol. 4. Academic Press, New York, pp. 609–664.

Lecuona, R.E. and Alves, S.B. (1988) Efficiency of *Beauveria bassiana* (Bals.) Vuill., *B. brongniartii* (Sacc.) Petch. and granulose virus on *Diatraea saccharalis* (F., 1794) at different temperatures. *Journal of Applied Entomology* 105, 223–228.

Lewis, L.C. and Bing, L.A. (1991) *Bacillus thuringiensis* Berliner and *Beauveria bassiana* (Balsamo) Vuillemin for European corn borer control: program for immediate and season-long suppression. *Canadian Entomologist* 123, 387–393.

Lewis, L.C., Berry, E.C., Obrycki, J.J. and Bing, L.A. (1996) Aptness of insecticides (*Bacillus thuringiensis* and carbofuran) with endophytic *Beauveria bassiana* in suppressing larval populations of European corn borer. *Agricultural Ecosystems and Environment* 57, 27–34.

Lewis, L.C., Bruck, D.J., Gunnarson, R.D. and Bidne, K.G. (2000) Colonization of entomopathogenic fungus *Beauveria bassiana* (Deuteromycotina:Hyphomycetes) on *Bt* transgenic corn (*Zea mays* Still) and their genetic isolines and assessement of possible plant pathogenicity. Crop Science (in press).

Li, D.P. and Holdom, D.G. (1994) Effects of pesticides on growth and sporulation of *Metarhizium anisopliae* (Deuteromycotina: Hyphomycetes). *Journal of Invertebrate Pathology* 63, 209–211.

Lindquist, R.K. (1993) Integrated insect, mite and disease management programs on greenhouse crops: pesticides and application methods. In: Robb, K. and Hall, J. (eds) *Proceedings, Ninth Conference on Insect and Disease Management in Ornamentals*, *Del Mar, California*. Society of American Florists, Alexandria, Virginia, pp. 38–44.

Lingg, A.J. and Donaldson, M.D. (1981) Biotic and abiotic factors affecting stability of *Beauveria bassiana* conidia in soil. *Journal of Invertebrate Pathology* 38, 191–200.

Liu, Y.T., Sui, M.J., Ji, D.D., Wu, I.H., Chou, C.C. and Chen, C.C. (1993) Protection from ultraviolet irradiation by melanin of mosquitocidal activity of *Bacillus thuringiensis* var. *israelensis*. *Journal of Invertebrate Pathology* 62, 131–136.

Lockwood, J.L. (1977) Fungistasis in soils. *Biological Reviews* 52, 1–43.

Lomer, C.J., Prior, C. and Kooyman, C. (1997a) Development of *Metarhizium* spp. for the control of grasshoppers and locusts. In: Goettel, M.S. and Johnson, D.L. (eds) *Microbial Control of Grasshoppers and Locusts*. *Memoirs of the Entomological Society of Canada* 171, pp. 265–286.

Lomer, C.J., Thomas, M.B., Douro-Kpindou, O.-K., Gbongboui, C., Godonou, I., Langewald, J. and Shah, P.A. (1997b) Control of grasshoppers, particularly *Hieroglyphus daganensis* in Northern Benin using *Metarhizium flavoviride*. In: Goettel, M.S. and Johnson, D.L. (eds) *Microbial Control of Grasshoppers and Locusts*. *Memoirs of the Entomological Society of Canada* 171, pp. 301–311.

Lopez-Llorca, L.V. and Olivares-Bernabeu, C. (1997) Growth inhibition of nematophagous and entomopathogenic fungi by leaf litter and soil containing phenols. *Mycological Research* 101, 691–697.

Lü, C.R. and Zhao, T. (1988) Study, production and application of *Beauveria bassiana*. In: Li, Y.W., Wu, J.W., Wu, Z.K. and Xu, Q.F. (eds) *Study and Application of Entomogenous Fungi in China,* Vol. 1. Academic Periodical Press, Beijing, China, pp. 15–17.

Magalhaes, B.P., Lecoq, M., De Faria, M.R., Schmidt, F.G.V. and Guerra, W.D. (2000) Field trials with the entomopathogenic fungus *Metarhizium anisopliae* var *acridum* against bands

of the grasshopper *Rhammatocercus schistocercoides* in Brazil. *Biocontrol Science and Technology* 10, 427–441.

Majchrocwicz, I. and Poprawski, T.J. (1993) Effects *in vitro* of nine fungicides on growth of entomopathogenic fungi. *Biocontrol Science and Technology* 3, 321–336.

Marcandier, S. and Khachatourians, G.G. (1987) Susceptibility of the migratory grasshopper, *Melanoplus sanguinipes* (Fab.) (Orthoptera: Acrididae), to *Beauveria bassiana* (Bals.) Vuillemin (Hyphomycete): influence of relative humidity. *Canadian Entomologist* 119, 901–907.

McCammon, S.A. and Rath, A.C. (1994) Separation of *Metarhizium anisopliae* strains by temperature dependent germination rates. *Mycological Research* 98, 1253–1257.

McCoy C.W., Samson, R.A. and Boucias, D.G. (1988) Entomogenous fungi. In: Ignoffo, C. and Mandava N.B. (eds) *CRC Handbook of Natural Pesticides*, Vol. 5. *Microbial Insecticides*, Part A: *Entomogenous Protozoa and Fungi*. CRC Press, Boca Raton, Florida, pp. 151–234.

Milner, R.J. (1994) Louis Pasteur's footsteps. *Pest Control Technology* 22, 84–85.

Milner, R.J. (1997) *Metarhizium flavoviride* (FI985) as a promising mycoinsecticide for Australian acridids. In: Goettel, M.S. and Johnson, D.L. (eds) *Microbial Control of Grasshoppers and Locusts*. *Memoirs of the Entomological Society of Canada* 171, pp. 287–300.

Milner, R.J. and Lutton, G.G. (1986) Dependence of *Verticillium lecanii* (Fungi: Hyphomycetes) on high humidities for infection and sporulation using *Myzus persicae* (Homoptera: Aphidae) as host. *Environmental Entomology* 15, 380–382.

Milner, R.J. and Staples, J.A. (1996) Biological control of termites: results and experiences within a CSIRO project in Australia. *Biocontrol Science and Technology* 6, 3–9.

Milner, R.J., Staples, J.A., Hartley, T.R., Lutton, G.G., Driver, F. and Watson, J.A.L. (1998a) Occurrence of *Metarhizium anisopliae* in nests and feeding sites of Australian termites. *Mycological Research* 102, 216–220.

Milner, R.J., Staples, J.A. and Lutton, G.G. (1998b) The selection of an isolate of the hyphomycete fungus, *Metarhizium anisopliae*, for control of termites in Australia. *Biological Control* 11, 240–247.

Mohamed, A.K.A., Bell, J.V. and Sikorowski, P.P. (1978) Field cage tests with *Nomuraea rileyi* against corn earworm larvae on sweet corn. *Journal of Economic Entomology* 71, 102–104.

Mohan, C.M., Lakshmi, K.A. and Devi, K.U. (1999) Laboratory evaluation of the pathogenicity of three isolates of entomopathogic fungus *Beauveria bassiana* (Bals.) Vuillemin on the American cockroach (*Periplaneta americana*). *Biocontrol Science and Technology* 9, 29–33.

Moore, D., Bridge, P.D., Higgins, P.M., Bateman, R.P. and Prior, C. (1993) Ultra-violet radiation damage to *Metarhizium flavoviride* conidia and the protection given by vegetable and mineral oils and chemical sunscreens. *Annals of Applied Biology* 122, 605–616.

Muller-Kogler, E. and Zimmerman, G. (1986) Viability of *Beauveria bassiana* (Bals.) Vuill. in contaminated soil under field and laboratory conditions. *Entomophaga* 31, 285–292.

Osborne, L.S., Storey, G.K., McCoy, C.W. and Walter, J.F. (1990) Potential for controlling the sweetpotato whitefly, *Bemesia tabaci*, with the fungus *Paecilomyces fumosoroseus.* In: *Proceedings of the Vth International Colloquium on Invertebrate Pathology and Microbial Control*, *Adelaide, Australia*, pp. 386–390.

Ouedraogo, A., Fargues, J., Goettel, M.S. and Lomer, C.J. (1997) Effect of temperature on vegetative growth among isolates of *Metarhizium anisopliae* and *M. flavoviride. Mycopathologia* 137, 37–43.

Pan, W.Y. and Zheng, H. (1988) Report on application of *Beauveria bassiana* against *Dendrolimus tabulaeformis* in arid forest region. In: Li, Y.W., Wu, J.W., Wu, Z.K. and Xu, Q.F. (eds) *Study and Application of Entomogenous Fungi in China,* Vol. 1. Academic Periodical Press, Beijing, China, pp. 7–76.

Pereira, R.M., Stimac, J.L. and Alves, S.B. (1993) Soil antagonism affecting the dose-response of workers of the red imported fire ant, *Solenopsis invicta*, to *Beauveria bassiana* conidia. *Journal of Invertebrate Pathology* 61, 156–161.

Petch, T. (1942) Notes on entomogenous fungi. *Transactions of the British Mycological Society* 25, 250–265.

Poprawski, T.J., Carruthers, R.I., Speese, J., Vacek, D.C. and Wendel, L.E. (1997) Early-season applications of the fungus *Beauveria bassiana* and introduction of the hemipteran predator *Perillus bioculatus* for control of Colorado potato beetle. *Biological Control* 10, 48–57.

Poprawski, T.J., Legaspi, J.C. and Parker, P.E. (1998) Influence of entomopathogenic fungi on *Serangium parcesetosum* (Coleoptera: Coccinellidae), an important predator of whiteflies (Homoptera: Aleyrodidae). *Environmental Entomology* 27, 785–795.

Quarles, W. (1999) Commercial biocontrol for termites. *IPM Practitioner* 21, 1–6.

Quintela, E.D. and McCoy, C.W. (1998) Conidial attachment of *Metarhizium anisopliae* and *Beauveria bassiana* to the larval cuticle of *Diaprepes abbreviatus* (Coleoptera: Curculionidae) treated with imidacloprid. *Journal of Invertebrate Pathology* 72, 220–230.

Ramakrishnan, R., Suiter, D.R., Nakatsu, C.H., Humber, R.A. and Bennett, G.W. (1999) Imidacloprid-enhanced *Reticulitermes flavipes* (Isoptera: Rhinotermitidae) susceptibility to the entomopathogen *Metarhizium anisopliae. Journal of Economic Entomology* 92, 1125–1132.

Ramoska, W.A. (1984) The influence of relative humidity on *Beauveria bassiana* infectivity and replication in the chinch bug *Blissus leucopterus. Journal of Invertebrate Pathology* 43, 389–394.

Ramoska, W.A. and Todd, T. (1985) Variation in efficacy and viability of *Beauveria bassiana* in the chinch bug (Hemiptera: Lygaeidae) as a result of feeding activity on selected host plants. *Environmental Entomology* 14, 146–148.

Rath, A.C. and Tidbury, C.A. (1996) Susceptibility of *Coptotermes acinaciformis* (Isoptera: Rhinotermitidae) and *Nasutitermes exitiosus* (Isoptera: Termitidae) to two commercial isolates of *Metarhizium anisopliae. Sociobiology* 28, 67–72.

Rath, A.C., Worledge, D., Koen, T.B. and Rowe, B.A. (1995) Long-term field efficacy of the entomogenous fungus *Metarhizium anisopliae* against the subterranean scarab, *Adoryphorus couloni. Biocontrol Science and Technology* 5, 439–451.

Rath, A.C., Bullard, G.K., Allsopp, P.G., Rogers, D.J. and Robertson, L.N. (1997) Persistence of *Metarhizium anisopliae* DAT F-001 in pasture soils for 7.5 years – implications for sustainable soil-pest management. In: Allsopp, P.G. and Rogers, D.J. (eds) *Soil Invertebrates in 1997 Proceedings of the Third Brisbane Workshop on Soil Invertebrates*. Brisbane, Australia, pp. 78–80.

Rembold. H. (1989) Azadirachtins: their structure and mode of action. *American Chemical Society Symposium Series* 387, 150–163.

Roberts, D.W. and Campbell, A.S. (1977) Stability of entomopathogenic fungi. *Miscellaneous Publications of the Entomological Society of America* 10, 19–76.

Roberts, D.W. and Hajek, A.E. (1992) Entomopathogenic fungi as bioinsecticides. In: Leatham, G.F. (ed.) *Frontiers in Industrial Mycology*. Chapman & Hall, New York, pp. 144–159.

Rombach, M.C. and Gillespie, A.T. (1988) Entomogenous Hyphomycetes for insect and mite control on greenhouse crops. *Biocontrol News and Information* 9, 7–18.

Rosengaus, R.B. and Traniello, J.F.A. (1997) Pathobiology and disease transmission in dampwood termites (*Zootermopsis angusticollis* (Isoptera: Termopsidae)) infected with the fungus *Metarhizium anisopliae* (Deutermycotina: Hyphomycetes). *Sociobiology* 30, 185–195.

Rosengaus, R.B., Maxmen, A.B., Coates, L.E. and Traniello, J.F.A. (1998a) Disease resistance: a benefit of sociality in the dampwood termite *Zootermopsis angusticollis* (Isoptera: Termopsidae). *Behavioral Ecology and Sociobiology* 44, 125–134.

Rosengaus, R.B., Guldin, M.R. and Traniello, J.F.A. (1998b) Inhibitory effect of termite fecal pellets on fungal spore germination. *Journal of Chemical Ecology* 24, 1697–1706.

Rosengaus, R.B., Jordan, C., Lefebvre, M.L. and Traniello, J.F.A. (1999) Pathogen alarm behaviour in a termite: a new form of communication in social insects. *Naturwissenschaften* 86, 544–548.

St Leger, R.J. (1993) Biology and mechanisms of insect-cuticle invasion by Deuteromycete fun-

gal pathogens. In: Beckage, N.E., Thompson, S.N. and Federici, B.A. (eds) *Parasites and Pathogens of Insects*, Vol. 2. Academic Press, San Diego, California, pp. 211–229.

Samson, R.A., Evans, H.C. and Latgé, J.-P. (1988) *Atlas of Entomopathogenic Fungi.* Springer-Verlag, Berlin.

Seifert, K.A. (2000) Molecules, morphology and classification: towards monophyletic genera in the Ascomycetes. In: *Studies in Microbiology.* CBS, Baarn, the Netherlands.

Shah, P.A. and Goettel, M.S. (1999) *Directory of Microbial Control Products and Services*. Society for Invertebrate Pathology, Gainesville, Florida.

Shah, P.A., Douro-Kpindou, O.K., Sidibe, A., Daffee, C.O., van der Pauuw, H. and Lomer, C.J. (1998) Effects of the sunscreen oxybenzone on field efficacy and persistence of *Metarhizium flavoviride* conidia against *Kraussella amabile* (Orthoptera: Acrididae) in Mali, West Africa. *Biocontrol Science and Technology* 8, 357–364.

Smits, N., Fargues, J., Rougier, M., Goujet, R. and Itier, B. (1996a) Effects of temperature and solar radiation interactions on the survival of quiescent conidia of the entomopathogenic hyphomycete *Paecilomyces fumosoroseus* (Wize) Brown and Smith. *Mycopathologia* 135, 163–170.

Smits, N., Rougier, M., Fargues, J., Goujet, R. and Bonhomme, R. (1996b) Inactivation of *Paeciliomyces fumosoroseus* conidia by diffuse and total solar radiation. *FEMS Microbiology and Ecology* 21, 167–173.

Sopp, P.I., Gillespie, A.T. and Palmer, A. (1990) Comparison of ultra-low-volume electrostatic and high-volume hydraulic application of *Verticillium lecanii* for aphid control on chrysanthemums. *Crop Protection* 9, 177–184.

Sprenkel, R.K. and Brooks, W.M. (1975) Artificial dissemination and epizootic initiation of *Nomuraea rileyi* and entomogenous fungus of lepidopterous pests on soybeans. *Journal of Economic Entomology* 68, 847–851.

Sprenkel, R.K., Brooks, W.M., van Duyan, J.W. and Deitz, L.L. (1979) The effects of three cultural variables on the incidence of *Nomuraea rileyi*, phytophagous Lepidoptera and their predators on soybeans. *Environmental Entomology* 8, 334–339.

Staal, G.B. (1987) Juvenoids and anti-juvenille hormone agents as IGRs. In: Delucchi, V. (ed.) *Proceedings, Symposium on Insect Pest Management: Quo Vadis? An International Perspective*. Geneva, Switzerland, pp. 277–292.

Steinhaus, E.A. (1958a) Stress as a factor in insect disease. In: *Proceedings International Congress of Entomology*, *Tenth Congress (Montreal),* Vol. 4, pp. 725–730.

Steinhaus, E.A. (1958b) Crowding as a possible stress factor in insect disease. *Ecology* 39, 503–514.

Storey, G.K. and Gardner, W.A. (1987) Vertical movement of commercially formulated conidia of the entomogenous fungus *Beauveria bassiana* in four Georgia soil types. *Environmental Entomology* 16, 178–181.

Storey, G.K. and Gardner, W.A. (1988) Movement of an aqueous spray of *Beauveria bassiana* into the profile of four Georgia soils. *Environmental Entomology* 17, 135–139.

Storey, G.K., Gardner, W.A. and Tollner, E.W. (1989) Penetration and persistence of commercially formulated *Beauveria bassiana* conidia in soil of two tillage systems. *Environmental Entomology* 18, 835–839.

Strasser, H. (1999) Beurteilung der Wirksamkeit des biologischen Pflanzenschutzpräparates Melocont® Pilzgerste zur Maikäferbekämpfung. *Der Förderungsdienst* 5, 158–164.

Studdert, J.P. and Kaya, H.K. (1990a) Water potential, temperature, and clay-coating of *Beauveria bassiana* conidia: effect on *Spodoptera exigua* pupal mortality in two soil types. *Journal of Invertebrate Pathology* 56, 327–336.

Studdert, J.P. and Kaya, H.K. (1990b) Water potential, temperature, and soil type on the formation of *Beauveria bassiana* soil colonies. *Journal of Invertebrate Pathology* 56, 380–386.

Studdert, J.P., Kaya, H.K. and Duniway, J.M. (1990) Effect of water potential, temperature,

and clay-coating on survival of *Beauveria bassiana* conidia in a loam and peat soil. *Journal of Invertebrate Pathololgy* 55, 417–427.

Tanada, Y. and Kaya, H.K. (1993) *Insect Pathology*. Academic Press, London.

Teerling, C.R., Pierce, H.D., Borden, J.H. and Gillespie, D.R. (1993) Identification and bioactivity of alarm phermone in the western flower thrips, *Frankliniella occidentalis. Journal of Chemical Ecology* 19, 681–697.

Thomas, M.B., Gbongboui, C. and Lomer, C.J. (1996) Between-season survival of the grasshopper pathogen *Metarhizium flavoviride* in the Sahel. *Biocontrol Science and Technology* 6, 569–573.

Thomas, M.B., Blanford, S. and Lomer, C.J. (1997) Reduction of feeding by the variegated grasshopper, *Zonocerus variegatus*, following infection by the fungal pathogen, *Metarhizium flavoviride. Biocontrol Science and Technology* 7, 327–334.

Thomas, M.B., Blanford, S., Gbongboui, C. and Lomer, C.J. (1998) Experimental studies to evaluate spray applications of a mycoinsecticide against the rice grasshoppers, *Hieroglpyhus daganensis*, in northern Benin. *Entomologia Experimentalis et Applicata* 87, 93–102.

Tomalek, M. (1994) Genetic improvement of *Steinernema feltiae* for integrated control of the western flower thrips, *Frankliniella occidentalis*. In: Smits, P.H. (ed.) *Proceedings, Fourth European Meeting on Microbial Control of Pests, IOBC Working Group on Insect Pathogens and Insect Parasitic Nematodes, 1993*. Bulletin 17, IOBC/WPRS, Zurich, Switzerland, pp. 17–20.

Vago, C. (1963) Predispositions and interrelations in insect diseases. In: Steinhaus, E.A. (ed.) *Insect Pathology: An Advanced Treatise.* Academic Press, New York, pp. 339–379.

Van der Schaaf, D.A., Ravensberg, W.J. and Malais, M. (1991) *Verticillium lecanii* as a microbial insecticide against whitefly. In: Smits, P.H. (ed.) *Proceedings, Third European Meeting on Microbial Control of Pests, IOBC Working Group on Insect Pathogens and Insect Parasitic Nematodes 1991*. Wageningen, the Netherlands, pp. 120–123.

Vanninen, I. and Hokkanen, H. (1988) Effects of pesticides on four species of entomopathogenic fungi. *Annales Agriculturae Fenniae* 27, 345–353.

Verhaar, M.A., Hijwegen, T. and Zadocks, J.C. (1996) Glasshouse experiments on biocontrol of cucumber powdery mildew (*Sphaerotheca fuliginea*) by the mycoparasites *Verticillium lecanii* and *Sporothrix rugulosa. Biological Control* 6, 353–360.

Vestergaard, S., Butt, T.M., Gillespie, A.T., Schreiter, G. and Eilenberg, J. (1995) Pathogenicity of the hyphomycete fungi *Verticillium lecanii* and *Metarhizium anisopliae* to the western flower thrips, *Frankliniella occidentalis. Biocontrol Science and Technology* 5, 185–192.

Vidal, C., Fargues, J. and Lacey, L.A. (1997a) Intraspecific variability of *Paecilomyces fumosoroseus*: effect of temperature on vegetative growth. *Journal of Invertebrate Pathology* 70, 18–26.

Vidal, C., Lacey, L.A. and Fargues, J. (1997b) Pathogenicity of *Paecilomyces fumosoroseus* (Deuteromycotina: Hyphomycetes) against *Bemisia argentifolii* (Homoptera: Aleyrodidae) with a description of a bioassay method. *Journal of Economic Entomology* 90, 765–772.

Wagner, B.L. and Lewis, L.C. (2000) Colonization of corn, *Zea mays* L., by the endophytic fungus, *Beauveria bassiana* (Balsamo) Vuillemin. *Environmental Microbiology* 66, 3468–3473.

Watson, D.W., Mullens, B.A. and Petersen, J.J. (1993) Behavioural fever response of *Musca domestica* (Diptera: Muscidae) to infection by *Entomophthora muscae* (Zygomycetes: Entomophthorales). *Journal of Invertebrate Pathology* 61, 10–16.

Watt, B.A. and LeBrun, R.A. (1984) Soil effects of *Beauveria bassiana* on pupal populations of the Colorado potato beetle (Coleoptera: Chrysomelidae). *Environmental Entomology* 13, 15–18.

Weber, D.C. and Ferro, D.N. (1993) Distribution of overwintering Colorado potato beetle in and near Massachusetts potato fields. *Entomologica Experimentalis et Applicata* 66, 191–196.

Wraight, S.P. and Carruthers, R.I. (1999) Production, delivery and use of mycoinsecticides for control of insect pests of field crops. In: Hall, F.R. and Menn, J.J. (eds) *Methods in Biotechnology,* Vol. 5, *Biopesticides: Use and Delivery*. Humana Press, Totowa, New Jersey, pp. 233–269.

Wraight, S.P., Carruthers, R.I., Bradley, C.A., Jaronski, S.T., Lacey, L.A., Wood, P. and Galaini-Wraight, S. (1998) Pathogenicity of the entomopathogenic fungi *Paecilomyces* spp. and *Beauveria bassiana* against the silverleaf whitefly, *Bemesia argentifolii. Journal of Invertebrate Pathology* 71, 217–226.

Wraight, S.P., Carruthers, R.I., Jaronski, S.T., Bradley, C.A., Garza, C.J. and Galaini-Wraight, S. (2000) Evaluation of the entomopathogenic fungi *Beauveria bassiana* and *Paecilomyces fumosoroseus* for microbial control of the silverleaf whitefly, *Bemisia argentifolii. Biological Control* 17, 203–217.

Young, E.C. (1974) The epizootiology of two pathogens of the coconut palm rhinoceros beetle. *Journal of Invertebrate Pathology* 24, 82–92.

Young, E.C. (1986) The rhinoceros beetle project: history and review of the research programme. *Agricultural Ecology and Environment* 15, 149–166.

Zelger, R. (1993) Maikäfer, in Versuchsbericht 1992 – Sektionen: Obstbau, Pflanzenschutz, Lagerung. (Land-und Forstwirtschaftliches Versuchszentrum Laimburg, Ed.). *Schriftenreihe Laimburg, Auer* 3, 126–138.

Zimmermann, G. (1994) Strategies for the utilization of entomopathogenic fungi. In: *Proceedings, VIth International Colloquium on Invertebrate Pathology and Microbial Control, 1994*. Montpellier, France, pp. 67–73.

4 Biology, Ecology and Pest Management Potential of Entomophthorales

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Introduction

The Entomophthorales are an order of fungi placed in the subdivision Zygomycotina, class Zygomycetes. Currently six families in the Entomophthorales are recognized: Entomophthoraceae, Neozygitaceae, Completoriaceae, Ancylistaceae, Meristacraceae and Basidiobolaceae (Humber, 1989). The two most important families, from the standpoint of entomologists and invertebrate pathologists, are the Entomophthoraceae and the Neozygitaceae. Recent phylogenetic analysis of the Entomophthorales based on ribosomal DNA suggests the Entomophthorales are monophyletic and that *Basidiobolus* spp. may not belong in the Entomophthorales (Jensen *et al.*, 1998). The generic classification in the Entomophthorales is generally clear at the *sensu lato* level. However, more detailed classification into genera *sensu stricto* with or without subgenera is unclear and an area of debate (Remaudière and Keller, 1980; Humber, 1981, 1989; Ben-Ze'ev and Kenneth 1982a, b; Balazy, 1993). There are 200–300 known species in the Entomopthoraceae and 15 species in the Neozygitaceae (Keller, 1997). Undoubtedly, many more species in these two families remain to be described. Figures 4.1–4.24 show examples of a number of insect and mite species from different orders and families with typical external signs of infection by Entomophthorales.

Both hyphomycete and entomophthoralean species have evolved to exploit the ecological niches in which they exist; similarities and differences between the groups are given in Table 4.1. In general, Entomophthorales tend to have narrow host ranges, close associations with foliar insect or mite hosts and conspicuous epizootics (Evans, 1989). In comparison, hyphomycete fungi tend to have wide host ranges and epizootics usually occur only in insect populations in soil (Keller and Zimmerman, 1989).

 Fungi as Biocontrol Agents (eds T.M. Butt, C. Jackson and N. Magan)

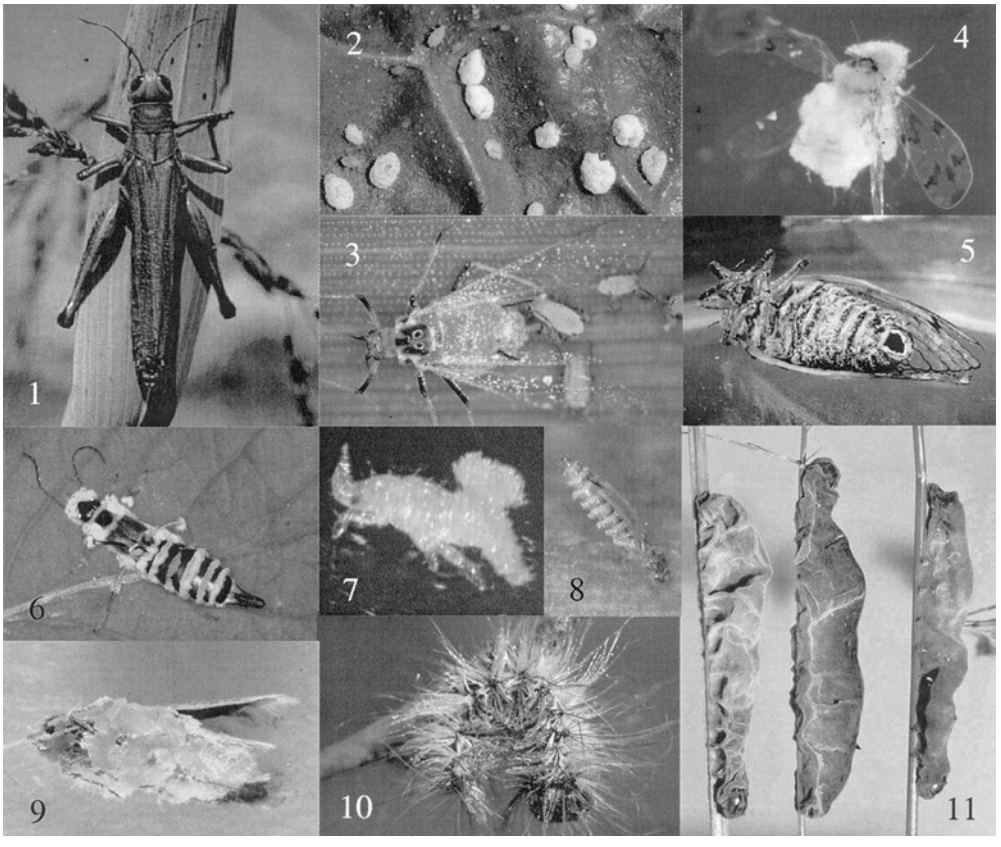

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Fig. 4.1. Differential grasshopper, *Melanoplus differentialis* (Orthoptera: Acrididae), infected with *Entomophaga grylli*. No external indication of infection is seen but the cadaver is filled with resting spores.

Fig. 4.2. Peach–potato aphids, *Myzus persicae* (Hemiptera: Aphididae), infected with *Erynia neoaphidis*.

Fig. 4.3. Alate grain aphid, *Sitobion avenae* (Hemiptera: Aphididae), infected by *E. neoaphidis*. The nymphs will become infected by conidia discharged from the infected adult.

Fig. 4.4. Bandedwinged whitefly, *Trialeurodes abutilonea* (Hemiptera: Aleyrodidae), infected with *Orthomyces aleyrodis*.

Fig. 4.5. Periodical cicada, *Magicicada trecassini* (Hemiptera: Cicadidae), infected with the resting spore stage of *Massospora cicadina*. The resting spores are spread in the environment through a hole in the abdomen of the still-living cicada.

Fig. 4.6. Earwig, *Forficula forficularia* (Dermaptera: Forficulidae), infected with *Zoophthora forficulae*.

Fig. 4.7. Onion thrips, *Thrips tabaci* (Thysanoptera: Thripidae), infected with *Entomophthora thripidum*. The conidiophores emerge in a bundle and discharge conidia from the still-living thrips. Photograph: Florian Freimoser and Anne Grundschober.

Fig. 4.8. Onion thrips infected with *Neozygites parvispora.* Photograph: Anne Grundschober.

Fig. 4.9. Adult diamondback moth, *Plutella xylostella* (Lepidoptera: Plutellidae), infected with *Zoophthora radicans*. Both adults and larvae are susceptible to *Z. radicans.*

Fig. 4.10. Larva of gypsy moth, *Lymantria dispar* (Lepidoptera: Lymantriidae), infected with *Entomophaga maimaiga*. Discharged conidia are seen on the hairs.

Fig. 4.11. Larvae of true army worm, *Pseudaletia unipuncta* (Lepidoptera: Noctuidae), infected with the conidial stage of *Erynia* (= *Furia*) *virescens*. The larvae die at the top of grass stems.

Table 4.1. General characteristics of the Entomophthorales and the Hyphomycetes.

Characteristic	Entomophthorales	Hyphomycetes
Conidia size and number per cadaver	>10 µm in length, relatively small numbers per cadaver	< 10 µm in length, relatively large numbers per cadaver
Conidia discharge	Active, with exceptions: *Massospora* spp., *Strongwellsea* spp. and some *Neozygites* spp.	Not active
Preformed mucus on conidia	Present	Not present with exceptions: *Verticillium* spp., *Hirsutella* spp., *Aschersonia* spp.
Rhizoids	Present in many species	Absent
Ability to modify host behaviour	Can modify behaviour, e.g, *Entomophora muscae*, *Entomophaga grylli*	Cannot modify behaviour with exceptions: *Sorosporella* spp.
Pre-death sporulation	Present in some species: *Entomophthora thripidum*, *Strongwellsea castrans* and *Massospora* spp.	Present in some species: *Verticillium lecanii*
Epizootics	Most common in foliar insects	Most common in soil insects
Host range	Generally narrow	Generally wide, with exceptions: *V. lecanii*, *Hirsutella thomsoni*
Toxin production	Not known	Observed in many species
Saprophytic	Not known, with exceptions: *Conidiobolus* spp.	Known in some species
Resting spores	Present in most species	Not present, with the exception of *Sorosporella* chlamydospores
Virulence	Few conidia required for infection	Many conidia required for infection, with exceptions: *V. lecanii*
Sporulation and germination rate	Fast	Slow
Higher-order conidium production	In all species	Only in *Aschersonia*

The most host-specific Hyphomycetes have life-history attributes similar to Entomophthorales and the Entomophthorales with widest host ranges have attributes similar to the Hyphomycetes (Table 4.1). In terms of exploitation in pest management some ecological attributes of the Entomophthorales are clearly very advantageous. Many species in the Entomophthorales play an important role in regulating host populations under certain conditions. They have caused spectacular epizootics in numerous insect populations and these epizootics commonly reduce the host population to near zero at a local scale: hence their great potential in pest management (Table 4.2).

Ecological, epizootiological and life-history attributes of each species–pest system must be understood to effectively exploit their potential, overcome their problems and design appropriate methods to integrate them into pest management strategies. In this chapter ecological case histories for a number of entomophthoralean species are described; the spatial and temporal distribution, persistence, dispersal and impact on host biology and behaviour are keys to their effective exploitation in specific environments. The use of these species in pest management is discussed with particular emphasis on future opportunities. Fungal species and insect hosts have been

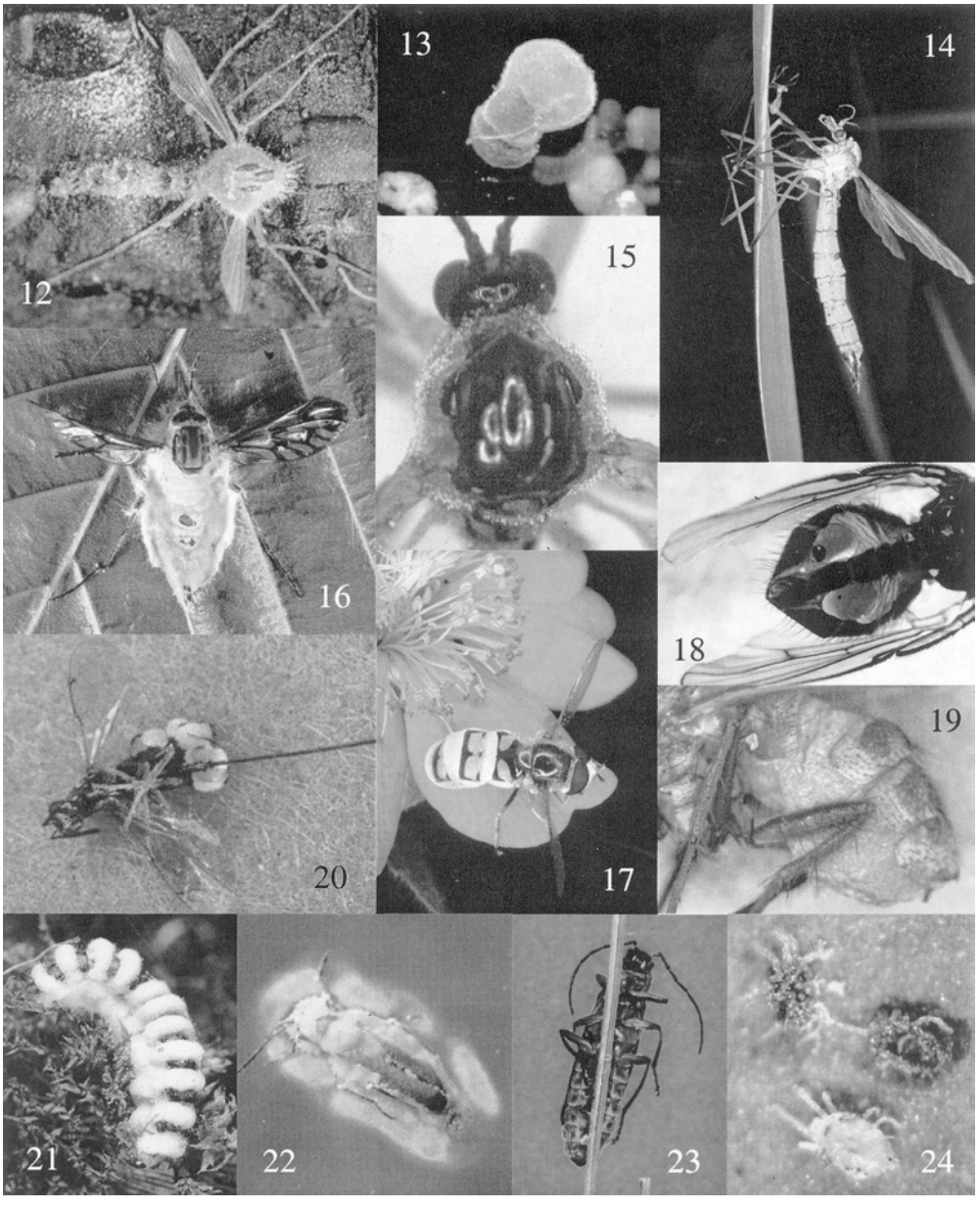

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Fig. 4.12. Nematoceous fly (Diptera: Chironomidae or similar), infected with *Erynia conica*. The cadaver is fixed by rhizoids to a piece of wood floating in the water. Discharged conidia are seen on the wood.

Fig. 4.13. Pupa of a snow-pool mosquito, *Aedes fitchii* (Diptera: Culicidae), infected with *Erynia aquatica*. Conidia are discharged from the floating pupa and infect newly emerging adults, which then die with the resting spore stage.

Fig. 4.14. Adult crane fly, *Tipula* sp. (Diptera: Tipulidae), grasping the vegetation with its legs. The cause of mortality is a fungus from the Entomophthorales (probably from the genera *Batkoa* or *Eryniopsis*). No fungal structures are seen before incubation in a moist chamber.

selected from complementary examples of pest management in agriculture, horticulture, forestry, livestock and rangelands.

Basic Biology

Entomophthoralean life cycles are often complex and usually involve at least two different types of spores: conidia and resting spores. The basic life cycle, as exemplified by *Entomophthora muscae*, is provided in Fig. 4.25. There are many elaborations and exceptions to the basic pattern, generally acting to increase the ability of a species to reach and infect its hosts and these are described in the following case histories.

Conidia are the spore forms responsible for infection during the season when hosts are active. Conidiophores emerge through membranous regions of the host, particularly through the intersegmental membranes, a hymenial layer of the fungus develops and copious numbers of primary conidia are actively discharged by hydrostatic pressure (Figs 4.26, 4.27, 4.30, 4.31). Tens of thousands of conidia can be produced from a single host, with numbers depending on cadaver biomass. While conidia are usually actively discharged from bodies of dead hosts, exceptions occur. For example, conidia are discharged from living thrips by *Entomophthora thripidum* (Fig. 4.7; Samson *et al.*, 1979) and conidia are not actively discharged from mealy-bug cadavers by some *Neozygites* species (B. Papierok, personal communication). Conidia of the cicada-infecting genus *Massospora* are produced within the abdomens of living adult hosts and, as adults fly, successive segments of the abdomen break off and conidia are dispersed (Fig. 4.5; Soper *et al.*, 1976). Species from the genus *Strongwellsea* produce conidia within the abdomens of living flies and an opening is formed in the ventral wall of the abdomen from which conidia are released as the living adult moves (Figs 4.18, 4.44–4.47).

Fig. 4.15. Adult *Ptychoptera contaminata* (Diptera: Ptychopteridae), infected with *Eryniopsis ptychopterae*. Conidiophores emerge on the sides of the thorax and on the abdomen.
Fig. 4.16. Snipe fly, *Rhagio mystaceus* (Diptera: Rhagionidae), infected with *Erynia* (= *Pandora*) *ithacensis*, attached by rhizoids to the underside of a beech-tree leaf.
Fig. 4.17. Hover fly (Diptera: Syrphidae), infected with a species from the *Entomophthora muscae* complex, presumably *E. syrphi*. Photograph: Holger Philipsen.
Fig. 4.18. Arctic fly, *Spilogone dorsata* (Diptera: Muscidae), infected with *Strongwellsea* sp. nov. Conidia are discharged from one or two abdominal holes in living individuals.
Fig. 4.19. Cabbage-root fly, *Delia radicum* (Diptera: Anthomyiidae), infected with *Strongwellsea castrans*. Bright orange resting spores fill the abdomen.
Fig. 4.20. Chalcid wasp, *Torymus druparum* (Hymenoptera: Torymidae), infected with *Entomophthora* sp.
Fig. 4.21. Larva of a beetle, *Lagria* sp. (Coleoptera: Tenebrionidae), infected with *Erynia* s. l. sp. Photograph: Neil Wilding.
Fig. 4.22. Adult cantharid beetle, *Rhagonycha fulva* (Coleoptera: Cantharidae), infected with *Erynia* (= *Pandora*) *lipai* and incubated in a moist chamber. Many discharged conidia are seen on the glass slide.
Fig. 4.23. Cantharid beetle, *R. fulva*, infected with *Tarichium rhagonycharum*. The specimen, filled with brown resting spores, is fixed by rhizoids to the vegetation.
Fig. 4.24. Cassava green mites, *Mononychellus tanajoa* (Acari: Tetranychidae), infected with *Neozygites* sp. The two cadavers at the top of the image have terminated sporulation. Photograph: Italo Delalibera.

Table 4.2. Examples of natural epizootics in insects and mites.

Host species	Main causative fungus	Reference
Acari: Tetranychidae		
Mononychellus tanajoa	*Neozygites floridana*	Delalibera *et al*., 1992; Elliot *et al*., 2000
Tetranychus urticae	*N. floridana*	Smitley *et al*., 1986
Hemiptera: Aphididae		
Myzus persicae	*Erynia neoaphidis*	McLeod *et al*., 1998
Aphis fabae	*E. neoaphidis*	Wilding and Perry, 1980
Microlophium carnosum	*E. neoaphidis*	Hemmati, 1999
Metopolophium dirhodum	*E. neoaphidis*	Hatting *et al*., 1999; Yeo, 2000
Diuraphis noxia	*E. neoaphidis*	Wraight *et al*., 1993
Sitobion avenae	*E. neoaphidis*	Schmitz *et al*., 1993; Nielsen *et al*., 2000a
Aphis gossypii	*Neozygites fresenii*	Silvie and Papierok, 1991; Steinkraus *et al*., 1991, 1995
Aphis citricola	*N. fresenii*	Kuntz, 1925
A. fabae	*N. fresenii*	Dedryver, 1978
Therioaphis trifolii	*Zoophthora radicans*	Kenneth and Olmert, 1975
Rhopalosiphon padi	*Entomophthora planchoniana*	Nielsen *et al*., 2000a
Elatobium abietinum	*N. fresenii*	Nielsen *et al*., 2000b
Hemiptera: Lachnidae		
Schizolachnus piniradiatae	*Erynia canadensis*	Soper and MacLeod, 1981
Hemiptera: Pseudococcidae		
Planococcus citri	*Neozygites fumosa*	Speare, 1922
Hemiptera: Cicadellidae		
Amrasca biguttula	*Batkoa amrascae*	Villacarlos and Keller, 1997
Empoasca kraemeri	*Z. radicans*	Galaini-Wraight *et al*., 1991
Empoasca fabae	*Z. radicans*	McGuire *et al*., 1987c
Hemiptera: Cicadidae		
Massospora levispora	*Okanagana rimosa*	Soper *et al*., 1976
Hemiptera: Aleyrodidae		
Trialeurodes abutilonea	*Orthomyces aleyrodis*	Steinkraus *et al*., 1998b
Thysanoptera: Thripidae		
Frankliniella occidentalis	*Neozygites parvispora*	Montserrat *et al*., 1998
Lepidoptera: Lymantriidae		
Euproctis chryssorhoea	*Entomophaga aulicae*	Speare and Colley, 1912
Lymantria dispar	*Entomophaga maimaiga*	Koyama, 1954; Hajek, 1997c
Orgyia vetusta	*E. aulicae*	Hajek *et al*., 1996c
Lepidoptera: Noctuidae		
Pseudaletia unipuncta	*Furia virescens*	Steinkraus *et al*., 1993c
Lepidoptera: Lasiocampidae		
Malacosoma disstria	*Furia crustosa*	MacLeod and Tyrrell, 1979
Lepidoptera: Geometridae		
Lambdina fiscellaria	*E. aulicae*	Otvos *et al*., 1973
Lepidoptera: Tortricidae		
Choristoneura fumiferana	*Z. radicans*	Vandenberg and Soper, 1978

Table 4.2. continued

Host species	Main causative fungus	Reference
Choristoneura fumiferana	*E. aulicae*	Perry and Regnière, 1986
Lepidoptera: Plutellidae		
Plutella xylostella	*Z. radicans*	Ullyett and Schonken, 1940; Kanervo, 1946; Aruta *et al.*, 1974; Ooi, 1981; Yamamoto and Aoki, 1983; Riethmacher and Kranz, 1994
P. xylostella	*Erynia blunckii*	Tomiyama and Aoki, 1982
Diptera: Muscidae		
Musca domestica	*Entomophthora muscae* and *Entomophthora schizophorae*	Mullens *et al.*, 1987; Steinkraus *et al.*, 1993b; Watson and Petersen, 1993b
Ophyra aenescens	*E. muscae*	Watson and Petersen, 1993b
Diptera: Psilidae		
Chamaepsila rosae	*E. muscae*	Eilenberg and Philipsen, 1988
	Conidiobolus pseudapiculatus	Eilenberg, 1988
Diptera: Fanniidae		
Fannia spp.	*E. muscae*	Mullens *et al.*, 1987
Diptera: Anthomyiidae		
Delia antiqua	*E. muscae*	Carruthers *et al.*, 1985
Delia coarctata	*E. muscae*	Wilding and Lauckner, 1974
Delia floralis	*E. muscae* and *Strongwellsea castrans*	Klingen *et al.*, 2000
Delia radicum	*E. muscae* and *S. castrans*	Eilenberg and Michelsen, 1999; Eilenberg, 2000; Thomsen and Eilenberg, 2000
Orthoptera: Acrididae		
Camnula pellucida	*Entomophaga grylli*	Carruthers *et al.*, 1997
Dissosteira carolina	*E. grylli*	Carruthers *et al.*, 1997
Coleoptera: Curculionidae		
Hypera postica	*Zoophthora phytonomi*	Harcourt *et al.*, 1974

Conidia are relatively fragile and short-lived but can germinate quickly. They are generally sticky, being covered with preformed mucus, aiding in host attachment (e.g. Fig. 4.32). As a general pattern, if primary conidia land on non-host surfaces, they can produce higher-order conidia, e.g. a primary conidium may germinate to produce and actively discharge a secondary conidium and a secondary conidium may germinate to produce and actively discharge a tertiary conidium (e.g. Figs 4.35, 4.36). Although it seems possible that this process could continue until the protoplasm is depleted, the infectivity of successive generations of supernumerary conidia beyond secondary has not been investigated. As a basic pattern, supernumerary conidia are similar in shape to primaries but each generation of supernumerary conidia is slightly smaller in size. The production of successive generations of supernumerary conidia greatly increases the ability of the fungus to reach and infect hosts. With some genera, e.g. *Neozygites*, primary conidia are not infective, secondary conidia are always

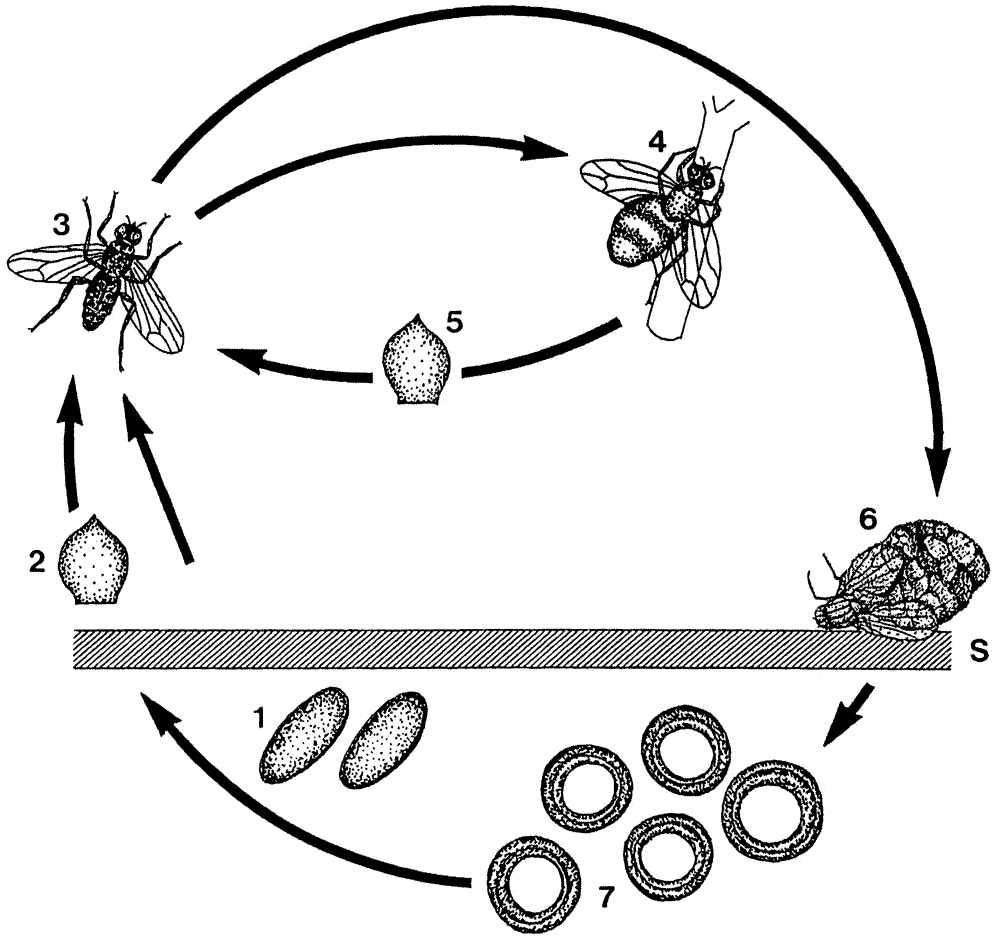

Fig. 4.25. Life cycle of *Entomophthora muscae* in the host, cabbage-root fly (*Delia radicum*, Diptera: Anthomyiidae).
1. Pupae of *D. radicum* overwinter in the soil. 2. During spring, infective conidia of *E. muscae* are produced and discharged from resting spores in the soil. 3. Adult *D. radicum* emerge from pupae during spring and become infected by conidia of *E. muscae*. 4. After the incubation period, *E. muscae* kills *D. radicum*. The dead fly is attached to the vegetation by rhizoids and legs. Conidiophores emerge from the abdomen and produce conidia. 5. Conidia are discharged from the cadaver and infect (eventually after replicative conidiation) other adult *D. radicum*. Several successive infection cycles can take place during the season in the host population. 6. After midsummer, some *E. muscae*-infected *D. radicum* develop resting spores instead of conidia. After the incubation period, flies die with resting spores and drop to the soil surface (S). The abdomen is filled with resting spores (azygospores). 7. Thick-walled resting spores survive in the soil surface layers during winter. In the next spring they will germinate and discharge primary conidia and the cycle is completed.

produced, and the infective secondaries may differ in shape and size from the primaries. In some genera, e.g. *Zoophthora*, higher-order conidia are not always actively discharged but rather produced on fine capilliconidiophores. The capilliconidium is borne on the top of the conidiophore some distance above the surface and remains attached until a host passes. The conidium is sticky and readily attaches to the host, breaking off from the conidiophore at a zone of weakness.

Little is known about host recognition, the first stage in the infection process. In general, conidia are not thought to require exogenous nutrients for growth. Typically, rounded appressoria are produced for host penetration (Fig. 4.37), although some studies report penetration without production of appressoria (Brobyn and Wilding, 1977; Lambiase and Yendol, 1977). Where fungal cells invade, the host cuticle is frequently delaminated and displaced or depressed, suggesting that mechanical pressure is used for cuticular penetration. In addition, histolysis of tissues beneath invading hyphae suggests the utilization of enzymes for penetration.

Once within hosts, some Entomophthorales initially grow as protoplasts, lacking sugar-rich cell walls. It is thought that this stage has evolved to escape detection by the host immune system (Beauvais *et al.*, 1989). Other species grow as unicellular hyphal bodies or coenocytic hyphae and do not have a protoplastic stage. Often, through most of the course of an infection, there are few overt symptoms, although host feeding may decrease as the infection progresses. Some infected insects display negative geotaxis directly prior to death; therefore, cadavers are located in elevated positions (e.g. *E. muscae* and *Entomophaga grylli*), allowing for more efficient dispersal of conidia after discharge. Cadavers of hosts killed by some species are fixed in place above the ground by rhizoids, which would also enhance chances for dispersal of conidia (Figs 4.28, 4.29). Host death is probably caused by physiological starvation of the host when the fungus has consumed all reserves. However, in one study of lepidopteran hosts infected with *Entomophaga aulicae*, a short-lived cell-lytic factor that was most active at or shortly after death is hypothesized as the cause of death (Milne *et al.*, 1994); the onset of cell-lytic activity was detected shortly before death, just as terminal behavioural changes occurred.

Many factors have been associated with the types of spores produced by cadavers but, in general, these factors act to synchronize fungal and host activity (e.g. Hajek, 1997b). The type of spore initiating an infection can play a part in subsequent fungal reproduction.

Resting spores are the most important way that Entomophthorales survive periods when hosts are not present or active. Resting spores of Entomophthorales are either formed by the union of two hyphae (zygospores) or by one hyphal cell rounding up at one end (azygospores). These spores are resistant, with a thick double wall. They are frequently dormant directly after production, often requiring a cold period of several months before germination is possible (Hajek, 1997a). Resting spores may germinate throughout the period of time that hosts are present in the field. Many resting spores do not germinate in the first year after production but survive for numerous years. This is thought to be how *Massospora* spp. infecting periodical cicadas persist during the extended intervals between insect outbreaks (e.g. 17 years). Once resting spores germinate they produce one to several forcibly ejected germ conidia or, in the case of *Neozygites* spp., capilliconidia, either of which is infective to new hosts. While knowledge of germ conidia is meagre, these spores are known to resemble primary conidia except that they can differ reproductively, e.g. by producing only conidia after infection, and thereby act to begin cycles of infection after a period of inactivity.

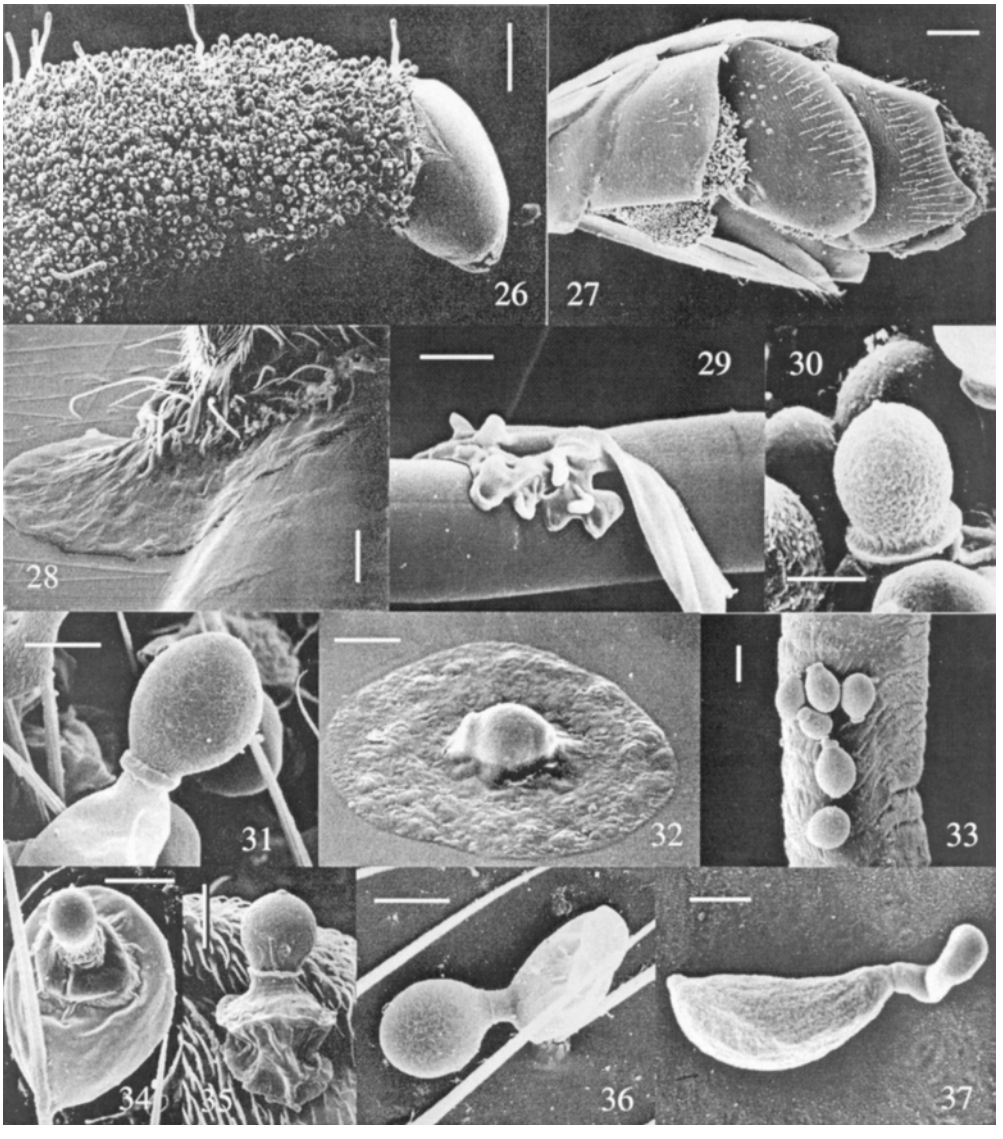

Fig. 4.26. Sciarid larva (Diptera: Sciaridae) infected with *Erynia* sp. Note cystidia above the conidiophore layer. Scale bar = 100 µm. SEM images, Figs 4.26–4.37: José Bresciani and Jørgen Eilenberg.
Fig. 4.27. Adult chalcid wasp, *Torymus druparum* (Hymenoptera: Torymidae), infected with *Entomophthora* sp. Conidiophores emerge only intersegmentally. Scale bar = 250 µm.
Fig. 4.28. Rhizoids and mucoid substances of *Entomophthora schizophorae* fix the mouthparts of an adult carrot fly, *Chamaepsila rosae* (Diptera: Psilidae), to the substrate. Scale bar = 10 µm.
Fig. 4.29. Rhizoid of *Zoophthora forficulae* attaching the earwig host, *Forficula forficularia* (Dermaptera: Forficulidae), to the vegetation. Scale bar = 10 µm.
Fig. 4.30. Primary conidium of *Entomophthora* sp. nov. being discharged from a cantharid beetle, *Cantharis livida* (Coleoptera: Cantharidae). Scale bar = 20 µm.
Fig. 4.31. Primary conidium of *Eryniopsis ptychopterae* formed at the end of the conidiophore. Scale bar = 20 µm.
Fig. 4.32. Discharged primary conidium of *Entomophthora schizophorae*. Note the material (wall laminae and mucus) surrounding the conidium, helping to attach the conidium to the insect cuticle. Scale bar = 10 µm.
Fig. 4.33. Discharged primary conidia of *Neozygites fresenii* attaching to aphid cuticle. Scale bar = 10 µm.
Fig. 4.34. Primary conidium of *E. schizophorae* attaching to the cuticle of a carrot fly and producing a secondary conidium. Scale bar = 20 µm.
Fig. 4.35. Fully formed secondary conidium of *Entomophthora muscae* formed by a primary conidium on the cuticle of a cabbage-root fly, *Delia radicum* (Diptera: Anthomyiidae). Scale bar = 20 µm.
Fig. 4.36. Primary conidium of *Enyniopsis ptychopterae* forming a secondary conidium on the cuticle of the host, *Ptychoptera contaminata* (Diptera: Ptychopteridae). Scale bar = 10 µm.
Fig. 4.37. A germinating capilliconidium (secondary conidium) of *N. fresenii*. Scale bar = 10 µm.

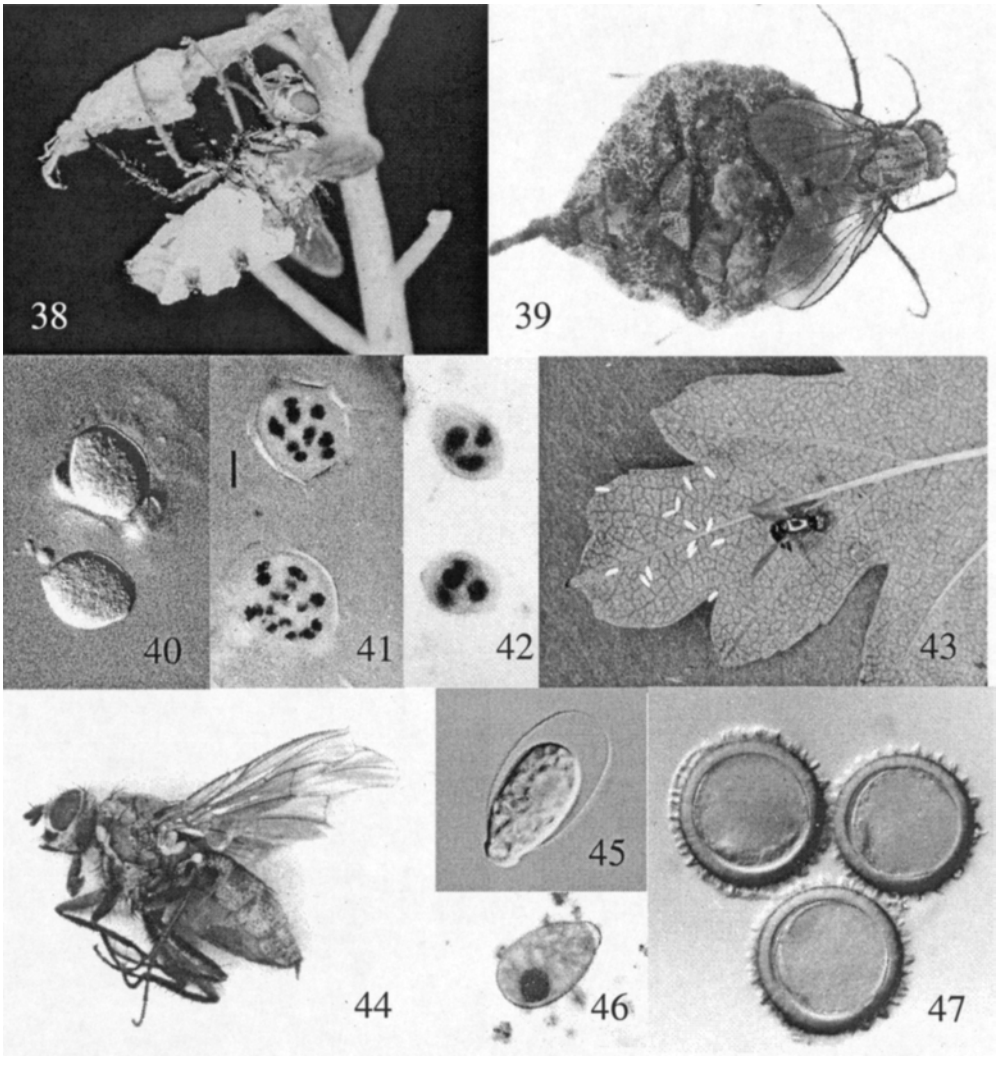

Fig. 4.38. Adult cabbage-root fly, *Delia radicum* (Diptera: Anthomyiidae), infected by *Entomophthora muscae*. The dead fly is attached to the vegetation and conidia are discharged.
Fig. 4.39. Cabbage-root fly, *D. radicum*, infected with *E. muscae*. The abdomen of the dead fly is filled with resting spores (azygospores).
Fig. 4.40. Primary conidia of *E. muscae*.
Fig. 4.41. Primary conidia of *E. muscae*. The nuclei are stained with aceto-orcein. Scale bar = 20 µm.
Fig. 4.42. Primary conidia of *Entomophthora schizophorae*. The nuclei are stained with aceto-orcein. Scale bar = 20 µm.
Fig. 4.43. Carrot fly, *Chamaepsila rosae* (Diptera: Psilidae), infected with *E. schizophorae*. The infected fly deposited eggs on leaves of *Crataegus* sp. 4 m above the soil surface before death due to infection.
Fig. 4.44. Cabbage-root fly, *D. radicum*, infected with *Strongwellsea castrans*. The primary conidia are discharged from living individuals though an abdominal hole.
Fig. 4.45. Primary conidium of *S. castrans*. Note detached spore wall layer. Scale bar = 20 µm.
Fig. 4.46. Primary conidium of *S. castrans*. The nucleus is stained with aceto-orcein. Scale bar = 20 µm.
Fig. 4.47. Resting spores of *S. castrans*. Note the spiny surface. Scale bar = 20 µm.

Ecological Case Histories

Entomophthora muscae, sensu lato *(Entomophthoraceae)*

Taxonomy and distribution

E. muscae was the first entomophthoralean fungus to be described (Cohn, 1855). In the last century, both the biology and potential of *E. muscae* for biological control of adult flies, especially the housefly, *Musca domestica*, have been studied extensively (reviewed by MacLeod *et al.*, 1976). The fungus is readily apparent as dead flies are attached to vegetation, walls, etc. by rhizoids emerging through the proboscis and by the legs of the dead flies (Fig. 4.38).

The genus *Entomophthora* is characterized by campanulate primary conidia (Figs 4.40, 4.41) and *E. muscae* is known from a range of dipteran hosts from different families of Cyclorrhapha, e.g. Muscidae, Anthomyiidae, Fanniidae. Keller (1984, 1987c) demonstrated that *E. muscae* was actually a complex of species and, at that time, *Entomophthora schizophorae* was described as a new species. *E. schizophorae* differs from *E. muscae* in only a few morphological features (e.g. fewer nuclei per conidium (Fig. 4.42)) but both have certain hosts in common. Recently *E. muscae sensu stricto* has been redescribed (Keller *et al.*, 1999). Both *E. muscae* and *E. schizophorae* are recorded in the field as infecting *M. domestica* and anthomyiid flies. While recent studies differentiate between the two species, earlier studies referring to *E. muscae* may indeed be studies of *E. schizophorae*. References referring solely to *E. schizophorae* are indicated in the text, although in most cases (life-cycle, growth *in vivo* and *in vitro*, use in biocontrol) the two species will be treated simultaneously as '*E. muscae*' *sensu lato*.

Host specificity

When considering both indoor and outdoor agricultural/livestock systems, observations indicate that *E. muscae* and *E. schizophorae* are not confined to one host species in specific situations, but may be transmitted to other dipteran species. First, during epizootiological studies of one dipteran host, other infected dipteran species were found at the same site and time (Mullens *et al.*, 1987; J. Eilenberg, unpublished). Secondly, transmission of the pathogen in the laboratory from one dipteran host to other dipteran hosts, including those in other families, has been successful in several cases (Kramer and Steinkraus, 1981; Mullens, 1989; Eilenberg *et al.*, 1990). It is therefore likely that several hosts can be involved in the epizootiology of the fungus, with transmission between different dipteran host species being common. Perhaps only some host species are involved in the entire life cycle; this could explain why resting spores are commonly found in only a limited number of host species. In addition to *E. muscae* and *E. schizophorae*, two similar species originally described as *E. muscae sensu lato* that attack higher Diptera have also been recognized: *Entomophthora syrphi* (Fig. 4.17) and *Entomophthora scatophagae*. They differ in morphological features and their host range in the field. The ability of these latter species to be transmitted between dipteran hosts has, however, not been examined thoroughly; *E. syrphi* was impossible to transmit to *M. domestica* in the laboratory and recent molecular data supports the hypothesis that *E. schizophorae* and *E. syrphi* are different from *E. muscae sensu stricto* (Jensen and Eilenberg, 2000).

The extent to which the natural host range of *E. muscae* and *E. schizophorae* goes beyond Diptera is still largely unknown. *E. muscae* has been recorded from Hymenoptera in the field and infection of Hymenoptera has been achieved in the laboratory. The hymenopteran species *Torymus druparum* occurs at the same time and at the same site as certain dipteran species and infections with fungal pathogens resembling *E. muscae* were found among *T. druparum*. In the laboratory it was possible to transmit *E. muscae* from *T. druparum* to *M. domestica* and *E. schizophorae* from *T. druparum* to *Chamaepsila rosae* (Eilenberg *et al.*, 1987; J. Eilenberg, unpublished). Cluster analysis of molecular data on *E. muscae* from *T. druparum* clustered among other *E. muscae* isolates from Diptera (Jensen and Eilenberg, 2000). The potential life cycle of *E. muscae* could, therefore, be even more complicated.

Epizootiology in annual cropping and livestock-related ecosystems

For some host–pathogen relationships the entire life cycle, including production of resting spores, has been observed in field populations: *Delia radicum*/*E. muscae* (Thomsen and Eilenberg, 2000), *Delia antiqua*/*E. muscae* (Carruthers *et al.*, 1985) and *Delia coarctata*/*E. muscae* (Wilding and Lauckner, 1974). For other host–pathogen relationships, resting spores occur rarely (e.g. *M. domestica*/*E. muscae* (Mullens *et al.*, 1987; Steinkraus *et al.*, 1993b)) or have never been seen in natural populations of the host, even during epizootics (e.g. *C. rosae*/*E. schizophorae* (Eilenberg and Philipsen, 1988)).

E. muscae and *E. schizophorae* have the ability to establish epizootics in populations of *M. domestica*, *Delia* spp. and *C. rosae*, with high prevalences of infection in the host population (*c.* 50–80%) developing within a few weeks (Table 4.2). Some epizootics show sharp peaks in infection and thus the epizootics occur rapidly, while other epizootics establish more slowly and are prolonged. Epizootics can be found both indoors and outdoors. Outdoor studies have shown that there is a tendency for epizootics during late summer and autumn to last longer than epizootics during spring and early summer.

Time to kill is strongly dependent on incubation temperature. At 21°C, *E. muscae* took 7 days to kill *D. antiqua* adults, and this time was extended to > 17 days at 10°C (Carruthers and Haynes, 1985). Similar patterns were observed for *C. rosae* infected with *E. schizophorae* (Eilenberg, 1987a) and *M. domestica* infected with *E. muscae* (Bellini *et al.*, 1992). Primary conidia are discharged from cadavers at a rate dependent on temperature. At 21°C, the peak discharge rate for *E. muscae* from *M. domestica* was 10–12 h post-mortem (Mullens and Rodriguez, 1985). For *C. rosae* infected with *E. schizophorae*, the peak discharge rate was approximately 8 h post-mortem at 20°C (Eilenberg, 1987a). The majority of primary conidia of *E. muscae* and *E. schizophorae* were discharged within 3.75 cm of the cadaver, although a few conidia were discharged as far as 8.75 cm (Six and Mullens, 1997). The total number of conidia discharged per infected individual was calculated to be 5.1×10^4 for *E. schizophorae* (Eilenberg, 1987a). The factors governing the total number of primary conidia per individual were cadaver weight and sex. Since females are larger than males, they produce more conidia (Mullens and Rodriguez, 1985). Secondary conidia of *E. muscae* are significantly more infective than primary conidia (Bellini *et al.*, 1992).

A number of studies have described significant behavioural aspects of the interaction between *E. muscae*/*E. schizophorae* and their hosts. During mating, males, of *M. domestica* are significantly more attracted to *E. muscae*-killed females compared with uninfected females (Møller, 1993). After contact, these males become infected and are

also capable of transmitting conidia to uninfected females during subsequent copulations (Watson and Petersen, 1993a). The fungus is favoured by these behavioural changes, which enhance the chances for dissemination of the fungus in the host population.

After infection, *E. muscae* induces further behavioural changes. *E. muscae*-infected *M. domestica* prefer higher temperatures than uninfected flies (the so-called 'behavioural fever'), with the result that the fungus may die and the fly survive (Watson *et al.*, 1993). This sort of self-cure or resistance based on behaviour is clearly disadvantageous for the fungus.

Host-plant recognition for ovipositing plant-feeding flies can be disturbed by fungus infection. Females of *C. rosae* infected with *E. schizophorae* did not recognize their host plants, even though they were physically capable of depositing eggs. This behaviour was documented in the field by Eilenberg (1987b) who found fertile eggs of *C. rosae* deposited on leaves in hedges at heights of several metres. The eggs were far away from the normal host plant, the carrot, with little chance of survival (Fig. 4.43). This behaviour will influence the effect of *E. schizophorae* on *C. rosae* populations, since infected females will not contribute to population growth.

Towards the end of an infection with *E. muscae*, *M. domestica* is severely affected. An infected fly passes through several phases governed by an endogenous clock, moving more and more sluggishly (Krasnoff *et al.*, 1995). The final result is that the flies attach themselves to the vegetation with their abdomen exposed and, after death, the fungus begins to discharge primary conidia.

These behavioural aspects are important for an understanding of host–pathogen interactions. The fungal-induced changes may eventually prove to be keys to success or failure in biological control programmes. Much has yet to be learnt, because all studies, except one, refer to *M. domestica*. No documentation of similar behavioural aspects in, for example, *Delia* spp. or comparative studies with other isolates of these fungi have been reported.

Neozygites fresenii *(Neozygitaceae)*

Taxonomy and distribution

The first member of this genus and family was described as *Empusa fresenii* from aphids by Nowakowski (1883). Then Witlaczil (1885) described an aphid infection as *Neozygites aphidis*, believing the pathogen to be a gregarine protozoan. Thaxter (1888), unaware of the description by Witlaczil, placed *E. fresenii* in a new subgenus, *Triplosporium*. Giard (1888) noted that the genera *Triplosporium* and *Neozygites* were synonymous. Remaudière and Keller (1980) replaced *Triplosporium* with *Neozygites*. Much of the literature prior to 1980 used the genus *Triplosporium*. Humber and Soper (1981) proposed conservation of the genus *Triplosporium* but this proposal failed, and 15 *Neozygites* species are currently recognized (Keller, 1997). More undescribed species of *Neozygites* from Collembola have recently been discovered (Dromph *et al.*, 1999).

N. fresenii has a worldwide distribution with reports of infected aphids from Africa (Silvie and Papierok, 1991), Australia (Milner and Holdom, 1986), Europe (Gustafsson, 1965a; Thoizon, 1970; Dedryver, 1978), India (Ramaseshiah, 1968), Israel (Bitton *et al.*, 1979), the South Pacific (Keller, 1997) and North America (Kuntz, 1925; Soper and MacLeod, 1963; Thaxter, 1888). This is considered the most common aphid pathogen of tropical regions (Remaudière, 1977). *Neozygites floridana* has

been reported from India (Ramaseshiah, 1971), West Africa (Yaninek *et al.*, 1996), Brazil (Delalibera *et al.*, 1992), Israel (Kenneth *et al.,* 1972), Poland (Mietkiewski *et al.*, 1993), and the USA (Brandenburg and Kennedy, 1981; Smitley *et al.*, 1986).

Host specificity

The members of the Neozygitaceae are specialized as pathogens of small arthropods, mainly mites (Acari), springtails (Collembola), thrips (Thysanoptera) and aphids (Hemiptera) (Keller, 1997). Because the hosts of the Neozygitaceae are small (Fig. 4.48), *Neozygites* spp. may be under-studied compared with species in the Entomophthoraceae, which infect larger arthropods, such as Lepidoptera, Diptera and Coleoptera.

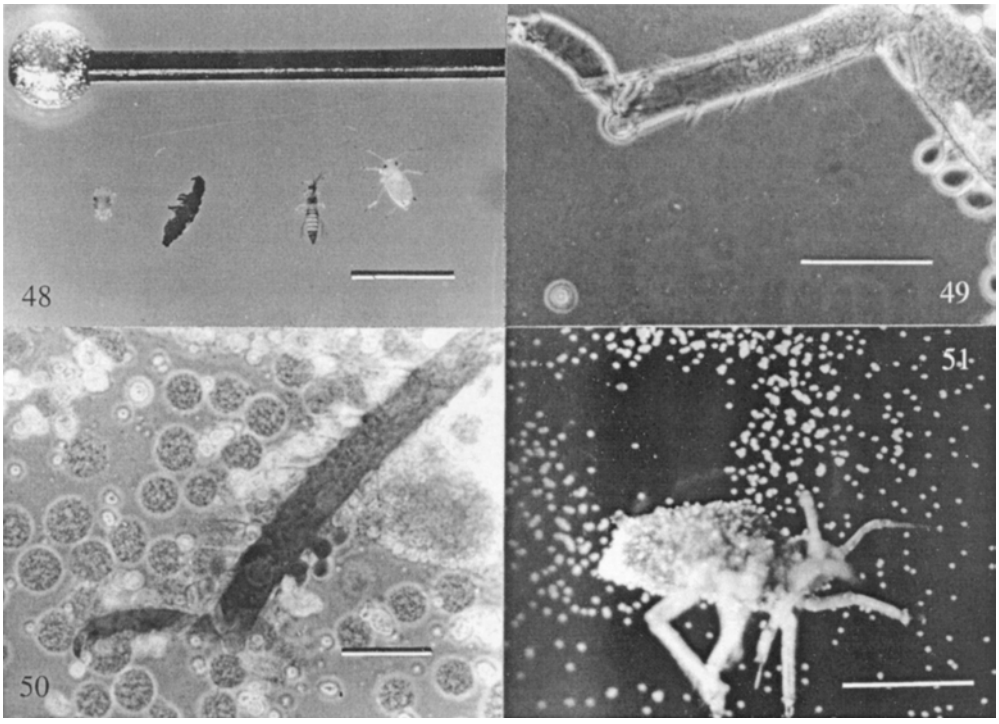

Fig. 4.48. Common hosts of Neozygitaceae next to an insect pin to show their small size. From left to right: spider mite, *Tetranychus urticae* (Acari: Tetranychidae); unidentified collembolan; unidentified thrips (Thysanoptera); cotton aphid, *Aphis gossypii* (Hemiptera: Aphididae). Scale bar = 2 mm.
Fig. 4.49. Leg of a cotton aphid showing infective capilliconidia (secondary spores) of *Neozygites fresenii* attached to the femur. The capilliconidia are the infective spore form and are tightly attached to the insect cuticle by the sticky mucoid hapteron on their apex. Scale bar = 100 μm.
Fig. 4.50. Cotton-aphid leg surrounded by protoplasts of *N. fresenii* liberated from the haemocoel of the host after squashing. Protoplasts are a wall-less vegetative form of the fungus and develop walls shortly before conidiophores are formed. Scale bar = 60 μm.
Fig. 4.51. *N. fresenii*-infected cotton aphid in conidial stage. Conidiophores have erupted through the host's integument, formed conidia and have explosively discharged conidia for several millimetres. Scale bar = 0.5 mm.

What limits the Neozygitaceae to small arthropods? Their common hosts, plant-feeding mites, aphids and thrips, are associated with plant structures. Ancestral *Neozygites* species may have made host shifts from mites to aphids or thrips, or vice versa, because of the close proximity of these various hosts. However, this does not explain why many other arthropods that occur in the same situations do not become infected. During *N. fresenii* epizootics in *Aphis gossypii* populations in Arkansas, many insects in a cotton field are contaminated with capilliconidia (the infective stage) of *N. fresenii* (D. Steinkraus, unpublished data), providing an opportunity for host shifts to occur. Yet hosts other than aphids have not been reported for *N. fresenii*. There also appears to be isolate specificity with respect to aphid host species (L.T. Villacarlos, unpublished).

Few experimental host-range studies have been conducted with *Neozygites* spp. At present it appears that *N. fresenii*, *Neozygites cinarae*, and *Neozygites microlophii* attack only aphids, while others, such as *Neozygites cucumeriformes* and *Neozygites parvispora*, are known only from Thysanoptera (Balazy, 1993) and *Neozygites sminthuri* only from Collembola (Keller and Steenberg, 1997). *N. floridana* and *Neozygites tetranychi* appear to be restricted to mites in the Tetranychidae. For more detailed discussion of host specificity of individual species in the Neozygitaceae, see Keller (1997).

Epizootiology in annual cropping ecosystems

The Neozygitaceae are the most important pathogens of Acari, Aphididae, Collembola and Thysanoptera in some geographical regions, particularly in tropical areas (Table 4.2). In addition to these specific examples, *Neozygites* spp. are recognized as important natural control agents of cassava green mite, *Mononychellus tanajoa,* in Brazil and West Africa (Fig. 4.24; Keller, 1997; Elliot *et al.*, 2000). Similar studies have shown the importance of *Neozygites* spp. in the natural control of mites on groundnuts (Boykin *et al.*, 1984), cotton (Carner and Canerday, 1968) and lima beans (Brandenburg and Kennedy, 1983). Their impact is also extremely fast; Klubertanz *et al.* (1991) found that a *Neozygites* sp. reduced two spotted spider mite, *Tetranychus urticae*, populations by up to 95% over a 6-day period on soybean.

There are a number of reasons why the Neozygitaceae are effective in causing epizootics in mites and aphids. First, their life cycles are rapid. The time from the initial aphid contact with a capilliconidium to the death of the host and fungal sporulation can be as short as 3 days (Steinkraus *et al.*, 1993a). Secondly, whereas some Entomophthorales attack only one host life stage, *Neozygites* spp. attack all stages, except eggs. Thirdly, a large number of primary conidia are produced per host, even though the hosts are small. The life cycle starts with the primary conidium, about 15 μm in diameter. Primary conidia germinate within 6–9 h to form capilliconidia, the infective stage. Almond-shaped capilliconidia are formed on the apex of capilliconidiophores and have a sticky mucoid hapteron. Capilliconidia are formed at the height of an aphid's femur. When aphids walk across a leaf, capilliconidia adhere tightly to the aphid (Fig. 4.49), rapidly germinate and penetrate the aphid's exoskeleton. Once within an aphid's haemolymph, the fungus initially reproduces vegetatively as protoplasts (Fig. 4.50) and, after 3 days, forms hyphal bodies. Three to four days after the aphid host is penetrated by the fungus, the aphid dies and about 3000 primary conidia of *N. fresenii* are explosively discharged per infected aphid (Fig. 4.51) (Steinkraus *et al.*, 1993a). Approximately 75% of these conidia enter the air, while 25% immediately hit the leaf on which the cadaver is located. Fourthly, during cotton aphid epizootics, the num-

ber of conidia of *N. fresenii* present in the air is immense. Conidia of *N. fresenii* are readily distinguished from other particulate matter in the air by their characteristic shapes, sizes and staining properties (Fig. 4.52) (Thaxter, 1888; Soper and MacLeod, 1963; Steinkraus *et al.*, 1991). Rotorod aerial spore traps (Fig. 4.53) collected up to 90,000 *N. fresenii* primary conidia per cubic metre of air during the night in a cotton field in Louisiana (Steinkraus *et al.*, 1999), similar to results reported previously from Arkansas (Steinkraus *et al.*, 1996b). When healthy sentinel *A. gossypii* were exposed overnight for 8 h to the air in a Louisiana cotton field during an epizootic, up to 50% of the aphids became infected (Steinkraus *et al.*, 1999).

The discharge of *Neozygites* conidia into the air appears to be strictly a mecha-

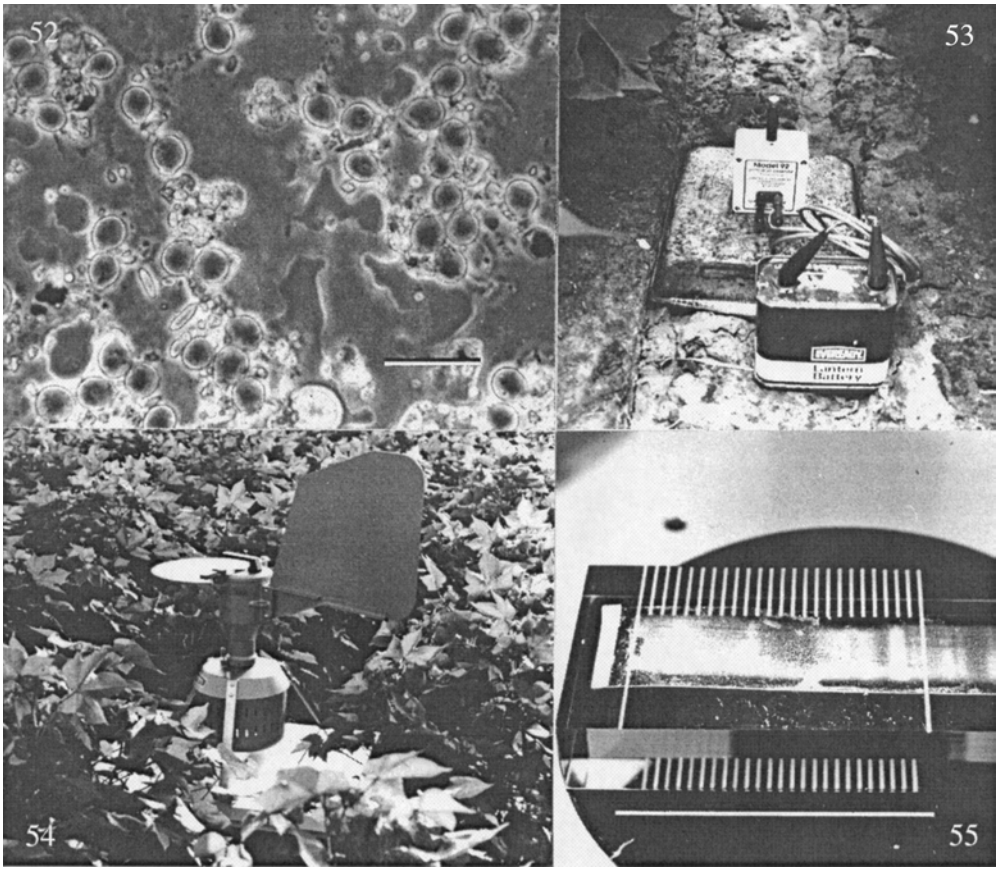

Fig. 4.52. Primary conidia of *Neozygites fresenii* trapped on the silicone grease-treated surface of a plastic rod from a rotorod aerial spore trap. The conidia were collected from the air in a cotton field in Louisiana during an epizootic in cotton aphids. Scale bar = 40 μm.
Fig. 4.53. Rotorod sampler in a cotton field. The battery-powered aerial spore sampler spins two plastic rods through the air, catching particles such as spores, dust and pollen.
Fig. 4.54. Burkard 7-day aerial spore trap in a cotton field. This trap sucks air through an orifice, aerial particles are impacted on a silicone grease-treated tape and can be quantified by hour for 7 days.
Fig. 4.55. A 24 h portion of the 7-day collection of aerial particles from a Burkard trap. Each 24 h section of the adhesive trap can be mounted on a slide, examined under a microscope and the spores present in the air quantified. Scale bar = 50 mm.

nism of dispersal, both short-range and long-range, and not directly involved in the infection process. Studies on the aerobiology of *N. fresenii* showed that most conidia in the air were primary conidia, with capilliconidia making up only 11.3% of the forms collected (Steinkraus *et al.*, 1996b). Most probably, capilliconidia found in the air were dislodged from the surfaces of leaves. Primary conidia of *N. fresenii* were damaged by relative humidities (RH) below 90%. Steinkraus and Slaymaker (1994) found that exposure of *N. fresenii* primary conidia to 75% RH for only 1 min significantly reduced subsequent capilliconidia formation to 29% versus 76% at 100% RH. The deleterious effect of low humidity on primary conidia is mitigated by the periodicity of discharge of *N. fresenii*. Counts of conidia caught in aerial spore traps (Figs 4.54, 4.55) indicated that active discharge of primary conidia occurred mainly between 0100 and 0300 h (Steinkraus *et al.*, 1996b, 1999). Capilliconidia are rapidly formed and are more resistant to lower humidities than primary conidia. Some capilliconidia remain infective for 2 weeks at 75% RH (Steinkraus and Slaymaker, 1994). Therefore, *N. fresenii* has the capability to kill its hosts shortly after nightfall, and then rapidly produce and discharge its primary conidia at night when the RH is high. Before daylight most of the low-humidity-sensitive primary conidia have germinated to form the more long-lived infective capilliconidia.

Some individual insects infected with *Neozygites* spp. form resting spores (zygospores) (Fig. 4.84). These spores are generally dark-walled and provide a long-lived spore form in the soil or on tree bark (Bitton *et al.*, 1979). In Israel resting spores of *N. fresenii* were synchronized to germinate in the spring concurrent with the build-up of *Aphis spiraecola* aphids on citrus trees (Bitton *et al.*, 1979). Thus, resting spores germinate under certain environmental conditions to produce capilliconidia that infect new hosts. Resting spores are not explosively discharged, and hosts infected with this stage of the pathogen generally become very fragile so that the host's cuticle is easily ruptured, dispersing resting spores on to plant surfaces and the soil.

The Neozygitaceae are unusual entomophthoraleans in that they appear to function best in hot weather. This is important because many crop pests, particularly mites and aphids, reproduce most rapidly during midsummer. For instance, *Erynia neoaphidis* has been found infecting *A. gossypii* on cotton in late autumn and *Myzus persicae* on winter spinach in Arkansas (Fig. 4.2), but has not been found during the summer in these areas (McLeod *et al.*, 1998). Thus, unlike *N. fresenii*, *E. neoaphidis* is of no importance in controlling the immense cotton aphid outbreaks that occur across the USA during June and July (Steinkraus *et al.*, 1995). The fact that *Neozygitaceae* are adapted to hot, humid, even tropical conditions has been reported by many researchers (Gustafsson, 1965a; Steinkraus *et al.*, 1991; Keller, 1997). In the Mississippi delta, epizootics of *N. fresenii* occur even in non-irrigated fields that have experienced no rain for up to a month before the epizootic. Epizootic development under these conditions is apparently supported by high night-time humidities.

Entomophaga maimaiga *(Entomophthoraceae) and other entomophthoralean fungi from forest Lepidoptera*

Several species of entomophthoralean fungi cause epizootics in outbreak species of forest-dwelling Lepidoptera in North America: *Entomophaga maimaiga* (Figs 4.56–4.60) from *Lymantria dispar*, *E. aulicae* and *Zoophthora radicans* from *Choristoneura fumiferana* and *Lambdina fiscellaria*, and *Erynia gastropatchae* (= *Furia crustosa*) from

Malacosoma disstria (Figs 4.61–4.63). *E. maimaiga* has been most studied and a recent review was published on this species (Hajek, 1999). In the following sections, comparisons are drawn among the four host–pathogen systems to investigate commonalities and differences in strategies developed by these different entomophthoralean species occupying similar ecological niches in forests.

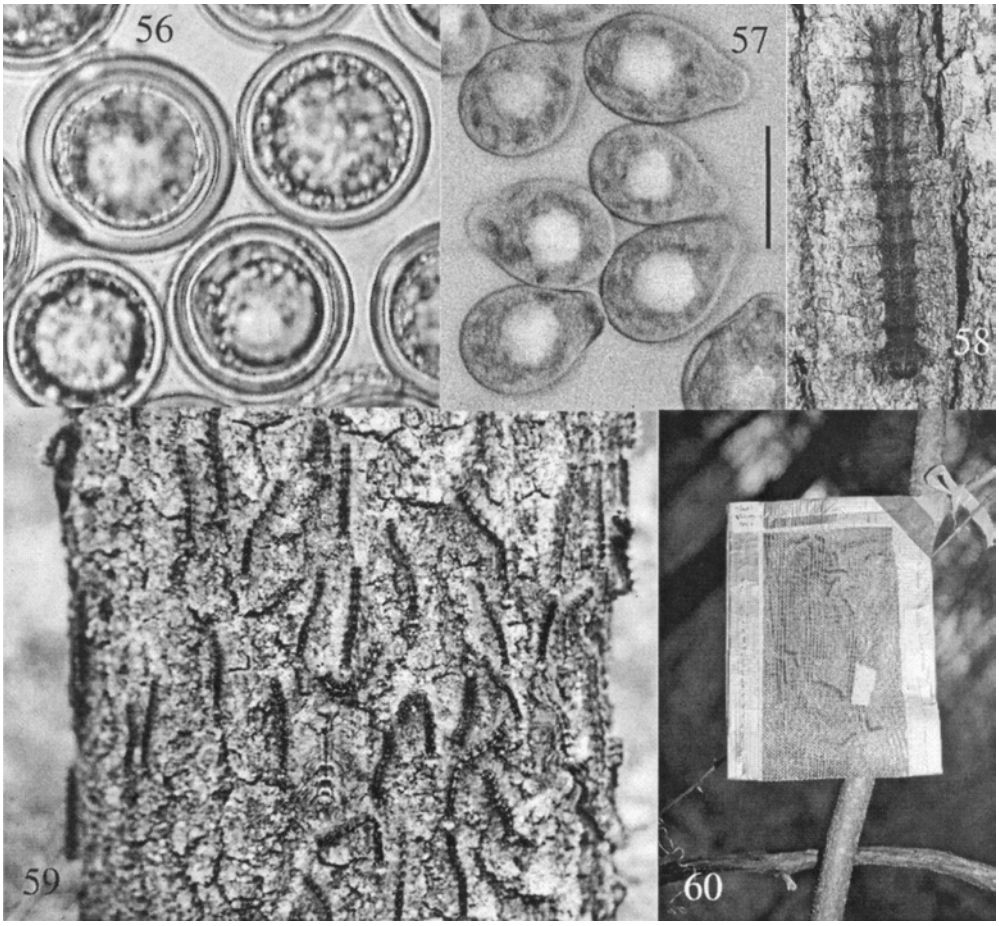

Fig. 4.56. Mature resting spores of *Entomophaga maimaiga.* Scale bar = 20 μm.
Fig. 4.57. Pear-shaped, primary conidia of *E. maimaiga*. The nuclei are stained with aceto-orcein. Scale bar = 20 μm.
Fig. 4.58. Cadaver of a late-instar larva of gypsy moth, *Lymantria dispar* (Lepidoptera: Lymantridae), infected with *E. maimaiga*. Although there is no external indication of fungal infection, this cadaver is filled with resting spores (azygospores) and chacteristically is attached to tree bark by prolegs, with head downwards. Earlier instars killed by *E. maimaiga* externally produce conidia (see Fig. 4.10). Photograph: Donald Specker.
Fig. 4.59. An epizootic caused by *E. maimaiga* in late-instar gypsy moth. Photograph: Gary Bernon.
Fig. 4.60. Window-screening cages used to expose gypsy-moth larvae in the field to evaluate location and activity of *E. maimaiga* inoculum.

Taxonomy and distribution

E. maimaiga is thought to be native to northern Asia (Japan, northern China, Korea and far-eastern Russia) but has also been reported establishing epizootics in North America since 1989 (Andreadis and Weseloh, 1990; Hajek *et al.*, 1990b; Table 4.2). Comparisons at the molecular level have shown that *E. aulicae* is a species complex, with *E. maimaiga* included in one of four groups. Fungi in the other three groups currently retain the name *E. aulicae* (Walsh, 1996). While this species complex is worldwide in distribution, one of the *E. aulicae* groups (group III) is known only from one strain isolated in Europe (only one European isolate was available for comparison at the molecular level). Among the two remaining *E. aulicae* groups, many isolates belong to group I, which is associated with a diversity of lepidopteran families and is known from both North America and Japan. Group II has been isolated only from Noctuidea in North America.

Host specificity

The species *E. maimaiga* was initially differentiated from the *E. aulicae* species complex, in part, because this is the only member that is known to infect *L. dispar* (Soper *et al.*, 1988). Host specificity of this species has been extensively studied due to its potential application for control. In the laboratory, *E. maimaiga* infected 36% of 78 non-target lepidopteran species tested at a concentration of conidia yielding > 50% infection in *L. dispar*, although per cent infection was usually low (Hajek *et al.*, 1995a). The only non-targets consistently infected at high levels were species of Lymantriidae (the family including *L. dispar*). To evaluate host specificity in the field, 1511 lepidopteran larvae of 52 species belonging to seven lepidopteran families were collected and reared during *E. maimaiga* epizootics in sympatric *L. dispar* populations. Throughout the field season only two non-target individuals were infected: one lasiocampid and one noctuid (Hajek *et al.*, 1996a). Due to the discrepancy between host specificity in the laboratory (physiological host range) and in the field (ecological host range), it is hypothesized that, in addition to physiological interactions between host and pathogen, temporal and spatial factors are also very important in determining the host specificity of *E. maimaiga*.

There are two main conditions under which *E. maimaiga* is unable to successfully attack hosts. First, *E. maimaiga* can develop in some non-host lepidopterans if injected into the haemocoel, but it cannot penetrate the insect cuticle (Hajek *et al.*, 1995a). For other non-host lepidopterans, the fungus cannot survive even when injected directly into the haemocoel (Hajek *et al.*, 1995a). *E. maimaiga* and *E. aulicae* both occur in the host haemocoel as protoplasts lacking cell walls, which is thought to aid in escaping detection by hosts. However, prolonged elevated levels of prophenoloxidase have been detected in *L. dispar* larvae inoculated with *E. aulicae* (Bidochka and Hajek, 1998), suggesting that this species is detected by the immune response. Plasma membranes of protoplasts contain glycoproteins that differ between *E. maimaiga* and *E. aulicae*; it is hypothesized that these surface glycoproteins may lead to detection of protoplasts in non-permissive hosts (Bidochka and Hajek, 1996).

While *Z. radicans* has been isolated from many different insects, bioassays have demonstrated that at least some strains of this species may be quite host-specific, even to the family or species levels. *E. gastropatchae* has been isolated only from members of the genus *Malacosoma* and appears to be very host-specific (Figs 4.61–4.63). In

Fig. 4.61. Mature resting spores of *Erynia gastropachae* having undulate epispores. Scale bar = 20 μm.
Fig. 4.62. Primary conidia of *E. gastropachae.* Scale bar = 20 μm. Photograph: Melanie Filotas.
Fig. 4.63. Cadavers of late instars of *Malacosoma disstria* (Lepidoptera: Lasiocampidae) infected and killed by *E. gastropatchae.* Cadavers are filled with resting spores (azygospores) and hang head downwards (arrow).

contrast, *E. aulicae* group I has been isolated from many lepidopteran families, though, as with *Z. radicans*, this does not preclude specificity of individual strains (Walsh, 1996).

Epizootiology in forest ecosystems

Forest ecosystems differ substantially from agricultural or pasture environments, where much of the research on entomophthoralean fungi has been conducted. Many forests are natural ecosystems, and the soil and leaf litter often remain largely undisturbed. After host death, cadavers of entomophthoralean-killed larval Lepidoptera containing resting spores (azygospores in the case of *E. maimaiga*) are often found attached to tree trunks (Figs 4.58, 4.59, 4.63). *E. maimaiga*-infected cadavers eventually fall to the ground, where resting spores are rapidly released from them, especially when it rains

(Hajek *et al.*, 1998b). The majority of resting spores remain in the organic layer of the soil, with mean densities of 4751 *E. maimaiga* resting spores g^{-1} dry soil at the bases of trees after an epizootic (Hajek *et al.*, 1998a). The greatest concentrations of resting spores occur directly around the bases of trees (Hajek *et al.*, 1998a). Resting spores are retained near the soil surface, where, upon germination, germ conidia can be actively discharged. Although some resting spores of *E. maimaiga* germinate the year after production, many do not (Hajek and Humber, 1997). Bioassays conducted by caging larvae on soil have demonstrated that *E. maimaiga* resting spores can persist in undisturbed forest soil for at least 6 years (Weseloh and Andreadis, 1997).

Forests also differ from habitats with only herbaceous vegetation due to the depth of the plant canopy. Many forest Lepidoptera feed on foliage quite distant from the soil, but the principal reservoir of entomophthoralean resting spores is in the soil. Therefore, either the hosts must travel to the soil for primary infection to occur, or infective entomophthoralean conidia must travel to the hosts. Both germ conidia from germinating resting spores and primary conidia from the surfaces of cadavers are actively discharged. The extent to which germ conidia produced from soil-borne resting spores become airborne is not known. Studies of this phenomenon are clearly difficult to undertake because germ conidia are morphologically identical to primary conidia, although they can differ reproductively (Hajek, 1997a). Infections initiated by resting spores produce only conidia, while infections initiated by conidia from cadavers can produce either conidia or resting spores (Hajek, 1997b). For infections initiated by conidia discharged from cadavers, production of resting spores versus conidia has been associated with a diversity of factors: host age or moulting status, environmental conditions, including temperature, season, light and humidity, fungal dose, isolate or combination of isolates and attenuation (Hajek and Shimazu, 1996). Cadavers of *L. dispar* larvae producing *E. maimaiga* conidia are generally found in the foliage (Hajek *et al.*, 1998b). Therefore, when conidia are discharged from cadavers, there is a good chance that they will become airborne. Sampling the air-spora and exposing caged larvae (Fig. 4.60) within a forest demonstrated that *E. maimaiga* conidia were abundant in the air at particular times (Hajek *et al.*, 1999). Abundance of *E. maimaiga* conidia in the air was autocorrelated and positively associated with leaf wetness and wind and negatively associated with temperature. These results emphasized the episodic nature of conidial abundance and the association with weather events of prolonged duration. The movement of wind carrying entomophthoralean conidia would differ between agricultural settings with a low plant canopy and forests with a much deeper plant canopy. Studies of the dispersal of *E. maimaiga* have shown that rates of spread differ on local and regional scales. Presumably different mechanisms for dispersal of conidia occur within forest stands and above the forest canopy where airborne conidia are transported long distances (Dwyer *et al.*, 1998).

Some forest lepidopteran larvae have a great tendency to wander, especially as late instars. For each of the four species of fungi being discussed, infection may be enhanced when hosts travel to the ground. For *E. aulicae* and *Z. radicans*, infection was not noted until after *C. fumiferana* became fifth instars (Vandenberg and Soper, 1978; Perry and Regnière, 1986). Perry and Regnière (1986) deduced an association between increased infection in late instars and the peak of larval wandering at the fifth to sixth instar; in fact, an epizootic caused by *E. aulicae* occurred during a season of defoliation, which apparently enhanced infection due to increased wandering on the soil by larvae searching for food. Similarly, *E. gastropatchae* epizootics have only been noted in the field after *M. disstria* became late instars and wandered on the forest floor (M.J.

Filotas and R.S. Soper, personal communciations). Similarly, late instars of *L. dispar* wander in the leaf litter and rest there during daylight hours, while second and third instars primarily feed in the foliage. Few *E. maimaiga* infections have been found among early instars of *L. dispar*, with infection prevalence only increasing once late instars were present (Hajek, 1997c). Empirical data from all of these host–pathogen systems have fostered the hypothesis that resting spores predominantly initiate cycles of infection once late instars wander on the forest floor. However, early instars also have contact with the forest floor, either during neonate dispersal (e.g. *L. dispar* ballooning) or when early instars fall or are blown from trees. Studies of the activity of *E. maimaiga* resting spores demonstrate initiation of germination just prior to *L. dispar* egg hatch and continued germination throughout the period of larval activity (Hajek and Humber, 1997; A.E. Hajek, unpublished data). It is not known whether resting spores of *E. aulicae*, *E. gastropatchae* or *Z. radicans* germinate for such a prolonged period. Germination of *E. maimaiga* resting spores is positively associated with soil moisture (Hajek and Humber, 1997).

A simulation model of *E. maimaiga* infection cycles in *L. dispar* has demonstrated that infections initiated by conidia produced on killed hosts are primarily responsible for the exponential increase in infection characteristic of epizootics. Therefore, epizootics develop due to cycles of conidial infections initiated throughout the season when larvae contact the soil (predominantly as mobile stages, such as late instars) and become infected. Information about differential exposure to fungal conidia based on larval behaviour has been incorporated into a simulation model for *E. maimaiga* (Hajek *et al.*, 1993). The model estimated that four to nine cycles of infection could occur during the yearly field season of *c.* 2 months, with ambient moisture levels strongly influencing the number of infection cycles.

L. dispar larvae held at 20°C die from *E. maimaiga* infection approximately 4–6 days after conidial inoculation (Hajek *et al.*, 1993). *E. maimaiga* (Hajek, 1989) and *E. aulicae* (Tyrrell, 1990) infections did not cause overt changes among infected larvae until weight gain decreased 2 days or 1 day prior to death, respectively. Changes in the behaviour of *C. fumiferana* larvae began with sluggishness at *c.* 5 h before death to near-immobility 4 h later (Tyrrell, 1990). For *E. maimaiga*, after larval death there is a lag of *c.* 17 h before conidia are produced from cadavers (Hajek *et al.*, 1990a) or > 2 days before mature resting spores are produced (Hajek and Humber, 1997). Either type or both types of propagule can be produced from an individual cadaver. The primary determinant of the type of spore produced is larval instar, with conidia predominantly produced from *E. maimaiga*-killed early instars and resting spores produced within later instars (Hajek and Shimazu, 1996). Third to sixth instars of *L. dispar* infected with *E. maimaiga* were more abundant in the understorey vegetation than high in the tree canopy (Hajek and Webb, 1999). Cadavers of *E. maimaiga*-killed *L. dispar* larvae producing conidia are most frequently found on the undersides of twigs and leaf petioles of understorey vegetation, while cadavers containing resting spores are generally found attached to lower regions of tree trunks (Hajek *et al.*, 1998b). The extent to which the differential distribution of cadavers within trees is determined by the fungus is not known. However, healthy early instars of *L. dispar* generally occur in the tree canopy, so the general location of conidia-producing cadavers of early instars does not differ markedly from the location of healthy early instars. Alternatively, late instars of *L. dispar* travel from dark, sheltered locations (leaf litter or under bark flaps) to the tree canopy and back daily and therefore spend at least part of this time on tree trunks. The predominant distribution of late-instar cadavers on tree trunks (and not

in the leaf litter or tree canopy) suggests a fungal-induced behaviour causing larvae to be located on tree trunks at the time of death, rather than in lower sheltered locations or in the tree canopy.

Entomophaga grylli *(Entomophthoraceae)*

Taxonomy and distribution

E. grylli was first collected in Europe from a *Gryllus* sp. and named by Fresenius (1858) and since then has been variously known as *Entomophthora grylli*, *Empusa grylli* and *Conidiobolus grylli* (Carruthers *et al.*, 1997). Since the first collections in Europe, *E. grylli* has been collected worldwide; however, it is now believed that these collections are not the same species as the original (Carruthers *et al.*, 1997). As with other entomophthoralean species, *E. grylli* exists as a complex of pathotypes, but none of these has been raised to species status. *E. grylli sensu stricto* appears to have a mainly European distribution. Pathotypes 1 and 2 are from North America, pathotype 3 is from Australia and pathotype 4 is from Japan and new pathotypes are continually being recorded (Carruthers *et al.*, 1989; Humber, 1989). Pathotypes 1, 2, 3 and 4 are sometimes referred to as *E. macleodii, E. calopteni, E. praxibuli* and *E. asiatica,* respectively, and we await formal descriptions. All pathotypes have been recorded causing epizootics (some examples in Table 4.2).

Host specificity

All members of the *E. grylli* species complex are pathogens of grasshoppers and locusts and, as such, are not pathogenic to other organisms (MacLeod, 1963; Carruthers *et al.*, 1989). Each of the two pathotypes/species from North America has a very different host range. Pathotype 1 infects band-winged grasshoppers in the Oedipodinae (e.g. Streett and McGuire, 1990), whereas pathotype 2 is associated with species in the Melanoplinae. These acridid species occur in the same habitat, but laboratory and field studies indicate that there is little or no cross-infection of the two pathotypes between these hosts (Pickford and Riegert, 1964; Carruthers and Soper, 1987; Ramoska *et al.*, 1988). The biology of these two pathotypes is also very different. Both pathotypes produce resting spores, which produce infective germ conidia to initiate the infection cycle in their hosts. In pathotype 1, this culminates in infected insects either producing actively discharged conidia for further cycling, or harbouring resting spores for overwintering. In pathotype 2, actively discharged conidia never occur, and resting spores are produced in all infected hosts. This pathotype is therefore thought to have only one infection cycle from each resting spore (Carruthers *et al.*, 1997). This has made interpretation of field sampling in the past very difficult.

Different grasshopper species have variable levels of susceptibility to the four different pathotypes and this seems to be resistance conferred at the level of the cuticle rather than after penetration (Ramoska *et al.*, 1988). Of all the pathotypes tested, pathotype 3 has the widest host range among North American grasshoppers (Ramoska *et al.*, 1988). This has raised concerns related to its release in classical control; however, in the field, only eight out of 20 species of grasshopper were found infected, and they were all pest species (Carruthers and Onsager, 1993). Further research has helped to confirm the ecological/physiological host range of *E. grylli*, pathotype 3 (Carruthers *et al.*, 1997).

Epizootiology in rangeland ecosystems

Infection with *E. grylli* is commonly called 'summit disease' as infected and dying insects exhibit abnormal behaviour and climb to the top of vegetation, dying in a head-up position, grasping the plant stems (Fig. 4.64; Evans, 1989). Insect death occurs in the late afternoon and early evening, synchronizing sporulation and infection with optimal conditions of high humidity, cool temperatures and zero ultraviolet radiation during the night (Carruthers and Soper, 1987; Carruthers *et al.*, 1988, 1992). Given suitable abiotic conditions, *E. grylli* is able to sporulate within hours of host death (Fig. 4.65). If abiotic conditions are not favourable, the cadaver desiccates, but the fungus remains viable for extended periods of time, with the ability to rehydrate, sporulate and desiccate repeatedly (Sawyer *et al.*, 1997). Spore trap data from areas adjacent to epizootics revealed very few *E. grylli* conidia, although many smaller conidia from other fungal species were collected (Chatigny *et al.*, 1979; Carruthers *et al.*, 1997).

The fungus proliferates within infected insects as protoplasts, only forming hyphal bodies 1–2 days prior to host death (Funk *et al.*, 1990). One study of the development of *E. grylli* pathotype 2 within grasshoppers demonstrated that the fungus only affected fat body and neural tissue in early stages of infection, and muscle tissue was penetrated only after host death (Funk *et al.*, 1993). Speed of kill is temperature-related but death usually occurs within 7 days, more slowly when resting spores are produced (Ramoska *et al.*, 1988; Carruthers *et al.*, 1992).

At the time that hyphal bodies are produced, disease symptoms also become more obvious; the host becomes sluggish, stops feeding and begins abnormal posturing behaviour. Egg laying and feeding are both retarded prior to death (Carruthers *et al.*, 1997). Recent studies with pathotype 1 infecting *Campanula pellucida* have shown that host behaviour also has an impact on pathogen survival and development in the host. Acridids naturally bask in direct sunlight, raising their body temperatures by up to 10–15°C above air temperature. This can raise the temperature in the haemocoel above the thermal limit of *E. grylli* (Carruthers *et al.*, 1992). This is the natural behaviour of the insect and is not encouraged by infection. However, in some areas of the USA, during some seasons, significant fungus mortality could occur due to host basking, a behaviour which, therefore, may cure infected insects.

In pathotype 1, differentiation to produce resting spores is favoured in late-stage instars at warmer temperatures (Carruthers *et al.*, 1997). Infected insects harbouring resting spores (Fig. 4.66) fall to the ground, releasing the spores into the soil at a variable rate, depending on rain and wind (Fig. 4.67). Here, spores may lie dormant for two or more seasons. The proportion of resting spores germinating depends on abiotic conditions, and germination of resting spores at a given site can continue for several weeks (Carruthers *et al.*, 1997). For pathotype 1 (and 3), intra-season cycles of infection, with epizootic potential, are possible from cadavers producing actively discharged conidia; these conidia are extremely pathogenic (Carruthers *et al.*, 1991) but also much more susceptible to extreme conditions, such as low humidity, high temperature and ultraviolet radiation (Carruthers *et al.*, 1988; Firstencel *et al.*, 1990). The average time for 50% of conidia-producing cadavers to disappear was 2.8 days in field experiments in Arizona and was clearly influenced by rainfall. The older a cadaver became without sporulating, the less likely it was to sporulate successfully. However, regardless of cadaver age, highest rates of sporulation were predicted when there was high humidity at night with associated long periods of leaf wetness (Sawyer *et al.*, 1997).

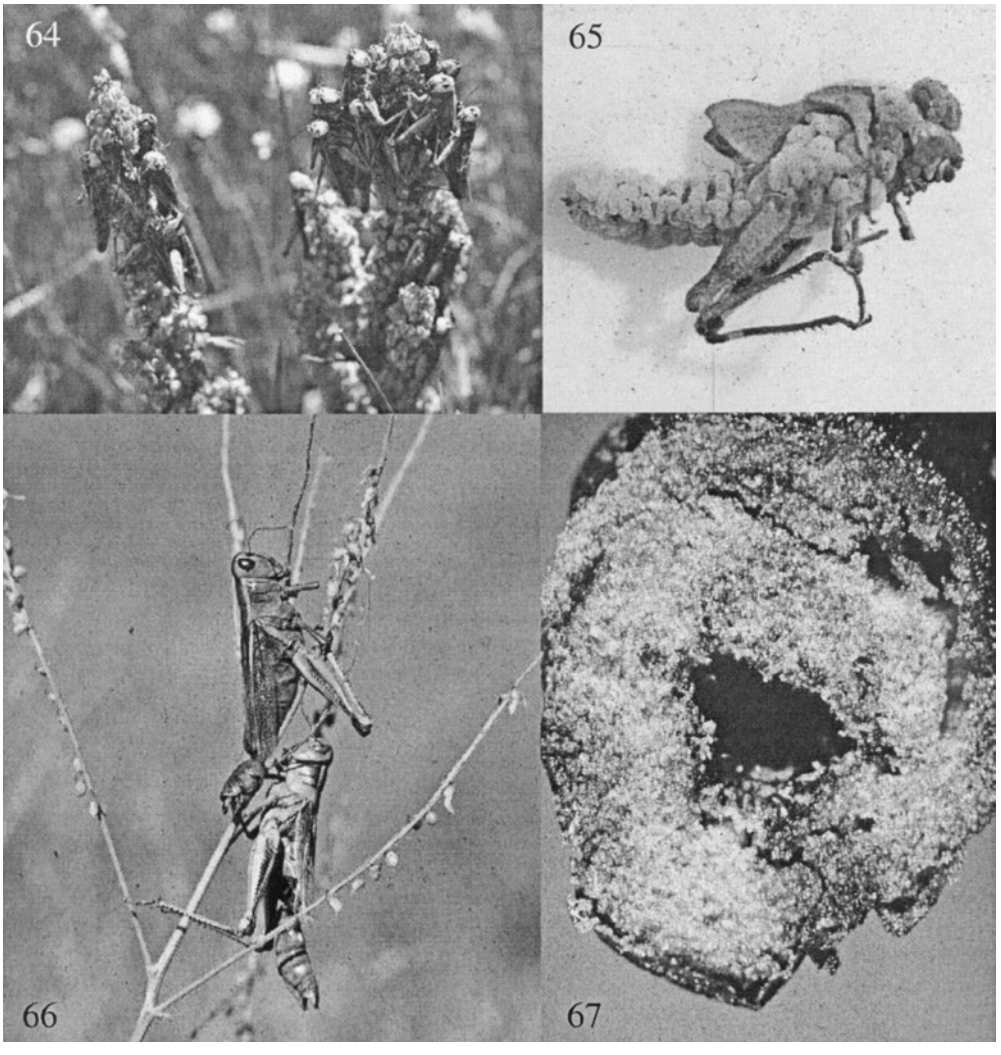

Fig. 4.64. Clear-winged grasshopper, *Campanula pellucida* (Orthoptera: Acrididae), infected with *Entomophaga grylli* pathotype 1. Infected cadavers sit in clusters at the top of the vegetation. Photographs Figs 4.64–4.67: Ray Carruthers.
Fig. 4.65. Dead *C. pellucida* with emerging conidiophores of *E. grylli* pathotype 1.
Fig. 4.66. Two-striped grasshopper, *Melanoplus bivittatus* (Orthoptera: Acrididae), infected with *E. grylli* pathotype 2. No conidiophores will emerge, but the bodies are filled with resting spores.
Fig. 4.67. Abdomen of *M. bivittatus* filled with resting spores of *E. grylli* pathotype 2.

Grasshoppers and locusts are highly mobile insects, and infection has little impact on their mobility during the early stages. Therefore, dispersing grasshoppers are likely to carry infection with them, at least over moderate distances. However, Carruthers *et al.* (1997) have suggested that long-range migration may, in part, have evolved as a way of escaping disease, particularly when the fungus can survive at a site for several seasons as resting spores. Carruthers *et al.* (1997) also noted that some species leave favoured feeding sites to lay eggs in more open areas, which may separate susceptible early instars from the overwintering sites of the fungus.

Erynia neoaphidis *(Entomophthoraceae)*

Taxonomy, distribution and host specificity

E. neoaphidis has a wide distribution, being recorded from Europe, Asia, Africa, North and South America, and Australasia (e.g. Wilding and Brady, 1984; Glare and Milner, 1991; Hatting *et al.*, 1999). Unusually, for such a common species, its genus remains a subject of debate, with different authors variously assigning it to *Erynia* (Keller, 1991), *Pandora* (Humber, 1989) and *Zoopthora* (Balazy, 1993), but in this text it will be considered as *Erynia. E. neoaphidis* has been recorded from > 70 species of aphids on annual and perennial crops, weeds and wild flowers (e.g. Wilding and Brady, 1984). Epizootics that contribute to the regulation of aphid populations are commonly recorded (Table 4.2) and, in some instances, the aphid population is reduced to near-zero on a local scale.

Virulence assays have tended to select highly pathogenic isolates under optimal conditions against a single life stage of a particular aphid species (e.g. Wilding, 1976). However, populations and biotypes of the same aphid species may differ significantly in their susceptibility; nymphs of the pea aphid *Acyrthosiphon pisum* are more susceptible than adults and alate adults are more susceptible than apterous adults (Milner, 1982, 1985a; Lizen *et al.*, 1985). Few studies have considered the relative susceptibility of a broader range of different aphid species or whether *E. neoaphidis* can move freely between different aphid species in the agroecosystem (Milner *et al.*, 1983). Glare and Milner (1991) suggest that those species that are most commonly found infected in the field are also those found most susceptible in laboratory assays. However, recent studies suggest this may not always be the case. Studies with an isolate of *E. neoaphidis* from the nettle aphid *Microlophium carnosum* showed that *A. pisum* was extremely susceptible, with an LC_{50} of 0.9 conidia mm^{-2}, while the cereal aphid *Sitobion avenae* was more resistant, with an LC_{50} of 34.9 conidia mm^{-2} (Hemmati, 1999). Yet epizootics of *E. neoaphidis* are common in both *A. pisum* and *S. avenae* in the UK, suggesting the possibility of pathotypes with specific host associations. Molecular techniques may be able to determine the genetic basis of some of these differences (Rohel *et al.*, 1997; Sierotzki *et al.*, 2000).

Epizootiology in annual cropping ecosystems

Infected aphids (Fig. 4.68) die at the end of the photophase, ensuring that sporulation occurs during the night, when conditions are humid, cool and free from ultraviolet radiation. The time of death is set by dawn, ensuring that aphids die at the end of the photophase (Milner *et al.*, 1984a). The position on the plant where infected aphids die appears to depend on the aphid species. For instance, in laboratory experiments, cadavers of *S. avenae* were found higher on wheat than uninfected individuals, but this was not the case for *A. pisum* cadavers (Roy, 1997). The effect in the field may be different. Cadavers are firmly attached to the foliage by discoid rhizoids and, at death, cystidia cause breaks in the cuticle around which the conidiophores emerge.

Conidiophores are branched and multinucleate, forming uninucleate lemon-shaped conidia, 18–35 × 10–15 μm in size (Fig. 4.69). They are actively discharged at an estimated velocity of between 5 and 19.5 m s^{-1} between 2 and 11 mm horizontally and 7 and 8 mm vertically away from the aphid cadaver, ensuring that they escape the boundary layer for dispersal. The maximum discharge distance and the total

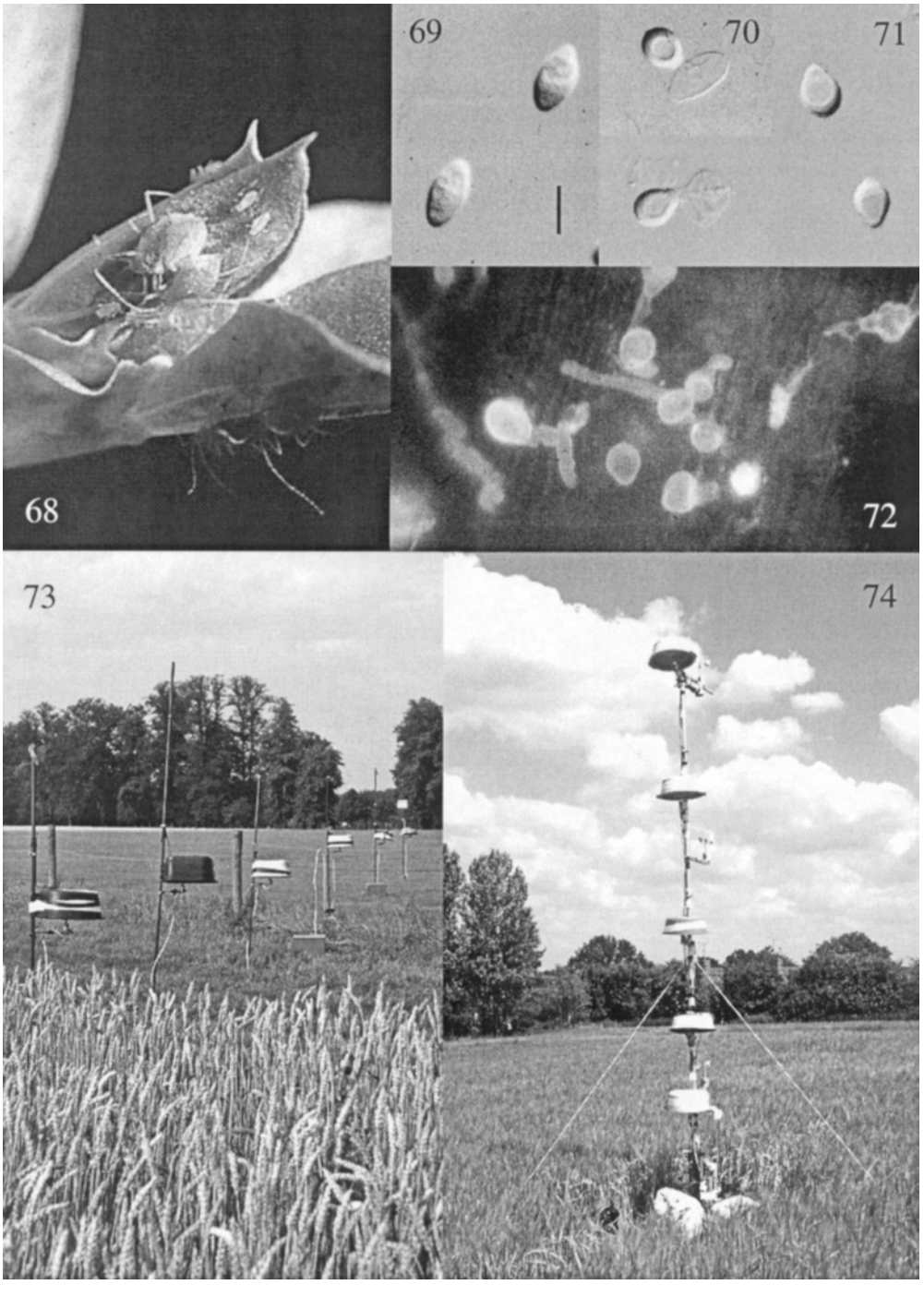

Fig. 4.68. *Erynia neoaphidis*-infected pea aphid, *Acyrthosiphum pisum* (Hemiptera: Aphididae). Conidiophores have erupted through the host's integument, have formed conidia and have explosively discharged conidia for several millimetres.
Fig. 4.69. Primary conidia of *E. neoaphidis.* Scale bar = 20 μm.
Fig. 4.70. Secondary conidia of *E. neoaphidis* being produced by primary conidia. Scale bar = 20 μm.

number of conidia produced are affected by temperature; greater numbers of conidia are discharged and they travel further at 18°C than at 10°C or 25°C (Hemmati *et al.*, 2001a). At 5°C, numbers of conidia produced are drastically reduced (Dromph *et al.*, 1997), as they are at 30°C (Glare and Milner, 1991). Infection of large aphid species results in the production of significantly more conidia than infection of small species (Glare and Milner, 1991); according to Hemmati (1999), *E. neoaphidis* produced 2.14 $\times 10^5$ conidia per cadaver of adult *A. pisum*, compared with 1.4 and 1.3 $\times 10^4$ conidia per cadaver of the smaller aphids, *S. avenae* and *M. persicae*, respectively.

Sporulation begins within 2 h of aphid death and the majority of conidia are produced within 24 h at 20°C (Glare and Milner, 1991; Hemmati, 1999). Sporulation is significantly affected by humidity, being almost completely inhibited below 93% RH (Wilding, 1969). If humidities fall below 93% but then rise again, sporulation can recommence (Glare and Milner, 1991). Conidia produced within the first 4 h of sporulation germinated faster and were more infective to *A. pisum* aphids than conidia produced after 30 h (Morgan, 1994). Both sporulation and germination are inhibited by green leaf volatiles (Brown *et al.*, 1995; P.A. Shah, unpublished data).

Once discharged, many conidia enter the airstream with the potential to be passively dispersed. In the UK, conidia were sampled from the air using a Burkard aerial spore trap (Fig. 4.54) between June and August, when infected aphid populations were evident in the field (Wilding, 1970a). Greatest numbers of conidia and the establishment of epizootics were associated with periods of rainfall or just after periods of rainfall (Dean and Wilding, 1971, 1973). Most conidia were collected between 0400 and 0800 h in the morning when temperatures were between 10 and 16°C and RH was greater than 90%. The majority of conidia collected were primary conidia (Hemmati *et al.*, 2001b).

Using rotorod samplers at different heights above and distances away from a cereal field in the UK where an epizootic of *E. neoaphidis* was noted, the vertical and horizontal profiles of conidia have been measured (Figs 4.53, 4.73, 4.74). The majority of conidia were found at the height of the crop, but some were trapped up to 4 m above the crop, where they would certainly have been dispersed long distances from their origin, with the potential to infect new aphid populations. Conidia were trapped up to 20 m horizontally away from field sources (Hemmati, 1999).

Conidia are deposited on to foliage or hosts through a combination of settling and impaction. Rain will wash conidia out of the air. The velocity at which they settle (settling velocity) relates to the distance that they may travel before deposition and has been determined for *E. neoaphidis*. *In vitro*-produced conidia of *E. neoaphidis* are significantly larger than *in vivo*-produced conidia (Morgan, 1994). While primary conidia are lemon-shaped, secondary conidia are smaller and can either be of a similar shape or more rounded, depending on the temperature at which they were produced

Fig. 4.71. Secondary conidia of *E. neoaphidis.* Scale bar = 20 μm.

Fig. 4.72. *E. neoaphidis* conidia on aphid cuticle showing germ tubes and appressoria. Scale bar = 20 μm.

Fig. 4.73. *E. neoaphidis* conidia profiles are assessed horizontally away from a wheat crop in the UK. The rotorod conidium samplers are protected beneath rain covers and supported at the height of the crops on canes.

Fig. 4.74. *E. neoaphidis* conidia profiles are assessed vertically above a wheat crop in the UK. The rotorod conidium samplers are protected beneath rain covers and supported on a mast.

(Figs 4.70, 4.71; Morgan *et al.*, 1995). For this reason *in vitro* conidia have a faster settling velocity than *in vivo* conidia which, in turn, settle faster than secondary conidia. In epizootiological terms, this suggests that *in vitro*-augmented fungus may not disperse far initially from the release site and that secondary conidia may be responsible for longer distance dispersal than primary conidia (Hemmati, 1999).

When conidia are deposited, they adhere to host or non-host surfaces. On the host, both primary and secondary conidia can germinate and penetrate under favourable abiotic conditions (Fig. 4.72; Butt *et al.*, 1990). At 100% RH, the mean time for infection is 5.3 h at 10°C and 4.5 hours at 20°C (Glare and Milner, 1991). *E. neoaphidis* proliferates within the aphid as protoplasts, only developing hyphal bodies at or just before death (Butt *et al.*, 1981; Kobayashi *et al.*, 1984). The time to kill is dependent on temperature; in *A. pisum*, infection did not occur at 0°C or at 30°C and the time to kill varied from 5 to 16 days at 20 and 10°C, respectively (Wilding, 1970b). Infection has little impact on *A. pisum* behaviour until just prior to death, when infected aphids are unable to respond to alarm pheromone, though they can still produce it (Roy *et al.*, 1999). If dislodged, infected aphids are less able to return to the plant (Roy *et al.*, 1999). On non-host surfaces, secondary conidia are produced within a few hours (Fig. 4.70); 6 h were required for 30% and 80% of primary conidia to germinate to produce secondary conidia at 10°C and 18°C, respectively (Morgan, 1994). Optimal temperatures for germination are between 18 and 21°C (Glare and Milner, 1991). At suboptimal temperatures, increasing numbers of rounded secondary conidia are produced compared with lemon-shaped ones. Conidia produced at suboptimal temperatures and then placed at optimal temperatures still germinate more slowly than those produced at optimal temperatures (Morgan *et al.*, 1995). Germination to produce secondary conidia is also affected by pH and osmotic potential (Morgan, 1994).

E. neoaphidis resting spores have not been recorded in the field, and the mechanism for overwintering is largely unknown. The fungus may persist during winter as hyphal bodies in cadavers (Feng *et al.*, 1992) or it may reproduce at a slow rate in overwintering aphids (Byford and Reeve, 1969; Feng *et al.*, 1991; McLeod *et al.*, 1998). Nielsen *et al.* (1998) have suggested that *E. neoaphidis* may overwinter in the soil as thick-walled 'loriconidia', which are similar to resting spores. Although conidia can survive up to 8 months at –3°C and +4°C on soil and at least 32 days at +5°C on foliage in the laboratory, these times are significantly reduced under winter field conditions (Morgan, 1994; Schofield, 1998). This suggests that field temperatures in the UK during the winter are not consistently low enough to prevent germination and prolong the survival of conidia. In regions where the temperature is consistently lower during the winter, these times may be extended.

During the summer in the UK, conidia are likely to be inactivated by ultraviolet radiation. Brobyn *et al.* (1985) demonstrated that survival of conidia on bean foliage was related to position on the plant; conidia on the upper surfaces of leaves survived < 3 days, whereas those on the undersides of leaves survived for > 7 days. In laboratory studies, humidity was implicated in conidial survival (Brobyn *et al.*, 1987). The role of rain in persistence of conidia and cadavers on foliage and soil was examined recently. Simulated heavy rainfall for > 30 min removed significant numbers of conidia, particularly from the upper surfaces of leaves. Sixty minutes of heavy rainfall were necessary before significant numbers of cadavers were removed from foliage. Cadavers on the soil were completely destroyed by this amount of rain. Lighter rain is likely to have little impact on conidial and cadaver persistence, and high humidity associated

with rainfall is likely to prove more beneficial for sporulation, germination and transmission and might compensate for the negative effects of inoculum removal (Pell *et al.*, 1997b).

Zoophthora radicans *(Entomophthoraceae)*

Taxonomy and distribution

Z. radicans was originally described from *Pieris brassicae* (Brefeld, 1870) as *Empusa radicans* and has subsequently also been known as *Entomophthora sphaerosperma* and *Erynia radicans. Z. radicans* has a worldwide distribution and has been recorded from numerous insect orders, including Coleoptera, Diptera, Hemiptera, Hymenoptera, Lepidoptera, Orthoptera, Thysanoptera and Trichoptera (Glare and Milner, 1991). Epizootics have commonly been recorded (Table 4.2).

Host specificity

Although *Z. radicans* is recorded from numerous different insect groups, the literature suggests that individual isolates are better adapted to infecting taxonomically related insect hosts (Papierok *et al.*, 1984; Milner and Mahon, 1985; Goettel *et al.*, 1990). Several strains, for example, were unable to infect species from orders other than the order from which they were isolated (Papierok *et al.*, 1984; McGuire *et al.*, 1987a; Magalhaes *et al.*, 1988). Others were unable to infect insects from different families within the same order (Mietkiewski *et al.*, 1986). However, some studies have identified isolates with seemingly broader host ranges. A Malaysian isolate of *Z. radicans* (reference NW250) from the diamondback moth *Plutella xylostella* (Figs 4.75, 4.76) is unable to infect one species of hymenopteran parasitoid, *Cotesia plutellae*, but did infect another parasitoid, *Diadegma semiclausum* (Furlong and Pell, 1996; Fig. 4.83). *D. semiclausum* was 100 times less susceptible than the original host, *P. xylostella*, and was never found infected in the field during epizootics, suggesting a physiological but not ecological susceptibility; the parasitoid is less likely to receive a sufficiently high dose in the field. Poprawski *et al.* (1992) reported that an isolate of *Z. radicans* from *Diuraphis noxia* was also able to infect the aphid parasitoid *Aphelinus asychis*. Some isolates of *E. muscae* from Hymenoptera can also infect Diptera (Eilenberg *et al.*, 1987, J. Eilenberg, unpublished) and Mietkiewski *et al.* (1986) also found that a *Zoophthora* sp. from a hymenopteran was able to infect a small percentage of inoculated Lepidoptera. Susceptibility demonstrated in the laboratory may not relate to infection in the field. Susceptibility relates both to the physiology of an insect and to its behaviour, which may encourage or discourage the acquisition of conidia (Goettel, 1994; Roy *et al.*, 1998). It is likely that pathotypes of *Z. radicans* exist, as with other entomophthoralean species, and that each pathotype has a limited host range.

Epizootiology in annual cropping ecosystems

In *Z. radicans*-infected spotted alfalfa aphid, *Therioaphis trifolii* f. *maculata*, day length affects the time of death, although not as precisely as seen in aphids infected with *E. neoaphidis* (Milner *et al.*, 1984a); Glare and Milner (1991) suggest that some aphids killed by *Z. radicans* are filled with resting spores, the production of which does not

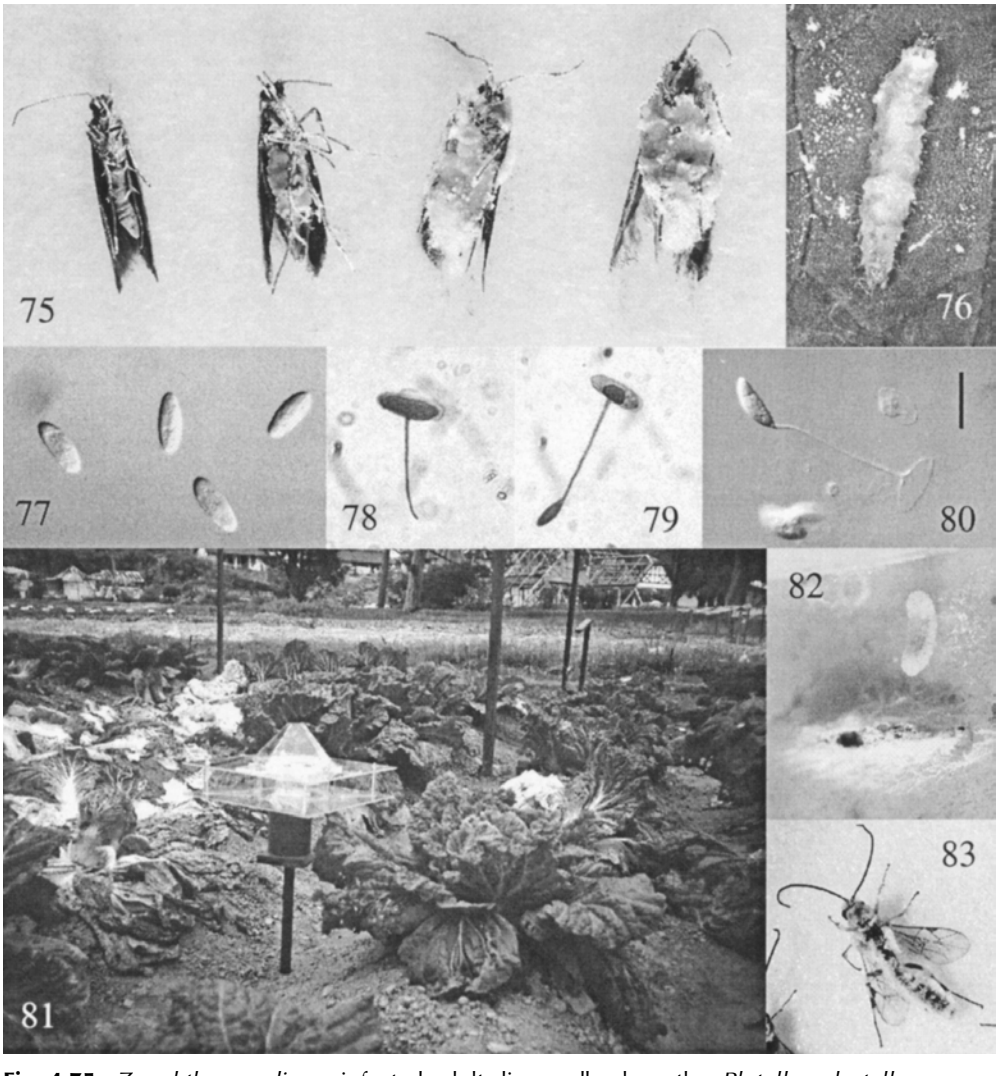

Fig. 4.75. *Zoophthora radicans*-infected adult diamondback moths, *Plutella xylostella* (Lepidoptera: Plutellidae), in the conidial stage. Conidiophores have erupted through the host's integument and formed conidia. The moth at the left of the image is uninfected.
Fig. 4.76. *Z. radicans*-infected larva of diamondback moth. The actively discharged conidia are seen as a halo around the cadaver.
Fig. 4.77. Primary conidia of *Z. radicans.* Scale bar = 20 μm.
Fig. 4.78. The formation of a capilliconidiophore from a primary conidium of *Z. radicans*. The conidium is stained in 10% cotton blue in lactophenol. Scale bar = 20 μm.
Fig. 4.79. The formation of a secondary conidum is initiated at the end of the capilliconidiophore of *Z. radicans*. The conidia is stained in 10% cotton blue in lactophenol. Scale bar = 20 μm.
Fig. 4.80. A fully formed capilliconidium of *Z. radicans* at the end of the capilliconidiophore. Scale bar = 20 μm.
Fig. 4.81. Autodissemination trap for *Z. radicans* in a vegetable crop, Cameron Highlands, Malaysia.

require a period of high humidity after host death. *Z. radicans* may produce actively discharged conidia or internal resting spores and, on occasion, both are produced from the same host.

Insects killed by *Z. radicans*, like those infected with *E. neoaphidis*, usually remain attached to the surface on which they have died by rhizoids or pseudorhizomorphs, which emerge from their ventral surface. A water-saturated or near-saturated environment is essential for active conidial discharge and subsequent germination and infection. Uninucleate, cigar-shaped primary conidia are discharged several millimetres from the cadaver with the potential to infect new hosts (Fig. 4.83).

Active discharge seems to follow a circadian rhythm, with more conidia being discharged in the dark than the light (Yamamoto and Aoki, 1983; Milner *et al.*, 1984a). In the spotted alfalfa aphid more conidia are produced at 25°C than at 15, 20 or 30°C and none are produced at 35°C (Milner and Lutton, 1983). Sawyer (1929) reported that sporulation was very low at 13°C and stopped entirely at 8°C. In contrast, Leite *et al.* (1996a) recorded reduced, but substantial, sporulation of *Z. radicans* on *Empoasca kraemeri* cadavers at 5 and 10°C compared with 15 and 20°C.

Discharged conidia are presumed to travel long distances in the air, although there have been no field measurements of aerial dispersal for *Z. radicans*. G.W. Riethmacher (personal communication) was able to collect conidia on microscope slides placed some distance from an epizootic occurring in *P. xylostella* on brassicas in the Philippines, suggesting that conidia were dispersed readily. In addition, the conidial settling velocities have been recorded for two isolates and these were less than those for species with larger conidia (Sawyer *et al.*, 1994; Hemmati, 1999).

Discharged conidia landing on appropriate hosts will infect them if the environment is saturated or near-saturated. Both primary and secondary conidia are thought to be infective (Wraight *et al.*, 1990; Pell *et al.*, 1993b) and the speed at which they germinate and infect is related to temperature (van Roermund *et al.*, 1984; Glare *et al.*, 1987). For isolates infective to the leafhopper *E. kraemeri*, conidia landing on a suitable host can develop either germ tubes with rounded or elongate appressoria for infection, long, slender capilliconidiophores bearing almond-shaped capilliconidia (Figs 4.78–4.80), or replicative conidiophores which actively discharge secondary conidia similar in shape to the primaries. Germination type is dependent on temperature; germ-tube production was greatest at 22°C (beginning within 0.7 h), capilliconidiophore production at 16.5°C (within 1.9 h) and replicative conidiophore production at 28–30°C (within 3.2 h) (Galaini-Wraight *et al.*, 1992).

The factors that affect the type of germination and subsequent development are clearly complex and differ depending on which type of conidia are formed and where they land on the host (Wraight *et al.*, 1990; Leite *et al.*, 1996b, c), what nutrients and pH are available (van Roermund *et al.*, 1984; Magalhaes *et al.*, 1990, 1991a, b) and the immune response of the host (Butt *et al.*, 1988). The type of secondary conidia produced can be dependent on whether the primary conidia were produced *in vivo* or *in vitro*; on 1% water agar capilliconidia were almost exclusively produced from *in*

Fig. 4.82. Dual infection and parasitization of diamondback moth larva: *Z. radicans* has emerged and is sporulating on the cadaver of the larva. In addition, the larva of the parasitoid, *Diadegma semiclausum* (Hymenoptera: Ichneumonidae), has also emerged and is about to pupate. Photograph: Mike Furlong.

Fig. 4.83. *Z. radicans*-infected parasitoid, *D. semiclausum* adult. Conidia are actively discharged from the dead host.

vitro-produced primaries, whereas greater numbers of actively discharged secondary conidia were produced from *in vivo*-produced primaries (J.K. Pell, unpublished).

After infection, *Z. radicans* proliferates throughout the host body as hyphae, with hyphal bodies forming near host death. No protoplast stages have been observed (Glare and Milner, 1991), although it is possible to produce them from walled stages *in vitro* (Glare *et al.*, 1989a). Time to kill varies between isolates and hosts and is dependent on temperature (Milner and Lutton, 1983; Leite *et al.*, 1996d). Fluctuating temperatures can extend the time to kill (Furlong *et al.*, 1995). *Z. radicans* isolates have been recorded as able to germinate and grow between 0 and 36°C, though most do not grow at or above 35°C (van Roermund *et al.*, 1984; Glare *et al.*, 1987; M.J. Furlong and J.K. Pell, unpublished data).

Infection may have an impact on host behaviour; food consumption by infected larvae of *P. xylostella* was not affected until the third day after infection (1 day prior to death). On the day of death, no food was consumed and, overall, infected larvae (inoculated as third instars) ate 44% less foliage than healthy larvae (Furlong *et al.*, 1997). The number of eggs laid by infected females (inoculated 3–6 h after eclosion) was also significantly reduced, even over the period of egg production prior to death (Furlong *et al.*, 1997). *Z. radicans* also inhibits the response to and production of sex pheromone in *P. xylostella*, thereby disrupting their mating behaviour (Reddy *et al.*, 1998). Changes in behaviour and reproductive rate contribute to pest management even before the host succumbs to disease.

Under certain circumstances, persistent resting spores (azygospores) are produced within the host in response to changing climatic factors, particularly low temperature and high humidity, high inoculum density, host age or inappropriate hosts (Ben-Ze'ev and Uziel, 1979; Shimazu, 1979; Perry *et al.*, 1982; McCabe *et al.*, 1984; McGuire *et al.*, 1987c; Glare *et al.*, 1989b). Resting spore production has not been observed in all isolates (Uziel *et al.*, 1982; Glare *et al.*, 1989b; Pell *et al.*, 1993), but the conditions for their production are not fully understood and may differ between isolates. Mixed infections with more than one isolate also encourage resting spore production, suggesting genetic recombination (Glare *et al.*, 1989b). Resting spore-filled cadavers are not attached by rhizoids and fall to the ground, where resting spores remain dormant during unfavourable climatic conditions. In the laboratory, they may remain dormant for 4 months at 4°C and 100% RH (Perry *et al.*, 1982). When resting spores germinate, in synchrony with host population increase (Perry and Fleming, 1989), they produce multiple, actively discharged, infective germ conidia.

Resting spores play a role in long-term persistence in the environment during unfavourable climatic conditions. The shorter-term survival of conidia between discharge and infection, necessary to ensure transmission, is also important in disease dynamics and can be affected by temperature, humidity, rain and ultraviolet radiation.

Conidia inoculated on to brassica foliage or soil in the field in Malaysia lost the ability to infect their host, *P. xylostella*, within 24 h (Furlong and Pell, 1997). This is similar to the time for conidial survival of a *Z. radicans* isolate from *T. trifolii* in Israel (Uziel and Shtienberg, 1993). In complementary laboratory studies at temperatures and day length simulating field conditions in Malaysia (12 : 12 h light : dark (L : D), 23 : 16°C), conidia survived for 16 days on both foliage and soil, suggesting that other factors reduced viability in the field (Furlong and Pell, 1997). Infectivity was lost faster on the upper than on the lower leaf surface and faster on dry than on moist soil, highlighting the importance of moisture in conidia survival (Furlong and Pell, 1997). Uziel and Kenneth (1991) also showed that *Z. radicans* conidia remained viable for extended

periods at high humidities. In a laboratory study, conidia viability, as measured using vital staining techniques, was lost most rapidly at RH < 95% and differed between isolates (Griggs *et al.*, 1999). One hour of simulated heavy rain on primary conidia on upper and lower leaves did not significantly reduce the ability of conidia to infect *P. xylostella* (Furlong and Pell, 1997). When this experiment was repeated with capilliconidia, the raindrops dislodged capilliconidia from their fine capilliconidiophores and washed them off the leaf within an hour. However, this is unlikely to have contributed to losses of inoculum observed in the field, particularly as persistence studies were repeated in the field (with and without rain) on several occasions with the same result (Furlong and Pell, 1997). Ultraviolet radiation is by far the most important factor in conidia mortality. Both primary conidia and capilliconidia are rapidly deactivated by ultraviolet radiation (within 3 min for primary conidia and 120 min for capilliconidia (Furlong and Pell, 1997). In simulated tropical sunlight, infectivity of primary conidia and immature capilliconidia fell to 10% within 8 h. Mature capilliconidia remained able to infect 40% of the larvae after 8 h exposure, corroborating the findings of Uziel and Shtienberg (1993) that mature capilliconidia were more resistant to ultraviolet radiation. The cadaver and the halo of conidia (high-density depositions) can provide some protection for conidia, and fungus within the host is also protected from ultraviolet radiation (M.J. Furlong, unpublished data).

Exploitation in Pest Management

The potential of Entomophthorales has largely been considered from the point of view of biological control approaches in isolation from other control measures. Although the potential to combine fungi with other strategies has always been accepted, this has only recently been practically addressed. Biological control is defined as:

> the use of natural enemies or competitor populations to suppress a pest population, making it less abundant and thus less damaging than it would otherwise be. It may be the result of purposeful actions by man or may result from the unassisted action of natural forces. Biological control may be employed either for suppression of crop or food pests, or for restoration of natural systems affected by adventive (nonnative) pests.
>
> (van Driesche and Bellows, 1996)

There are three types of biological control: introduction (or classical biological control), conservation and augmentation. Here, successful and unsuccessful examples where these three strategies have been applied are described, identifying opportunities and bottlenecks. Approaches that are moving towards integration of biological, chemical and cultural strategies (i.e. integrated pest management (IPM) in the true sense) are also discussed and future prospects evaluated.

Biological control: introduction of exotic Entomophthorales

When pest species are introduced to a new area, their natural enemies are often absent. In these cases, introductions of natural enemies may decrease populations of the new pest. Natural enemies for introduction can be collected from an exotic area, usually where the pest host is indigenous, and released in the area of introduction after

appropriate quarantine and evaluation (van Driesche and Bellows, 1996). This strategy is called classical biological control. Alternatively, exotic natural enemies may be introduced against a native pest, a strategy that has been called neoclassical biological control or use of a new association. As fungal isolates within a species can differ significantly in a number of characteristics, including host specificity (= pathotypes), the introduction of an exotic pathotype can be considered as an introduction of a 'new' natural enemy even if the fungal species is already present. Unfortunately, difficulties in identification and separation of species, or pathotypes within a species, can make it difficult to determine whether a pathotype is already present or not. In addition, many pest species have been transported worldwide over many centuries and it is difficult to determine whether pests have been introduced or are indigenous.

In any case, the goal of pathogen introductions is to release the natural enemy for establishment so that it will eventually create a self-maintaining system, which, in the long run, can be much less expensive than other control approaches. In some instances, exotic isolates have been introduced to test inoculative or inundative augmentation where the objective was to cause epizootics; such examples will be described later, because the methods used were appropriate for immediate control and not solely establishment. Several examples are described where exotic entomophthoralean species have been introduced for establishment against pests, whether native or introduced.

Entomophaga maimaiga *and* Lymantria dispar

In 1909, an entomophthoralean fungus found infecting *L. dispar* in Japan was brought to the northeastern USA, where *L. dispar* had been introduced. This fungus was introduced in 1910/11 by releasing infected insects but it was not considered to have become established at that time (Speare and Colley, 1912). Surprisingly, in 1989, an entomophthoralean fungus was found causing epizootics in *L. dispar* populations in seven northeastern states (Andreadis and Weseloh, 1990; Hajek *et al.*, 1990b). This fungus was identical to isolates from Japan (named *E. maimaiga*), but it was not at all certain whether the fungus found in 1989 had originated from the 1910/11 releases or from a hypothetical, more recent accidental introduction (Hajek *et al.*, 1995b). Since 1989, *E. maimaiga* has repeatedly caused epizootics, resulting in widespread maintenance of *L. dispar* populations at low densities. From 1989 to 1992, the spread of this fungus across much of the distribution of *L. dispar* in northeastern North America was documented (Elkinton *et al.*, 1991; Hajek *et al.*, 1996b). Some of the spread was due to human activity, including purposeful point introductions but possibly also accidental movement of soil containing resting spores. However, the seemingly simultaneous appearance of *E. maimaiga* over large areas where it had not previously been detected under appropriate conditions (i.e. host population present and adequate rainfall) suggests that much of the spread could be attributed to airborne movement of the conidia.

L. dispar was introduced to the Boston area of North America in 1868 or 1869 and has increased in distribution ever since. This early-spring, univoltine species has a great potential for outbreaks and yet, for periods of time, it can remain largely undetected in the forest. For several years after 1989, populations of *L. dispar* were dense enough to be noticeable in forests, and an outbreak was feared. At that time, *E. maimaiga* did not occur throughout the area infested with *L. dispar* and the fungus was not considered capable of spreading extensively on its own. Therefore, programmes were developed to introduce this fungus into areas where it did not occur, but a reli-

able method for *in vitro* production was not available. In a preliminary study, *E. maimaiga* was effectively redistributed by collecting resting spore-laden soil from the bases of trees where epizootics had occurred and releasing that soil around the bases of trees where control of *L. dispar* was required (Hajek and Roberts, 1991). In addition, weekly watering of the soil at the bases of the trees increased infection rates. To quantify the numbers of resting spores released, wet-sieving and density-gradient centrifugation were followed by spore counts (Hajek and Wheeler, 1994). Using this methodology, a redistribution programme was conducted in four states along the leading edge of *L. dispar* spread, where surveys had demonstrated that the fungus did not occur. *E. maimaiga* resting spores were released at 34 plots of 0.1 ha in 1991 and seven plots in 1992 (Hajek *et al.*, 1996b). During 1991, *E. maimaiga* infections were found in 28 of the 34 release plots with infection levels of > 40%. Once again, weekly watering of the soil at the bases of trees increased host infection. Infections were also found in four of the 15 control plots but with an average infection of only 0.5%. During 1992, an average of 72% infection was found in the 1992 release plots, and infection levels were high at all of the 1991 release sites.

Redistribution was also evaluated in central Michigan, using release of soil-borne resting spores and laboratory-infected larvae (Smitley *et al.*, 1995). In the second year after introduction, *E. maimaiga* caused 9–40% infection in release plots and infection was inversely correlated with defoliation. In the third year after fungal release, epizootics of *E. maimaiga* occurred at the two release sites. Throughout the study, there was no significant difference between the two methods for introduction. While quantification of resting spores in soil to be introduced is time-consuming, inoculation of larvae is much more so. Therefore, releases of *E. maimaiga* in other locations have predominantly focused on releasing resting spores.

Between 1990 and 1994, *E. maimaiga* was released at a total of 146 sites in eight states (A.E. Hajek, unpublished data). The principal method for redistribution has been movement of soil containing *E. maimaiga* resting spores. Releases of this fungus have continued along the leading edge of the ever-increasing distribution of gypsy moth. In areas newly colonized by gypsy moth, damage can be severe and the time for *E. maimaiga* to invade on its own cannot be predicted. Due to concerns about the many organisms that could be moved along with *E. maimaiga* resting spores when moving soil, redistribution now uses field-collected cadavers containing resting spores. This new method is not without drawbacks; it requires collecting cadavers from epizootics during the 3+-week period after larval mortality, when the majority of cadavers remain attached to tree-trunks (Hajek *et al.*, 1998b). After this time, many of the cadavers fall to the ground and decompose to release resting spores into the soil. Because the timing and location of epizootics are not predictable, cadaver collection requires a large effort during a relatively brief period to find and visit epizootic sites for cadaver collection. Cadaver collection is mostly done before the pest oviposits. Therefore, at-risk sites cannot be identified for immediate release. Methods have yet to be developed to ensure fungal survival during storage of resting spore-laden cadavers until such time that release sites have been identified. In addition, if the fungus is to be stored throughout winter, resting spores must receive conditions that satisfy their constitutive dormancy requirements. Studies are currently being conducted to identify these conditions. At present, although *E. maimaiga* occurs throughout a major part of the *L. dispar* distribution area, there is still a strong demand for inoculum along the leading edge of spread of this pest, where land managers and residents are unsure how long it will take for this fungus to become well established.

Zoophthora radicans *and* Therioaphis trifolii *f.* maculata

Spotted alfalfa aphid became an important pest of pasture legumes, particularly lucerne, when it was introduced into Australia in 1977. Although six species of fungi were found in aphids, they were at extremely low levels in spotted alfalfa aphid; *Z. radicans* was not recorded from this aphid (Milner *et al.*, 1980). In other countries *Z. radicans* was known to cause epizootics in spotted alfalfa aphid populations (Hall and Dunn, 1957; Kenneth and Olmert, 1975) and so was considered to have good potential for introduction. An isolate from Israel was chosen because of the similarities in climate between its area of origin and the site for release in Australia. The fungus was released either by the placement of sporulating *in vitro* cultures over lucerne plants infested with aphids or introducing laboratory-infected living and dead aphids at four sites (Milner *et al.*, 1982). Although infection levels were very low in the first few samples made at release positions, it was clear within 5 weeks that epizootics were in progress. Highest levels of infection were recorded close to the release points (3 m), extending horizontally as far as 15 m, with occasional infected aphids found throughout the field. No rain occurred during the epizootic but humidity remained high for prolonged periods each night. The fungus was able to survive as resting spores, enabling it to persist from year to year and subsequently spread on its own (Milner *et al.*, 1982). Although some isolates of *Z. radicans* can infect Hymenoptera, this fungus was never found infecting the spotted alfalfa aphid parasitoid *Trioxys complanatus* (Glare and Milner, 1991).

Zoophthora radicans *and* Empoasca fabae *in the USA*

The potato leafhopper, *Empoasca fabae,* is an important pest of lucerne, potatoes and other crops in the midwestern and northeastern USA and epizootics of *Z. radicans* were regularly observed in Wisconsin but rarely in states further south. In Illinois, *Z. radicans* had never been recorded from *E. fabae* despite intensive investigations between 1960 and 1985. Lack of this species in Illinois was considered to be due to the higher temperatures in this state. Isolates from Wisconsin were released against *E. fabae* in Illinois in 1984 using a method similar to that described by Milner *et al.* (1982). Initial infections were observed at the release sites but no subsequent horizontal transmission was recorded (McGuire *et al.*, 1987c). However, epizootics did occur widely the following year around the original release site and it was suggested that one of the isolates released might have become established and caused these epizootics. Observations in 1986 indicated that *Z. radicans* was again present in the same area and contributed to reductions in *E. fabae* populations (McGuire *et al.*, 1987b). No further research has been done to confirm the establishment of this pathogen and so the success of the introduction is not confirmed. The released isolate was also highly infective to *E. kraemeri*, but had limited virulence against aphids and did not infect Lepidoptera (McGuire *et al.*, 1987a).

Neozygites fresenii *and* Aphis gossypii *in California*

Classical biological control introductions of *N. fresenii* were made in cotton, in the San Joaquin Valley of California in 1994 and 1995 (Steinkraus and Rosenheim, 1995; Steinkraus *et al.*, 1998a). Cotton aphids in California lacked fungal pathogens, leaving a niche that could be filled by *N. fresenii*. Mid-August releases of isolates from

Arkansas, using dried infected aphids (Fig. 4.85), were moderately successful, resulting in the spread of the pathogen from the release areas. Fungus activity continued until September or October, despite environmental extremes and heavy predator activity (D.C. Steinkraus, unpublished data). Levels of infection were considered higher than those used to predict initiation of epizootics in the southeastern USA, but epizootics did not develop. Whether the pathogen will persist in California and cause long-term suppression of cotton aphid populations is unknown. Low RH in the San Joaquin Valley may limit the survival and spread of the fungus.

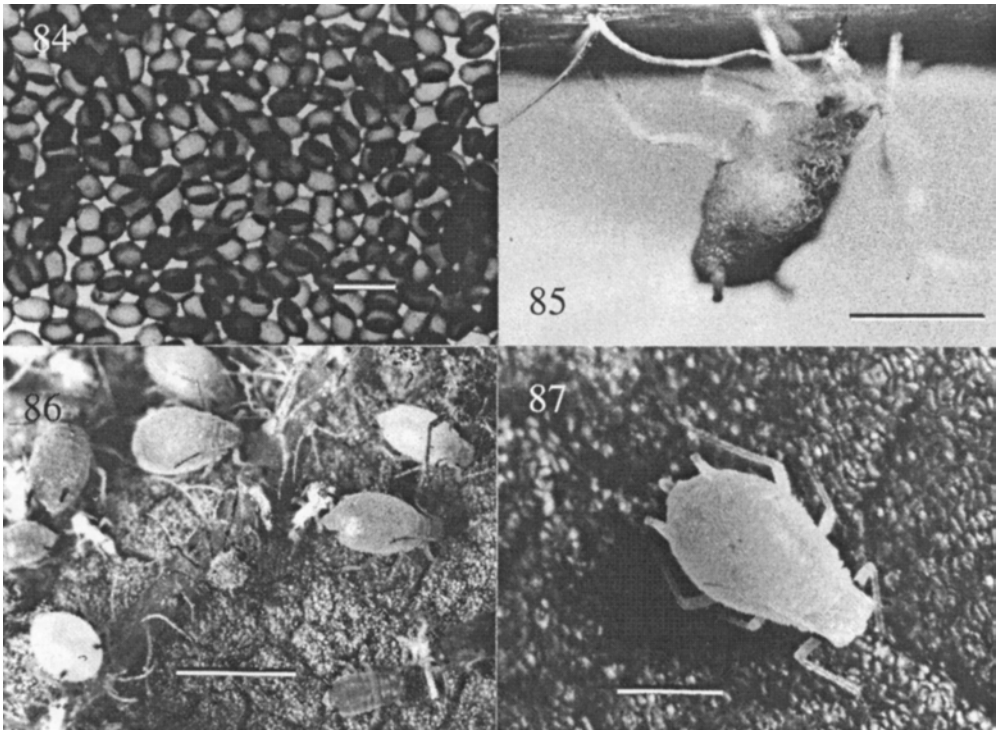

Fig. 4.84. Resting spores of *Neozygites fresenii* from *Aphis gossypii*. Scale bar = 50 μm.
Fig. 4.85. *N. fresenii*-infected cotton aphid. Infected aphids can be dried prior to formation of conidia, frozen and stored for years. Within an hour of being placed at room temperature in a humid chamber, conidia will form and be discharged from such aphid cadavers. Dried infected aphids are useful in laboratory and field experiments.
Fig. 4.86. Underside of a cotton leaf from a field in which an epizootic caused by *N. fresenii* is in progress. Several aphids killed by the fungus are visible, as well as several infected aphids, which are shiny and swollen and which will be killed by the fungus within hours. During sporulation each infected aphid cadaver will discharge conidia on to the leaf, into the air and on to surrounding aphids.
Fig. 4.87. Cotton aphid infected with *N. fresenii*. Three days after infection the aphid host becomes whitish, slightly swollen and sometimes shiny. When such an aphid is squashed on a slide and examined, thousands of protoplasts or hyphal bodies will be revealed. Scale bar = 0.5 mm.

Entomophaga grylli *pathotype 3 and grasshoppers*

E. grylli, pathotype 3, from Australia causes disease epizootics in Australia in areas similar to the rangelands of the western USA. In addition, although its general life cycle is very similar to that of pathotype 1, its host range is wider than that of either pathotype 1 or 2 from the USA. In fact, its host range is similar to the combined host range of the other two isolates (Ramoska *et al.*, 1988). Pathotype 3 sporulates more rapidly than pathotype 1, allowing it to produce conidia and infect hosts during a shorter dew period, although it is less able to undergo successive cycles of desiccation, rehydration and sporulation. Conidia of pathotype 3 are morphologically distinguishable from pathotype 1, so their spread in the field could be monitored easily. It was therefore considered to have great potential for introduction into the USA. An initial release of pathotype 3 was made in McKenzie County in North Dakota in 1989 (500 laboratory-infected fifth-instar nymphs were released in a 45-acre field site), but it was late in the season and, although low levels of sporulation occurred, little secondary transmission was observed. As the release was late in the season, resting spores were probably produced rather than infective conidia. Earlier-season releases (synchronized with early-instar grasshoppers and a high likelihood of high humidity) were made on five occasions in 1990 at the same site (500 infected insects released on each occasion). Sampling during the following 4 years indicated that pathotype 3 had become established with successive seasons of overwintering and reinfection (1991–1993). This was associated with significantly reduced populations of a number of pest grasshopper species in the release areas. This included nearly 100% infection of fifth-instar nymphs (i.e. prior to egg laying) of *C. pellucida* and *Melanophus sanguinipes* in 1991 (Carruthers and Onsager, 1993). DNA probes were used to evaluate persistence and spread (Bidochka *et al.*, 1996). In 1992, 23% infection was found with no infection > 1 km from the release sites. By 1993 and 1994, the Australian pathotype was rare or not found at all and researchers questioned the long-term survival of this pathotype in North America. Further releases over a wider area were not made due to concerns for the survival of indigenous non-pest grasshoppers (Lockwood, 1993a, b), although mycosis was only observed in eight out of 20 grasshopper species found at the release site and all eight species are pests of rangeland (Carruthers and Onsager, 1993; Carruthers *et al.*, 1997). The reverse situation was also tried, i.e. release of *E. grylli* pathotype 1 into Australia (Milner and Soper, 1983; Milner, 1985b). The fungus did cause initial infection, but did not establish outside the release cages.

Biological control: conservation of Entomophthorales

Biological control through conservation seeks to identify effective indigenous natural enemies and adopt management practices that conserve and promote them in the field. Fundamental is the assumption that the natural enemies exist locally and that they have the potential to suppress the pest. Management practices that favour entomophthoralean fungi may include provision of increased moisture and provision of overwintering sites or alternative hosts. Here we describe examples where conservation of Entomophthorales has been observed and/or attempted to encourage their activity.

Use of irrigation

Epizootics have been encouraged in several systems through irrigation to ensure high relative humidities for sporulation and infection. Wilding *et al.* (1986b) demonstrated that irrigation of field beans greatly increased the proportion of black bean aphids (*Aphis fabae*) infected with *E. neoaphidis* compared with plots that were not irrigated. *Erynia ithacensis* exists at enzootic levels in populations of the mushroom gnat, *Phoradonta flavipes*, in hothouses in China. Huang *et al.* (1992) found that they could increase the 14% background level of infection to 37% within 2 days and ultimately to 59% by spraying water on to wall corners, mushroom-bed racks and ceilings. Pickering *et al*. (1989) compared infection of *A. pisum* aphids in two areas of a pecan orchard in Georgia; sprinkle irrigation resulted in high levels of infection (74%) with *E. neoaphidis*, but no fungal mortality was noted in the area that was drip-irrigated. Similar results were also recorded by Hall and Dunn (1957) in irrigated lucerne.

Providing reservoirs/alternate hosts

In Switzerland, Keller and Suter (1980) recorded that economically unimportant aphid species developing in spring in meadows (lucerne) were important for the multiplication of entomophthoralean fungi. If aphid populations in these meadows were high in the spring, *E. neoaphidis* and *Conidiobolus obscurus* rapidly built up to sufficient levels to regulate aphid populations in adjacent fields of annual crops. When aphids were scarce in the spring, this did not happen. Feng *et al.* (1991) suggested that the earlier infections observed in *Metopolophium dirhodum*, an aphid feeding in exposed locations on cereal leaves, provided an increase in entomophthoralean inoculum throughout the field, with subsequent increases in infection of an aphid inhabiting sheltered locations (*D. noxia*) and a more xerophilic species (*S. avenae*). Similarly Powell *et al.* (1986b) found that entomophthoralean fungi were more common at the weedy edges of fields, and they presumed that this was because alternative aphid hosts were present and the weed canopy afforded a better environment for transmission than in the wheat fields. Other aphid-pathogenic species, such as *Zoophthora aphidis*, *Zoophthora phalloides* and *Entomophthora planchoniana*, are also known to overwinter in hosts in hedges and forest borders (Keller, 1987a, b, 1998; Nielsen *et al.*, 2001a)

The presence of hedgerows is vital to the persistence and spread of *E. schizophorae* and *E. muscae* in dipteran populations (Eilenberg, 1985, 1988, 1999). The prevalence of *E. schizophorae* in carrot fly, *C. rosae*, was always significantly greater in insects sampled from hedges than from carrot fields. Hedges are the preferred sites for flies to rest and therefore cadavers become attached there prior to sporulation, making hedges the sites where uninfected flies are more likely to receive inoculum (Eilenberg, 1987b). The same phenomenon, though less obvious, was also seen for the cabbage root fly, *D. radicum*, and infections of *E. muscae*; the prevalence of *E. muscae* was always greatest amongst flies caught in the hedges or flowers around the field (Eilenberg, 2000).

Biological control: augmentation of Entomophthorales to initiate early-season epizootics

When natural enemies are missing (in glasshouses, for example), late in arriving at new plantings or simply too scarce to provide control, their numbers may be increased

through releases or applications; this is termed augmentation. 'Inoculative releases' are those in which small numbers of the natural control agent are added early in the season so that they will reproduce and effect control for an extended period. 'Inundative release', or the mycoinsecticide approach, is used when pest control is required rapidly and exclusively by the natural enemy released and not by natural enemy progeny. There are several examples where natural levels of entomophthoralean fungi were supplemented to effect control in one of these ways. However, for Entomophthorales it is more usual that the intention is to inoculate the crop with the fungus early in the season so that the fungus can establish and multiply, retaining pest populations below the damage threshold. Whether an inoculative or inundative release, the intention is to effect control that season and to repeat applications in subsequent seasons. In many examples the trials are on a small scale and are not repeated, making the differentiation between the two approaches more difficult.

Usually, the isolate of fungus being released at a site was not collected from the same site. Due to the variability among entomophthoralean isolates within a species, it is then questionable whether the natural population is being 'augmented' or a novel isolate is being released for control. Regardless, these instances are included under the umbrella term of 'augmentation' when the objective is immediate or delayed pest control within a single crop season and not long-term control through self-perpetuating establishment (introduction). Generally, studies included in this section are those where an isolate being released is indigenous to the area or, if not indigenous, is not known to have characteristics significantly different from the native isolate, e.g. significant differences in specificity.

Entomophaga maimaiga *and* Lymantria dispar *in the USA*

Inoculative augmentation of the lepidopteran pathogen *E. maimaiga* was conducted to evaluate the potential of this method to cause earlier initiation of epizootics in areas where this fungus had already established (Hajek and Webb, 1999). A programme to investigate inoculative augmentation utilizing resting spores collected from the organic layer of soil directly at the bases of trees in locations where epizootics had occurred (Hajek *et al.*, 1998a) was established. Movement and release of resting spore-bearing soil required obtaining permissions from state and federal authorities, collection of soil from areas where quarantines of plant pathogens did not exist and screening collected soil to exclude larger organisms.

In spring, a relatively small number of resting spores were released around bases of trees in treatment sites (1×10^6 resting spores per site). Larvae were sampled throughout the field season, both from understorey vegetation and from the upper tree canopy. Larval survival was lower for treatment versus control plots on four of 12 sampling dates. While infection by *E. maimaiga* was always greater in treatment than in control plots, differences were not statistically significant due to variability among plots. At the end of the season, the only plots with severe defoliation were control plots where numbers of fifth-instar larvae were associated with defoliation levels. It was concluded that a treatment effect had been seen and this study was repeated in a second year to confirm results. However, during the second season, epizootics unexpectedly occurred throughout the region, causing a complete collapse in host populations and subsequently no defoliation. While the strategy of releasing resting spores of *E. maimaiga* shows promise, methods for *in vitro* production of resting spores are needed before such a technique could be considered operational. Efficient production meth-

ods would facilitate and improve such releases and make it possible to release standardized material and higher doses than previously possible.

Zoophthora radicans *and* Empoasca fabae *in the USA and Brazil*

In New York state, Wraight *et al.* (1986) applied a dry mycelial preparation of a *Z. radicans* isolate from central Brazil against populations of *E. fabae* on lucerne, on two occasions in September 1984. *Z. radicans* was not found infecting leafhoppers in surveys made in the area prior to release, and so technically this also constituted a classical introduction. However, the intention was to test the potential for inoculative/inundative augmentation on a pest population in an area free of any potentially contaminating fungi and so this study is included in this section. Dry particles of mycelium were broadcast late in the evening directly on to dew-covered foliage in 2 m × 2 m plots at a rate of 10 g m^{-2}. For the first release, temperatures were low for the first day and it was dry on the following 2 days, after which time, rain removed all the inoculum; fewer than 10% of nymphs became infected. On the second occasion, temperatures and humidities were higher, ensuring good sporulation; 80 and 64% of nymphs became infected in the two treatment plots, compared with 0% in the untreated plot. Wraight *et al.* (1986) suggested that, although the doses in the treatment were too high for large-scale application, the method showed promise as a way of initiating epizootics in leafhopper populations when climatic conditions were favourable. This was confirmed by subsequent trials (July 1986) in which applications of *Z. radicans* dry mycelium to small 'inoculation plots' in two fields of lucerne resulted in field-wide epizootics that decimated the leafhopper populations (Wraight and Roberts, 1987).

In 1990 and 1991 seven additional isolates of *Z. radicans* (all from Serbia) were released in test fields near Ithaca, New York, for experimental control of *E. fabae*. On each occasion, only three infected leafhoppers were found in extensive searches made immediately after release. Isolations were made from each of these six insects. Using random amplification of polymorphic DNA polymerase chain reaction (RAPD-PCR), it was possible to determine that five of these isolates were very similar to the released isolates and different from any other isolates included in molecular analysis, suggesting that the fungus had caused a low level of disease. The sixth isolate was similar to an isolate from aphids and was thought to represent a population endemic at the release site (Hodge *et al.*, 1995). Although infection by the released isolates could be confirmed, the prevalence was very low, and it was uncertain whether the isolates would eventually be able to establish epizootics.

Releases of *Z. radicans* against *Empoasca* sp. on bean plants (*Phaseolus*) in 1990 in São Paolo, Brazil (Leite, 1991), followed the same methodology for fungal mycelium production and release as used in central New York, USA, in 1984 (Wraight *et al.*, 1986). In this region, *Z. radicans* was already known to cause epizootics, but natural control was often achieved too late to prevent damage. The objective of this study was to initiate epizootics earlier in the crop cycle. An isolate of *Z. radicans* from Yugoslavia was selected for the test; it was considered to have exceptional biological control potential, because it was originally isolated from an epizootic in *Empoasca vitis* under drought conditions. Applications of 0.25–2.0 g of dried mycelium m^{-2} were made in 12 small subplots (2 m × 2 m) within a single 0.5 ha field. Over the range of concentrations applied, per cent infection was generally greater in plots receiving more fungus, although this was not always the case. On day 5 post-application, the average rate of infection in the treated plots was 25% compared with only 6% in untreated plots

located 5–36 m from the treatments. After 8 days, infection in the treated plots was still greater (34%) than in untreated plots (18%); however, the fungus spread rapidly during the following week and similar levels of infection were recorded in all plots (60–64%) after 15 days. The field-wide epizootic reduced leafhopper numbers to below the economic threshold (from 4.8 to 0.7 hoppers per trifoliate). In summary, infection foci were successfully established in the small plots, and the fungus spread rapidly through the entire field. A few cadavers of infected leafhoppers were found in part of the field at the time of the release. The proportion of infections caused by the resident versus introduced fungus was not determined, but researchers concluded that the epizootic was caused primarily by the released pathogen.

Zoophthora radicans *and* Plutella xylostella

Epizootics of *Z. radicans* in *P. xylostella* populations are common but unpredictable and often fail to prevent increase in pest densities above the economic threshold. In a similar experiment to that of Wraight *et al.* (1986), Pell and Wilding (1994) introduced a Taiwanese isolate of *Z. radicans* into caged populations of *P. xylostella* in the UK to test the potential for establishing early-season epizootics. *Z. radicans* had not previously been recorded from *P. xylostella* in the UK and so the problem of confusion from contamination by local isolates was removed. Between 36 and 68% of larvae were infected in treated cages compared with 0% in untreated cages, demonstrating the potential for augmentation of *Z. radicans*.

Entomophaga grylli *pathotype 1 and grasshoppers in the USA*

A number of 'probable' releases of *E. grylli* were made into North America in the early part of the last century. In many cases it is likely that the fungus was misidentified, and many of the releases were exotic material, including material from Natal, South Africa. Carruthers *et al.* (1997) question whether pathotypes 1 and 2 are actually native to North America, as is commonly accepted. However, regardless of whether the pathotype is native or exotic, augmentation was attempted in 1983 in New Mexico. Approximately 400 field-collected *C. pellucida* were injected with pathotype 1 (*in vitro*-produced protoplasts of an isolate originally collected from an epizootic in Arizona) and released in a 10 ha area near Underwood Flat, New Mexico. Intensive monitoring was carried out in the following years to determine infection levels in grasshopper populations. During the season of release, only 5% of the grasshoppers became infected, but, in the following season, infection levels reached 40%. No parallel control sites were available, but, as no infection was found prior to release, it was assumed that this level of infection was due to the release (Carruthers *et al.*, 1997). To make augmentative releases on a larger scale, methods for *in vitro* production of mycelial material possessing the capacity to sporulate are needed, especially as such methods would remove the constraint of having to inoculate by injection. To date this has not been possible, and so attempts to augment *E. grylli* pathotype 1 in rangeland sites were abandoned (Carruthers *et al.*, 1997).

Entomophthora muscae/Entomophthora schizophorae *and Diptera*

The use of *E. muscae* or *E. schizophorae* for biological control has been considered for a number of dipteran pests. So far, a number of augmentations for control of indoor

populations of *M. domestica* have been tried (Geden *et al.*, 1993; Kramer and Steinkraus, 1993; Shimazu and Kuramoto, 1994; Six and Mullens, 1996; Kuramoto and Shimazu, 1997). The releases, on dairy farms, at poultry facilities or in large cages, took place either by dispersing cadavers of fungus-killed flies or by releasing living fungus-infected *M. domestica*, or both. *E. muscae/E. schizophorae* was established in populations of *M. domestica* and, in certain cases, the fungus was more prevalent than in control sites. In no case, however, was it possible to document a decrease in *M. domestica* populations sufficient for control (Mullens *et al.*, 1987).

Releases using *E. muscae* or *E. schizophorae* are difficult to perform for various reasons. First, adult flies are difficult to monitor, precisely due to their behaviour, which is greatly influenced by external factors. This also hampers the possibilities of finding or establishing control plots close to treated plots and monitoring the prevalence of the fungus. For this reason, all release experiments have been done indoors on farms, e.g. poultry houses, with *M. domestica* as the target (Steinkraus *et al.*, 1993b; Six and Mullens, 1996; Kuramoto and Shimazu, 1997). Another problem is the lack of *in vitro* material for release.

Both *E. muscae* and *E. schizophorae* have been kept in culture *in vivo* in various hosts: *M. domestica*, *D. radicum*, *D. antiqua* and *C. rosae* (Kramer and Steinkraus, 1981; Carruthers and Haynes, 1985; Eilenberg, 1987a; Mullens, 1989). The disease was transmitted from sporulating cadavers to uninfected hosts and it was possible for the authors: (i) to keep cultures *in vivo* for a prolonged period of time; (ii) to study host–pathogen relationships, e.g. the effect of temperature on the mortality rate; and (iii) to determine the host range of the fungus. *In vivo* cultivation methods have been used for the production of large numbers of infected flies to be released alive or distributed as cadavers for biocontrol experiments in stables (Geden *et al.*, 1993; Steinkraus *et al.*, 1993b; Shimazu and Kuramoto, 1994).

Although early initiation of epizootics could be achieved artificially, it has proved difficult to control *M. domestica* indoors, even in a confined environment. This is due to a number of factors. Behavioural fever, for example, can influence the development of epizootics in animal houses (Kalsbeek *et al.*, 1999). The simultaneous presence of the two species *E. muscae* and *E. schizophorae* may have seriously biased several of the attempts, since the two species may have different biologies and epizootiology and therefore different requirements for the establishment of epizootics. Without doubt, there is a potential for the control of dipteran insects with *E. muscae* and *E. schizophorae*, but more cost-efficient and reliable *in vitro* systems for production require development.

Erynia neoaphidis *and* Neozygites fresenii *in aphids*

On some occasions, rates of epizootic development are enhanced by extended dew periods and rain and can follow the build-up of aphid populations very closely, retaining that population below the threshold at which spraying of synthetic chemical insecticides is necessary (Glare and Milner, 1991; J.K. Pell, personal observation). However, in many years, the development of epizootics is too late to prevent crop damage, particularly in high-value crops (e.g. salads), where the damage threshold is low. Inoculative augmentation has been attempted on several occasions to encourage the early impact of *E. neoaphidis* and *N. fresenii* in glasshouses and in the field. These trials have given variable results.

In the glasshouse Dedryver (1979) dispersed local isolates of *N. fresenii* in living

infected aphids on two occasions among *A. fabae*-infested bean plants. The glasshouse was maintained at a constant 18°C and was irrigated by misting, ensuring saturated or near-saturated conditions for 20 h day^{-1}. In untreated areas, aphid numbers reached a mean maximum of 1300 per plant after 19 days, whereas treated plants reached a mean maximum of 490 aphids per plant after 8 days. Between 80 and 90% infection in treated aphids was observed after 24–27 days. Latgé *et al.* (1982) and Silvie *et al.* (1990) applied fresh or dry mycelium of *E. neoaphidis* to aphid-infested plants as an aqueous spray in glasshouses. Although the fungus sporulated and was able to infect some aphids, population suppression through further horizontal transmission was not achieved. More recently, glasshouse trials of *E. neoaphidis* formulated as dried mycelium or in alginate beads and applied as a mycoinsecticide have shown some promise for control of *Macrosiphon euphorbiae* (Shah *et al.*, 2000).

Latteur and Godefroid (1983) introduced *in vitro*-produced mycelia of *E. neoaphidis* into populations of aphids on cereals at three field sites in France. Although the inoculum sporulated, there was no population suppression. In this study and in those of Latgé *et al.* (1982) and Silvie *et al.* (1990), tropical isolates of *E. neoaphidis* were used, although their intention was clearly to augment the existing fungal population, rather than to effect classical biological control. This is acceptable because there is no reason to believe that the exotic isolate represented a different pathotype of the fungus, although its temperature optimum was higher. In contrast, *E. grylli* pathotypes occurring on different continents have different biological and molecular characteristics.

Under field conditions, Wilding (1981) released local British isolates of *E. neoaphidis* and *N. fresenii* in fungus-killed aphid cadavers into *A. fabae* populations and was able to cause an early-season crash in aphid populations with both fungal species in 2 out of 4 years. The two species of fungi spread rapidly and some yield improvements were observed in 1 year. Both the successful years had below-average temperatures and above-average rainfall. In a further trial, plots were treated with *E. neoaphidis* in a similar way and, although 70% of aphids became infected, the fungus failed to multiply rapidly enough to protect the crop adequately. Irrigation increased the proportion of infected aphids, confirming that the fungus can be limited by dry conditions (Wilding *et al.*, 1986b). Similar effects were seen in cereals (Wilding *et al.*, 1990), with *E. neoaphidis* causing increased infection in treated plots but acting too slowly and unpredictably.

In a recent study by Poprawski and Wraight (1998) in Idaho, small pieces of sporulating *E. neoaphidis* mycelium were inserted directly into approximately 200 rolled wheat leaves with large colonies of Russian wheat aphid, *D. noxia*. After 5 days, inoculated tillers were sampled. The fungus had sporulated profusely and 18% of aphids were infected. The fungus spread rapidly to uninoculated tillers within subplots but was slow to spread to the rest of the field.

Bottlenecks in exploitation in biological control – mass production and formulation

Mass production is the most important bottleneck in the use of Entomophthorales as mycoinsecticides (inundative augmentation), when large quantities would be required, and, to a lesser extent, for smaller-scale use in inoculative augmentation and classical introduction. For all these strategies the quantity and, more importantly, the quality

of mass-produced materials must be reliable. Members of the Entomophthorales present a spectrum of nutritional requirements for growth *in vitro* (Latgé, 1981). Based on their nutritional requirements for growth, the Entomophthorales can be divided into four groups. *Conidiobolus* spp. grow quickly on standard media. *Batkoa, Erynia* and *Zoophthora* spp. are a little more difficult but can generally be grown on standard media with some supplements. *Entomophthora* and *Entomophaga* spp. need more complex media and *Strongwellsea* and *Neozygites* have proved difficult to grow in anything but tissue culture media (Keller, 1997).

Therefore, complexity regarding *in vitro* mass production can differ significantly among genera. This poses problems related to mass production. With respect to formulation and application, what are the options with respect to the propagule to be released: conidia, resting spores, hyphal bodies, dried mycelium? Cells of the Entomophthorales are relatively large, posing problems for conventional use in spray equipment; some sprayers have filters as fine as 10 µm, although most conventional hydraulic sprayers have openings of at least 150 µm. Conidia are also sticky, making them difficult to harvest from cultures and suspend uniformly in water. In addition, if, during formulation or application, the mucus surrounding conidia is lost, a vital adhesion mechanism could be lacking. As conidia are considered ephemeral, most work has considered only hyphal material and resting spores. To date, technology for economic mass production has not been developed to enable marketing any Entomophthorales for large-scale application. However, numerous studies have been conducted to develop methods for mass production; these have primarily targeted hyphal stages or resting spores.

Production of hyphal stages

The general hypothesis behind mass production of hyphal stages is that, after application in the field, these stages would rehydrate and produce conidia *in situ*. With this strategy as a goal, McCabe and Soper (1985) developed a mass production and drying method for *Z. radicans*, named the 'marcescence process'. The fungus was grown in 25 l fermentation vessels and then sprayed with 10% maltose and dried. Fungal mats were then milled, and hyphal fragments were subsequently released in the field against *E. fabae*. An unknown proportion of hyphal fragments stored in the freezer (–20°C) were viable for at least 1 year. Li *et al.* (1993), using an isolate of *Z. radicans* under development for *P. xylostella* control and a slightly modified marcescence process, found that dried mycelia could be stored at 4°C for up to 80 days, but did not survive freezing. These researchers suggested that the mucilage observed in scanning electron microscopy of mycelium was an important protectant during drying. Cold-temperature (4°C) drying of mycelia of this isolate was essential for storage; if the mycelia were dried under ambient conditions, they lost the ability to sporulate within 2 weeks (Pell *et al.*, 1998).

Nolan (1985) worked on developing media and conditions to optimize mass production of *E. aulicae* hyphal bodies that would be able to produce conidia. The formation and yield of protoplasts and hyphal bodies under varying conditions was investigated using several media including Grace's insect tissue culture media plus fetal calf serum. A protein-free defined medium including eight amino acids was developed that supported growth of protoplasts in both stationary and shaken cultures (Nolan, 1988). Subsequently, enhancement of hyphal body production through loose binding of early protoplast stages to a substrate was investigated. Hyphal bodies constituted

94% of fungal cells in the presence of substrates but only 42% in the absence of substrates. Using 14 l fermentation vessels, addition of a neutral or positively charged disc improved hyphal body production, especially by the fourth day. A disc with a net neutral charge (mylar) demonstrated greater yields than a positively charged disc (polypropylene) (Nolan, 1990). A negatively charged disc (Teflon) was found to inhibit the transition from protoplasts to hyphal bodies, with a recycling of protoplast developmental stages occurring instead (Nolan, 1991). Further studies in 14 l fermenters compared the effects of three different media (a basal medium consisting of 13 amino acids supplemented with either fetal calf serum, tryptic soy broth plus albumin or tryptic soy broth plus calcium caseinate) on amino acid uptake and production and glucose and oxygen utilization by *E. aulicae* (Nolan, 1993b). The medium supplemented with fetal calf serum (a major component of standard media for growing entomophthoralean cells in tissue culture) delayed protoplast growth in *E. aulicae*, possibly due to inhibition by free fatty acids, and supported the lowest hyphal body yield after 9 days. This basal medium supplemented with tryptic soy broth and calcium caseinate greatly increased yields to 3×10^8 hyphal bodies l^{-1}, while inducing some production of conidia during fermentation growth, demonstrating that conidia could be produced from these hyphal bodies. Hyphal bodies may be easily separated from the medium and they withstand applications as sprays. The medium supported growth of *E. aulicae* isolates from numerous geographical areas and is relatively inexpensive (Nolan, 1993a, 1998).

E. neoaphidis grows readily on complex solid media containing egg yolk and milk (Latgé *et al.*, 1978; Wilding and Brobyn, 1980). On a larger scale, mycelium has also been produced in 10 l batch fermenter culture in yeast extract, glucose and milk (Li *et al.*, 1993). More recently, a semi-defined medium containing oleic acid has been developed (Gray *et al.*, 1990). In this medium, oleic acid was required at low concentrations (0.02% v/v) but was toxic at high concentrations (0.2% v/v). The consequence of toxicity was that cultures in semi-defined liquid media only grew if the initial inoculum density was high. The presence of baffles in fermenters assisted growth and also resulted in greater wall growth (Nielsen *et al.*, 2000a). Gray and Markham (1997) developed a model to describe the growth kinetics of *E. neoaphidis* in liquid culture and, by applying predictions from the model, were able to grow *E. neoaphidis* in continuous culture for the first time. This model was able to account for some of the inconsistent results that were observed in simple batch fermenter culture. More work is required, particularly with respect to the C and N requirements during growth, if consistent, good-quality *E. neoaphidis* is to be produced on a large scale.

The most successful culture of *E. muscae* and *E. schizophorae in vitro* has been obtained using liquid media (Carruthers *et al.*, 1985; Eilenberg *et al.*, 1990, 1992; A.B. Jensen, unpublished) with growth as protoplasts (Latgé *et al.*, 1988). Conidia production from *in vitro* cultures has only been achieved on a very small scale, although these conidia are infective. Currently, material for experimentation and release is produced *in vivo* in various hosts: *M. domestica*, *D. radicum*, *D. antiqua* and *C. rosae* (Kramer and Steinkraus, 1981; Carruthers and Haynes, 1985; Eilenberg, 1987a; Mullens, 1989).

The *Neozygitaceae* are among the most advanced of the Entomophthorales in terms of host specialization and growth requirements. Numerous attempts to grow *Neozygites in vitro* have failed (Gustafsson, 1965b; Mietkiewski *et al.*, 1993) or reports are questionable (Kenneth *et al.*, 1972, in Keller, 1997); this led Keller (1997) to state that there is no growth in standard media. However, Butt and Humber (1989) successfully

cultured the vegetative cells of an unidentified *Neozygites* sp. from an infected *T. urticae*, in Grace's insect tissue culture medium and mammalian tissue culture medium 199. Delalibera (1996), Leite *et al.* (1996e) and others reported *in vitro* culture of *Neozygites* sp. isolated from mites, but growth was limited to the hyphal body stage. Grundschober *et al.* (1998) have recently successfully growth *N. parvispora in vitro*, which augurs well for future research with other *Neozygites* spp. They cultured two isolates of *N. parvispora* (from *Thrips tabaci*) using Grace's insect tissue culture medium supplemented with fetal bovine serum and pretreated lepidopteran haemolymph. Both the fetal bovine serum and the haemolymph were essential for sustained growth of hyphal bodies. These hyphal bodies were able to act as conidiophores and produce primary conidia. Interestingly, *E. thripidum*, another species from *T. tabaci*, which was also previously impossible to culture *in vitro*, grew in a similar medium but without the requirement for haemolymph (Freimoser *et al.*, 2000).

Successful *in vitro* cultures of *Neozygites* spp. have been reported only for species isolated from mites and thrips, all of which apparently do not form protoplasts. *N. fresenii* from aphids does form protoplasts prior to hyphal body formation, and this may be one factor influencing the lack of *in vitro* success with *Neozygites* spp. from aphids. At present, the prospects of producing *Neozygites* species *in vitro* for use as mycoinsecticides are uncertain. However, Grundschober *et al.* (1998) are determining the vital components in haemolymph necessary for the growth of hyphal bodies and may be able to apply this knowledge to other species. The production of mycelia of good quality in sufficient quantities for commercial applications requires further research in order to define the media requirements during production. For some entomophthoralean species, e.g. *E. aulicae* and *E. neoaphidis*, certain fatty acids, particularly oleic acid, promote growth and development (Nolan, 1988; Gray *et al.*, 1990). Often variation between fermenter batches of mycelium is high, even when conditions are as constant as possible, suggesting that vital components are missing (Pell *et al.*, 1998).

Dried insect cadavers containing entomophthoralean hyphal bodies are known to survive well at 4°C (Kenneth *et al.*, 1972; Wilding, 1973; Tyrrell, 1988; Pell and Wilding, 1992). Aphids infected with *N. fresenii* and dried in the hyphal body stage (Fig. 4.85) have been dried and frozen for years and the fungus still formed primary conidia within an hour of being rehydrated (Steinkraus *et al.*, 1993a). Entomophthoralean conidia produced *in vivo* are often more pathogenic than those produced *in vitro* (Papierok, 1982; Morgan, 1994). Through an understanding of the *in vivo* nutrient requirements of these fungi and their intrinsic ability to survive desiccation, it may be possible to improve the pathogenicity and storage potential of *in vitro*-produced fungus. For example, Nolan and Dunphy (1979) demonstrated that the incorporation of fungal sex hormones and insect growth hormones into *in vitro* cultures of *E. aulicae* (= *Entomophthora egressa*) encouraged the development of thick-walled hyphal bodies. The promotion of specific fungal structures more suited to the drying process by the addition of specific components to the culture medium could offer an opportunity to optimize production methods in the future.

Formulation of hyphal material

For application, mycelium produced in liquid culture must be formulated. Recent studies by Shah *et al.* (1998) have identified algination as a way of formulating *E. neoaphidis* mycelium for application. An optimal concentration of 1.5% sodium alginate in either

0.1 or 0.25 mol l^{-1} calcium chloride as a gelling agent was used to trap hyphae (220–620 μm × 7–19 μm fragments only) in a matrix. Granules containing 40% fungal biomass produced significantly more conidia than those containing 20% or 10% biomass and the algination process had no impact on infectivity of conidia produced compared with those from unformulated mycelia. Significantly more conidia were produced from granules supplemented with sucrose, starch or chitin (Shah *et al.*, 1999). Granules dried at 95% RH retained more activity than those dried at lower humidities suggesting rate of drying is critical. Storage for short periods was better at 10°C than 23 or 4°C, and a storage humidity of 65% was better than either 80 or 90% (Shah *et al.*, 1998). Although only small fragments could be formulated in this way, requiring filtration during harvesting, this still represents a promising formulation technique and a significant step forward for the practical application of *E. neoaphidis* and other Entomophthorales. Recent glasshouse trials using alginate granules and unformulated mycelia have given promising results (Shah *et al.*, 2000). In addition, hyphal bodies of *N. parvispora* produced *in vitro* could be entrapped in alginate from which they were capable of producing capilliconidia (Grundschober *et al.*, 1998). Commercial application methods for Entomophthorales are still required.

Production of resting spores

Some mass-production research programmes have focused on resting spores. However, only some species of Entomophthorales are known to produce resting spores *in vitro* (Table 4.3). Principal efforts have focused on aphid pathogens in the genus *Conidiobolus*, due to the ease with which members of this genus produce resting spores both in hosts and *in vitro*. Studies have been conducted to optimize production of resting spores using solid media. Soper *et al.* (1975) produced kilogram quantities of *Conidiobolus thromboides* (= *Entomophthora* sp. nr. *thaxteriana*) resting spores using media containing egg yolks, with yields approximating 2–3 g of spores per egg yolk. While the cost of labour and supplies precluded production on a commercial scale, resting spores could easily be produced for small-scale field trials. As part of this study, germinability of *in vitro*-produced resting spores was evaluated. The presence of selected chemicals, such as ethanol, *cis*-cinnamaldehyde, D-limonene, and *n*-nonanol, enhanced resting spore germination. Significant increases in germination were obtained after high-speed blending of resting spores or sonication. These studies were extended to investigate storage of the resting spores; storage of dry resting spores at 4°C for 1 year resulted in no change in germination.

A greater effort has been focused on *in vitro* production of resting spores by *Conidiobolus* spp. using liquid media. Initially, several species of Entomophthorales were used to test the effect of various sources of carbon (Latgé, 1975a), nitrogen (Latgé, 1975b) and lipids and fatty acids (Latgé and de Bievre, 1976) on fungal growth and sporulation. Fungal species or isolates within a species differed in their propensity to produce resting spores *in vitro*. This information helped with the development of semi-defined media for the production of resting spores by *C. thromboides* (= *Entomophthora virulenta*) (Latgé *et al.*, 1977, 1978) and *Conidiobolus obscurus* (= *Entomophthora obscura*) (Latgé, 1980). Darkness, 25°C and a pH of 6.5, with yeast extract as the nitrogen source and a 15% total nutrient concentration, maximized production of *C. thromboides* resting spores (Latgé *et al.*, 1978). For *C. obscurus*, which has more complex nutritional requirements than *C. thromboides*, culturing in aerated media containing 4% glucose and 1% yeast extract produced 0.5–1 × 10^6 resting spores ml^{-1}

Table 4.3. Species of Entomophthorales reported as producing resting spores *in vitro*.

Species	Host group of isolate	Reference
Basidiobolus ranarum	Aphididae	Latgé, 1975a
20 species of *Conidiobolus*	Various	King, 1977
Conidiobolus coronatus	Aphididae	Gustafsson, 1965b
Conidiobolus obscurus	Aphididae	Latgé and Perry, 1980
Conidiobolus pseudapiculatus	Tenthredinae	Keller, 1987c, 1991
Empusa sp.	Tortricidae	Sawyer, 1929
Entomophaga aulicae	Geometridae	Nolan *et al.*, 1976
Entomophaga conglomerata	?	Tyrrell, 1970
Entomophaga gigantea	Tipulidae	Keller, 1987c
Entomophaga limoniae	Limoniidae	Keller, 1987c
Entomophaga maimaiga	Lymnatriidae	Kogan and Hajek, 2000
Entomophaga tipulae	?	Tyrrell, 1970
Entomophthora culicis	Diptera	Gustafsson, 1965b
Entomophthora muscae[a]	Diptera	Schweizer, 1948
Entomophthora schizophorae	Diptera	Eilenberg *et al.*, 1990
Erynia aquatica	Nematocera	Keller, 1991
Erynia athaliae	Tenthredinidae	Keller, 1991
Erynia blunckii	Plutellidae	Keller, 1991
Erynia dipterigena	Diptera	Keller, 1991
Erynia neoaphidis	Aphididae	Uziel and Kenneth, 1986
Erynia virescens	Noctuidae	Keller, 1991
Furia crustosa	Lasiocampidae	MacLeod and Tyrrell, 1979
Pandora nouryi	Aphididae	Gustafsson, 1965b
Zoophthora elateridiphaga	Elateridae	Keller, 1991
Zoophthora phytonomi	Curculionidae	Ben Ze'ev and Jaques, 1990
Zoophthora radicans	?	Gustafsson, 1965c
Zoophthora viridis	Miridae	Keller, 1991

[a]The validity of these observations is in question.

within 6–8 days (Latgé, 1980). Chemically defined media have been developed for these two species (Perry and Latgé, 1980; Latgé and Sanglier, 1985).

Resting spores ($1–3 \times 10^6$ spores ml^{-1}) of *C. obscurus* were produced in 20 l fermenters using semi-defined media, but problems were encountered in larger fermenters (Remaudière, 1983). When batches in large fermenters (100–1000 l) were successful, yields matched those from small-scale laboratory studies. However, during many runs, fermenter batches failed due to contamination. Resting spore production is a relatively slow process, requiring 7–8 days, and, over this long time period, standard sterilization procedures for fungal fermentation were often not adequate. Once prespores are formed by the fourth day of fermentation, nutrients were no longer necessary and, to avoid contamination, prespores could be extracted from fermenters, washed in water and mixed with humid clay. After production, these spores require a period of 3 months at 4°C in a humid atmosphere before germination is possible. Therefore, the time necessary to evaluate the quality of fermentation products was judged prohibitive by industry (Latgé *et al.*, 1983). In addition, numerous scientists attempted to use *in vitro*-produced resting spores of *C. obscurus* for aphid control, and all of these attempts failed (Wilding *et al.*, 1986a). Reasons given for the failure included low germination of resting spores, asynchronous germination, and the fact that all field trials were

conducted in a single year during which aphid populations were low and weather conditions might have been detrimental for the development of epizootics.

Recently, *E. maimaiga* was found to produce resting spores in cell culture, and production of resting spores is being investigated *in vitro* (Kogan and Hajek, 2000). In Grace's insect tissue culture medium plus fetal calf serum, resting spore production requires 7–21 days, much longer than *C. obscurus*. *In vitro* resting spore production varies dramatically by fungal isolate, requires a large surface area : volume ratio in stationary tissue culture flasks, and can be inhibited by > 5% fetal calf serum or glycerol. At present, resting spores have only been produced in low volumes, with a maximum of 3×10^4 resting spores ml^{-1}, but studies are under way to investigate the potential for greater yields in larger volumes of less complex media. Studies of dormancy requirements and per cent germination of *in vitro*-produced resting spores are being conducted simultaneously (A.E. Hajek, unpublished data).

Integrated pest management – the way forward for exploitation of Entomophthorales

The potential of Entomophthorales in pest population regulation lies in the fact that they are common and effective natural antagonists in many insect and mite populations and can decimate those populations at some times of the year, surviving as dormant resting spores or in alternate hosts at times when host populations are absent or low. They have relatively narrow host ranges and a huge capacity for natural dispersal but are difficult to mass-produce.

There are trends in the ecological case histories described here, and in the details of how those species have been used. Classical biological control has been more successful in perennial crops compared with short-term annual cropping systems. In both the *E. maimaiga*/*L. dispar* system and the *Z. radicans*/lucerne aphid system, long-term control was assisted by the ability of the fungus to survive as resting spores and, in the case of the *Z. radicans*/lucerne aphid system, the careful selection of an isolate able to survive under the prevailing abiotic conditions. Undoubtedly, rapid disease spread was achieved through the active discharge and dispersal mechanisms of the two pathogens. In the forest system, long-term control was also enhanced by the leaf litter, which was hardly disturbed from year to year (aiding long-term persistence), and also through conservation (providing moisture in spring in areas where resting spores were released (Hajek and Roberts, 1991; Hajek *et al.*, 1996b)) and augmentation at the leading edge of spread. The presence of at least semi-permanent reservoir sites was also important for the *E. grylli*/grasshopper system.

Where pests are indigenous, existing in annual crops in fragmented agroecosystems, successful approaches learnt from strategies used in perennial crops may still be applied. In annual crops, conservation approaches have potential, particularly by the provision of permanent or semi-permanent refuges in the agroecosystem, i.e providing an environment similar to that in perennial crops. Inoculative augmentation has been attempted but even where infection levels were increased, spread was often unpredictable or too late to prevent crop damage. This is largely because, even though the pathogens have effective mechanisms of persistence and dispersal, the environment is transient and optimal conditions for disease transmission are unpredictable within it. Continued studies on the epizootiology of these fungi will contribute to our understanding of key parameters associated with epizootic establishment so that we can

manipulate conditions to make their occurrence more predictable and intense. Both Wilding (1982a) and Milner (1997) identified this problem and the possible solutions: use fungi as one component of an integrated management strategy or use them as inundative mycoinsecticides, in which case there is a severe bottleneck with respect to mass production and formulation for the Entomophthorales, as previously discussed. Mass production, formulation and application techniques present significant technological difficulties that will only be overcome through fundamental studies of fungal physiology. The mycoinsecticide strategy has greatest potential in glasshouse and high-value systems and even then should be integrated with other approaches. For annual, broad-acre, low-value crops, IPM holds the greatest potential.

IPM is:

> a pest management system that in the socioeconomic context of farming systems, the associated environment and the population dynamics of the pest species, utilizes all suitable techniques in as compatible a manner as possible and maintains the pest population levels below those causing economic injury.
>
> (Dent, 1995)

The spotted alfalfa aphid is no longer a significant pest in Australia for a number of reasons, only one of which was the classical introduction of *Z. radicans*, which undoubtedly contributed to its downfall (R.J. Milner, personal communication). Other control methods used were resistant cultivars, chemical insecticides and the build-up of natural enemies in unsprayed areas, i.e. using the principles of IPM. Today, the aphid is rare (R.J. Milner, personal communication).

IPM seeks to integrate multidisciplinary methodologies to develop pest management strategies that are practical, effective, economical and protective of both public health and the environment (Smith and Reynolds, 1966; Smith *et al.*, 1976). IPM systems will only be successful if they are designed based on an understanding of the key interactions between the pest, its natural enemies and the environment. The range of control measures available for use in IPM are pesticides, host-plant resistance, biological control, cultural control and interference methods (e.g. semiochemicals) (Dent, 1995). More information is required on the integration of Entomophthorales as biological control agents in IPM to exploit them predictably against a number of crop pests.

Fundamental to implementing IPM is the development of reliable pest monitoring methods that relate to an economic damage threshold. Assuming that the conventional use of an insecticide is the first method of control likely to be attempted, these thresholds allow the farmer to make informed decisions on whether to spray, i.e. only when pest damage has reached the predetermined 'action level' (van Emden, 1989; Dent, 1995). The likelihood of the pest population reaching the action threshold is related to other parameters, such as natural enemy abundance (including Entomophthorales), host-plant resistance, cultural practices and climatic variables, all of which are exploited in IPM. If pesticide applications can be reduced in this way, the farmer can make financial and environmental savings. This will also maintain the effectiveness of the currently available narrow-spectrum insecticides by reducing the selection pressure for resistance and so contributing to resistance management.

Compatibility between Entomophthorales and chemical pesticides?

Regardless of whether an entomophthoralean fungus is to be classically introduced, conserved or augmented in an environment as part of an IPM programme, it is essential

to understand how it might interact with the chemical insecticides and fungicides most commonly used for crop protection, in order to determine whether pesticide applications need to be temporally or spatially separated from the most susceptible life stage of the entomophthoralean natural enemy. Susceptibility of fungi to pesticides, particularly fungicides, can vary among fungal species, among isolates within a species and among chemicals (Poprawski and Majchrowicz, 1995; Hermann, 2000), and entomophthoralean fungi are generally more adversely affected by fungicides than hyphomycetes (Majchrowicz and Poprawski, 1993).

Numerous laboratory studies have examined the impact of fungicides on growth (Hall and Dunn, 1959; Soper *et al.*, 1974; Lagnaoui and Radcliffe, 1998), germination, sporulation, infectivity and persistence of Entomophthorales (e.g. Wilding and Brobyn, 1980). These laboratory studies are useful in indicating which chemicals are most likely to have an impact in the field, and need to be continually updated as new products come on to the market. Chemicals which have deleterious effects in the laboratory then need to be tested for field impact.

Fungicides applied to crops can interfere with entomophthoralean fungi, with resulting increases in pest populations. Nanne and Radcliffe (1971) found that potatoes sprayed with captafol, mancozeb and Bordeaux mixture had 1.7–2.6 times more aphids (*M. persicae*) late in the season compared with controls not receiving fungicide treatments. The proportion of aphids infected with entomophthoralean species was 4–5 times greater in the control plots than in the treated plots. Smith and Hardee (1996) found more cotton aphids (*A. gossypii*) and a reduced prevalence of *N. fresenii* in plots treated with carboxin compared with untreated control plots. During years of intensive fungicide spraying, increases in *M. persicae* numbers and potato leaf roll virus were reported by farmers in Minnesota (Lagnaoui and Radcliffe, 1998). In contrast, the numbers of *A. fabae* on field beans were not affected by weekly applications of maneb, benomyl, captafol, mancozeb and tridemorph, even though benomyl significantly decreased aphid mortality due to *E. neoaphidis* (Wilding, 1982b).

Pesticide resistance and the environmental and economic impacts of pesticides have been the greatest driving force behind the development of IPM. The generally accepted view of incompatibility between chemicals and biological control agents has particularly focused attention on understanding and evaluating the impact of natural enemies when assessing the need to apply a chemical (Kogan *et al.*, 1999). One criterion for such an assessment is the concept of 'inaction levels' of natural enemies, which was proposed by Sterling (1984) and has been applied to avoid negative impacts of chemicals on entomopathogenic fungi from the Hyphomycetes (Kogan *et al.*, 1977). Decisions on whether to apply an insecticide were made not only on the economic injury level of the pest, i.e. the 'action level/threshold', but also on the effective population density of the natural enemies or 'inaction level/threshold'. Mathematical models can also be used to predict expected populations of natural enemies and therefore the likelihood of a necessity to use an insecticide. Entomophthoralean fungi have also been used, or have the potential to be used, in combination with semiochemicals, insect natural enemies, host-plant resistance and other pathogens, which are all techniques moving towards true IPM and will be described below.

Use of 'inaction thresholds' and predictive models for IPM with Entomophthorales

NEOZYGITES FRESENII AND *APHIS GOSSYPII* IN THE USA. Since 1989 fungal epizootics

in cotton aphids have been documented in the mid-south USA (Steinkraus *et al.*, 1995). In 1991, the causal agent was identified as the fungus *N. fresenii* (Steinkraus *et al.*, 1991). Studies in the USA have shown that *N. fresenii* epizootics in cotton aphids occur yearly between June and August over wide areas of cotton production (Steinkraus *et al.*, 1995). Further research has indicated that epizootics can be predicted by careful diagnosis of aphid samples (Hollingsworth *et al.*, 1995). When prevalence reaches 15%, aphid declines caused by epizootics are usually imminent. This can be used to limit the use of chemical insecticides and thus save money and potentially assist in the conservation of both fungal and insect natural enemies of aphids.

Declines are more rapid in fields with large aphid populations. Field scouting for fungus-killed aphids is the simplest practical method for detection of fungus during early stages of epizootics (Figs 4.86, 4.87). However, it is not very accurate, even if a hand-lens or stereo-microscope is used. Aphids killed by pesticides may superficially resemble aphids killed by *N. fresenii*. With experience, it is possible to recognize recently killed aphids. Infected aphids are pale grey or tan in colour, somewhat crystalline in appearance, stand on their heads and are attached to the leaf by their mouth-parts. After aphids have been dead for several days, they are often overgrown by saprophytic fungi. These aphids are more noticeable because they are covered with brownish green, woolly fungi. Such aphids are often a good indicator of an ongoing epizootic. However, diagnosis of individual aphids with a phase microscope is preferable and much more accurate. Based on an understanding of the epizootiology of *N. fresenii*, it has been possible to avoid unnecessary applications of insecticides by monitoring *A. gossypii* populations, as discussed below.

In 1993, an extension-based service was established to determine the prevalence of the fungus in cotton aphids collected from fields in Arkansas (Steinkraus *et al.*, 1996a, 1998a; Steinkraus and Boys, 1997). In 1997, the service was expanded to include Louisiana and Mississippi and then expanded again in 1998 to include Alabama and Georgia. Prior to planting cotton each spring, extension agents and consultants are chosen in each state to participate in the programme. Participants are mailed aphid-sampling kits consisting of vials containing 70% ethanol, labels, instructions and pre-addressed express-mail envelopes. During the season, participants sample aphids whenever aphids are considered a problem. The method for determining fungus prevalence in a field is to collect aphids from five areas of a cotton field by rolling up infested leaves or cutting off leaf strips containing aphids and placing these in vials of 70% ethanol. The aphid samples are shipped by express mail to the diagnostic laboratory, where they are squashed and analysed microscopically.

In the laboratory, a random subsample of 50 aphids from each field is squashed in lactophenol–acid fuchsin stain on microscope slides. It is essential that the subsample be a random sample of aphids. No matter what the condition or size, each aphid must have a chance of being selected. With a phase microscope, each individual aphid is diagnosed for signs of the fungus at 200× magnification. Each aphid is carefully scanned for the presence of protoplasts, hyphal bodies, resting spores, conidiophores, conidia, secondary conidia or a combination of saprophytic fungi and *N. fresenii* structures. It takes about 1 h for an experienced operator to examine the aphids from one field.

The success of this service is based on the persistence of *N. fresenii* structures and the small, soft-bodied nature of *A. gossypii*. These attributes make it possible to identify both internal and external signs of infection in squashed aphids (Figs 4.88, 4.89). With training, each of the important structures of the pathogen is easily recognized.

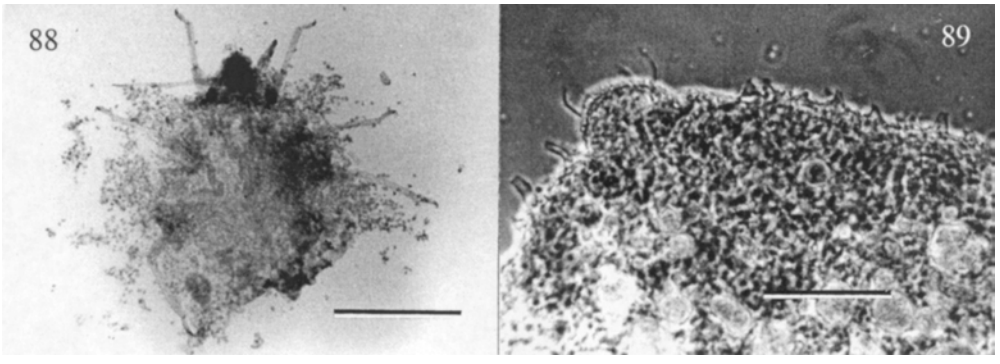

Fig. 4.88. *Neozygites fresenii*-infected cotton aphid squashed on a slide. This aphid contains the protoplast stage of the fungus. Scale bar = 0.5 mm.
Fig. 4.89. Abdominal region and cauda of an *N. fresenii*-infected cotton aphid immediately after sporulation. Remnants of conidiophores indicate that this aphid was killed by the fungus and had discharged conidia. Scale bar = 75 μm.

Capilliconidia, the infective stage, are extremely firmly attached to the legs and antennae of aphids (Figs 4.33, 4.49). Protoplasts and hyphal bodies are released from within infected squashed aphids (Fig. 4.50). Resting spores (Fig. 4.84) and conidiophores (Figs 4.51, 4.89) are also clearly visible. Therefore, when a random sample of aphids is diagnosed, an accurate estimate of the prevalence of *N. fresenii* in the aphid population can be determined.

Results are reported to senders within 24 h. If fungal prevalence is 15% or higher, there is a strong likelihood that the aphid population will decline within a week, due to the occurrence of an epizootic. If it is 50%, there is a strong likelihood that the aphid population will decline within a few days. The critical moment for scouting for the fungus is when a grower is considering an insecticide application for aphid control. If permanent plant damage is being caused by an aphid population and scouting reveals no fungus or very low levels of fungus, growers must use their best judgement in making crop management decisions. The presence or absence of the fungus in the field is an additional piece of information cotton crop consultants can use when making management decisions. This service has been considered useful by the majority of participants. When disease prevalence is 15% or more, growers frequently decide not to use an insecticide, thus saving money, preserving beneficial insects and reducing environmental contamination by pesticides (Steinkraus *et al.*, 1996a, 1998a; Steinkraus and Boys, 1997).

ZOOPHTHORA PHYTONOMI AND THE ALFALFA WEEVIL. Management practices to exploit the natural control potential of *Zoophthora phytonomi* against alfalfa weevil, *Hypera postica*, have been developed. In initial economic assessments, Brown and Nordin (1982) predicted that, in a lucerne field that has not received insecticide applications, the economic benefits from a naturally occurring epizootic were larger than the cost of insecticide use.

A model of this host/pathogen system was used to develop alfalfa weevil control recommendations that take advantage of the activity of *Z. phytonomi*. An early harvest date was recommended so that first cut coincided with the time of first incidence of

disease in weevils, resulting in earlier and more intense epizootics (Brown, 1987). First incidence of the fungus could be accurately predicted with the model. Harvesting the lucerne was thought to stimulate epizootics because larvae became concentrated in warm and humid wind-rows, optimizing chances for disease transmission (Brown and Nordin, 1986). Adequate weevil control resulted, with insecticide applications required only early in the season. While the early harvest date may reduce the profit from the first cutting, epizootics of *Z. phytonomi* could reduce larval weevil populations affecting the second cutting (Nordin, 1984). Benefits from epizootics were greater in wet years than dry years.

Entomophthora muscae and *Delia antiqua*, the onion fly, in the USA. Six biological components were found to be important in the levels of *E. muscae*-induced control of the onion fly, *D. antiqua*. These included parameters related to the pathogen and primary host, the secondary host (the seed-corn maggot, *Delia platura*), the onion crop and adjacent crops and border plants (Carruthers and Soper, 1987; Carruthers *et al.*, 1985). A simulation model was developed and verified using laboratory and field data to describe the parameters affecting pest *D. antiqua* dynamics in the onion agroecosystem (Carruthers and Soper, 1987). Adding the alternate host significantly increased the prevalence of *E. muscae*; emergence of the seed-corn maggot earlier in the season than the onion maggot allowed *E. muscae* to reach higher levels earlier in the season. Close proximity of secondary hosts, in borders, etc., would aid in the natural control of onion maggot. The simulation model, in combination with laboratory and field experiments, also predicted deleterious impacts of pesticide applications on *E. muscae*. This information was used to advise onion growers in Michigan and led to a reduction in pesticide use while maintaining or increasing onion maggot control (Carruthers *et al.*, 1985; Carruthers and Soper, 1987).

Integrating Entomophthorales with behaviour-modifying chemicals (semiochemicals)

Autodissemination. The hypothesis behind autodissemination is that pest behaviour can be manipulated using semiochemicals to encourage the spread of pathogens to susceptible conspecifics on the crop earlier in the season than they would normally spread. In this way disease epizootics would establish and decimate small early-season pest populations before the crop was damaged.

The strategy has been applied to the control of the diamondback moth, *P. xylostella*, using *Z. radicans*. Male moths are attracted into a specially designed fast-entry slow-exit trap in response to synthetic female sex pheromone. Whilst inside the trap, they become contaminated with infective conidia from a sporulating source of *Z. radicans*. On habituation to the pheromone, contaminated moths leave the trap and return to the crop, disseminating the pathogen among their own populations (= autodissemination). The benefits of this strategy over the inundative mycoinsecticide approach are threefold. Use of a specific sex pheromone targets the inoculum to the diamondback moth, as this is the only insect entering the trap. Only small quantities of fungal inoculum and pheromone are required, thereby limiting problems associated with mass production, formulation and storage. Whilst inside the trap, the fungus can be protected from the damaging effects of ultraviolet radiation, and an abiotic environment that favours sporulation and infection can be provided. If an epizootic develops, insecticide applications will be reduced.

Prototype traps have been designed and evaluated in the laboratory and the field to test this strategy, and this work is described here. Complementary laboratory studies have selected isolates, originating from the diamondback moth, with high virulence in the country where the strategy was to be tested (Pell *et al.*, 1993b) and low virulence against non-target organisms (Furlong and Pell, 1996). The prototype trap allows rapid entry of the insects to a central inoculation arena but delays departure, thereby ensuring heavy contamination of moths with infective conidia. The central arena of the trap contained the sporulating fungus and pheromone lure and was protected from rain and ultraviolet radiation by a pyramid of plastic placed above it. A water reservoir was connected by a wick to the central arena to ensure that the humidity remained high within the trap. Moths entered and left the central arena through baffles (Pell *et al.*, 1993a). The lure comprised a polythene vial impregnated with the synthetic pheromone, and fungus production could be adapted from the methods described by McCabe and Soper (1985), Li *et al.* (1993) and Pell *et al.* (1993b, 1998). The trap was placed either at the height of the crop or on the soil between rows of the crop during evaluation (Fig. 4.81).

In field evaluations made in the Cameron Highlands, Malaysia, moths responded to the synthetic sex pheromone and entered the trap at all times of the day and night. Moths entering the trap spent a mean of 88 s in the central inoculation arena. Parallel laboratory studies showed that when a sporulating source of *Z. radicans* was placed above and below the pheromone lure in the trap, the LT_{50} for a single visit by male moths to the inoculation arena was less than 60 s (Furlong *et al.*, 1995; Pell and Furlong, 1998). Therefore, the duration of the visits observed in the field would guarantee infection if appropriate quantities of inoculum were placed in the trap (Furlong *et al.*, 1995). Under field conditions, infected moths succumbed to *Z. radicans* within 4 days of inoculation. This was within the active lifetime of the healthy moths, which was vital as *Z. radicans* must develop within a living host and cannot grow and sporulate on an insect that has died before the fungus has fully completed its development (Furlong *et al.*, 1995).

The prototype trap was both complex in design and expensive to construct and therefore impractical, particularly for use by resource-poor farmers in Africa and Asia, the targeted users of the technology. Simpler traps have now been designed, based either on the standard Delta trap (Oecos, Kimpton, UK) or on materials such as soft-drink cans, which are readily available to farmers in these regions. A Delta trap with a Petri-dish insert (inoculation arena) and the pheromone lure (= Delta–Petri trap) was more effective at attracting moths (16.2 ± 4.2 moths entered in 15 min) than the prototype trap (7.9 ± 2.8 moths entered in 15 min) when tested in kale in Kenya (Pell and Furlong, 1998; J.K. Pell and M.J. Furlong, unpublished data). The duration of visits to the central inoculation arena of the Delta–Petri trap (79.9 ± 19.2 s) was less than to the same region of the prototype trap (122.2 ± 19.4 s) but was still within the time necessary to ensure infection of the moth (Pell and Furlong, 1998; J.K. Pell and M.J. Furlong, unpublished data). Simple traps also required modifications to compensate for the removal of the water reservoir in the prototype trap. To maintain high humidities within the inoculation chambers, the inner surfaces of the traps were coated with a layer of 1% tap-water agar. Simple traps incorporating a water reservoir and water agar surfaces are currently being tested in Australia (J.K. Pell and R. Vickers, unpublished data).

Once effective traps were designed that reliably contaminated insects with fatal doses of inoculum, it was essential to quantify dispersal and transmission of inoculum

from those insects to the remaining population. This has mainly been examined in laboratory studies. In a preliminary field trial, a fluorescent marker was placed in the inoculation arena. Moths became contaminated and were observed depositing the marker on foliage up to 5 m away from the trap, demonstrating that fungal dispersal would occur over at least that distance and almost certainly much further (Pell *et al.*, 1993a).

Dispersal of inoculum from contaminated moths to conspecifics can occur in two ways: passive transfer from contaminated males to other adults and larvae on the crop or, upon the death of the contaminated moth, transfer of fungal inoculum from the resulting cadaver to larvae and adults on the crop. In laboratory studies, although the dose of *Z. radicans* conidia acquired in the inoculation arena was sufficient to kill 90–100% of the moths, passive mechanical vectoring of *Z. radicans* to other adults and larvae was extremely limited; a mean of 15% of larvae on plants in contact with *Z. radicans*-contaminated moths became infected (Pell and Furlong, 1998). Larvae that die from infection are characteristically attached to the leaf surface by rhizoids and the sticky conidia were actively discharged, forming a halo around the dead insect. These cadavers caused disease in a significant proportion of the surrounding larval population (M.J. Furlong and J.K. Pell, unpublished data). In addition to ensuring the death of the male moths entering the trap, with the resulting potential to initiate epizootics, *Z. radicans* has detrimental impacts on its host prior to death (e.g. decreased feeding in infected larvae), which also contribute to a reduction in crop damage. If an epizootic is to establish, good levels of transmission are essential. Disease epizootiology requires further study in the field to better understand how to enhance the development of epizootics. In addition, extensive experiments to determine the number of traps and quantity of inoculum required to effect control are necessary. The number of traps required per hectare and the position and timing of their use require particular examination. Autodissemination also has potential in other systems, particularly with flies, and may overcome the problems associated with a highly mobile host and a fungus that is difficult to mass-produce. Using autodissemination will only require a small amount of inoculum (*in vivo* or *in vitro*) and would exploit the natural behaviour of flies. *M. domestica* adults are attracted to fungus-killed cadavers, thus encouraging their entry into inoculation devices, and adults are known to passively transfer disease in subsequent matings (Møller, 1993; Watson and Petersen, 1993a).

THE 'PUSH–PULL' STRATEGY. The 'push–pull' strategy or 'stimulo-deterrent diversionary strategy' (Miller and Cowles, 1990; Pickett *et al.*, 1991) aims to reduce pest colonization on the crop by reducing the attractiveness of that crop in a number of ways, including the use of antifeedants and semiochemicals, such as oviposition deterrents. At the same time that pests are 'pushed' away from the crop, they are 'pulled' into a highly attractive sacrificial or 'trap' crop nearby. The attractiveness of the sacrificial area is maximized by visual cues and semiochemicals, such as enhanced plant-derived attractants and insect-derived attractants, such as aggregation pheromones. Simultaneously, predators and parasitoids of the pest are also attracted into this area, or highly selective control agents could be used, such as entomopathogenic fungi. This strategy is currently being developed for protection of oil-seed rape crops in the UK and includes the use of the hyphomycete fungus *Metarhizium anisopliae* (Pickett *et al.*, 1995). However, there is no reason why entomophthoralean fungi could not be encouraged in the sacrificial area in a similar way, in conjunction with habitat management and the encouragement of insect natural enemies (see below).

Integrating Entomophthorales with insect natural enemies

Entomophthoralean fungi generally have limited host ranges and are therefore unlikely to directly infect insect natural enemies that are active in the same agroecosystem. This makes them suitable for integration with insect natural enemies for pest management. Apart from direct infection of insect natural enemies, other interactions can occur and have the potential for exploitation in IPM.

The presence of foraging predators and parasitoids in insect populations significantly increases transmission of entomophthoralean fungi (Furlong and Pell, 1996; Roy *et al.*, 1998). When starved, the predatory beetle *Coccinella septempunctata* fed on *E. neoaphidis*-infected *A. pisum* cadavers, although they rarely consumed entire cadavers (Pell *et al.*, 1997a; Roy *et al.*, 1998). Disease transmission from damaged and intact cadavers was not significantly different. However, the presence of the foraging predator on the plant significantly increased infection levels in aphids on that plant compared with plants where there was no predator foraging. *A. pisum* exhibits very strong escape responses in the presence of a predator, which is thought to encourage contact with inoculum and therefore increase transmission (Roy *et al.*, 1998). Complementary to the study of predator and *E. neoaphidis* interactions, Fuentes-Contreras *et al.* (1998) showed that the presence of foraging parasitoids, *Aphidius rhopalosiphi*, encouraged *E. neoaphidis* infection in *S. avenae* populations. In addition, the presence of the foraging parasitoid, *D. semiclausum*, significantly enhanced transmission of *Z. radicans* in *P. xylostella* larval populations (Furlong and Pell, 1996).

Pest populations are patchy in their distribution within the agroecosystem, especially early in the season, and so fungus dispersal between those populations is essential if fungi are to be effective control agents. Aerial dispersal clearly plays a role in passive movement of inoculum between aphid populations. More directed movement will occur through the movement of infected alate aphids, as with *Z. radicans* (Milner *et al.*, 1982). However, these are not the only dispersal mechanisms. For example, the aphid predator *C. septempunctata* can passively carry inoculum of *E. neoaphidis* between *A. pisum* populations while foraging and can thereby establish disease within those populations. This has been demonstrated in laboratory and field studies; in the laboratory, passive vectoring occurred even when the predator foraged on plants with only a single sporulating cadaver present (Pell *et al.*, 1997a; Roy *et al.*, 1998). These studies demonstrate that, with appropriate management, these interactions could be manipulated and encouraged to favour the fungus. This would be particularly appropriate in conjunction with conservation strategies and semiochemicals to manipulate the behaviour of the predators so as to favour dispersal of the pathogen (Roy and Pell, 2000). Parasitoids are not effective as vectors of *E. neoaphidis* to *S. avenae* or *Z. radicans* to *P. xylostella* larvae (Furlong and Pell, 1996; Fuentes-Contreras *et al.*, 1998). In both these cases the parasitoids were contaminated naturally during foraging. If they had been contaminated artificially with more inoculum, it might have been sufficient to facilitate vectoring. Poprawski and Wraight (1998) describe a release of the parasitoid *A. asychis* contaminated with *E. neoaphidis* for control of Russian wheat aphid, *D. noxia*. Aphids were parasitized by the wasp but did not become infected with the fungus. Although this trial was unsuccessful, there remains a potential that parasitoids could be useful in vectoring disease, given appropriate management systems.

Parasitoids and pathogens can interact antagonistically when they compete for resources within their host. For example, the parasitoid wasp *A. rhopalosiphi* continues to attack *E. neoaphidis*-infected cereal aphids until 1 day before fungus-induced

death (Brobyn *et al.*, 1988). Parasitoid development takes longer than fungus development, meaning that, at 20°C, parasitoid oviposition must occur at least 4 days before fungal infection if the parasitoid is to emerge (Powell *et al.*, 1986a). However, since the preferred weather conditions for parasitoids and fungi are contrasting (parasitoids are more active in warm dry conditions and entomophthoralean fungi are more active in cool humid conditions), Milner *et al.* (1984b) suggest that a combination of the two agents is still likely to afford the best control option. Similarly, in the *P. xylostella* natural enemy complex, *Z. radicans* has the potential to interact with two hymenopteran parasitoids, *C. plutellae* and *D. semiclausum*. As already discussed, *D. semiclausum* is susceptible to the fungus, though significantly less so than *P. xylostella*, and infected parasitoids do oviposit significantly fewer eggs than uninfected ones. Neither parasitoid can recognize infected larvae and therefore they oviposit into those larvae. Simultaneous infection and parasitism results in competition within the host. When parasitoid eggs are laid into infected larvae, the eggs are unable to develop successfully. However, if a parasitoid has completed most of its development in the larva prior to infection, it will emerge successfully (Furlong and Pell, 2000). On some occasions the fungus and the parasitoid can emerge from the same host (Fig. 4.82). All these experiments are laboratory-based, and so conclusions must be limited by the context in which the data were collected. In the field, the interactions may be spatially or temporally prohibited; parasitoids can be active under different climatic conditions from fungi, for example. In addition, with appropriate management systems in place, antagonistic interactions can be spatially or temporally avoided.

Habitat manipulation

Biological control using Entomophthorales has been most successful in perennial crop systems (e.g. forestry) or in systems where the pest spends significant periods of time in permanent habitats. This is in part due to the stable nature of the habitats, which act as reservoirs for the fungal natural enemy. In annual cropping systems, foliage is removed and the soil ploughed repeatedly, removing inoculum from the environment. Habitat manipulation could provide permanent or semi-permanent reservoirs in the agroecosystem, with the specific purpose of assisting persistence and early-season multiplication of entomophthoralean fungi. These reservoirs could be managed field margin strips adjacent to hedgerows (Roy and Pell, 2000). The potential of arthropod natural enemies to be conserved in the same areas would improve their chances of encouraging local transmission and vectoring to pest populations in adjacent crops (Roy and Pell, 2000). The success of environmental manipulation to encourage natural enemies relies on the characteristics of the pest, the natural enemy and the ecosystem (Fuxa, 1998). For example, the entomopathogen must be able to replicate extensively, must persist for long periods of time in the reservoir and/or have a sufficiently broad host range to exploit alternate hosts in the reservoir and must be able to disperse from the reservoir into pest hosts on crops. The potential of managed field margins as reservoirs for *E. neoaphidis* is currently under development in the UK (J.K. Pell, unpublished).

Integrating Entomophthorales with host-plant resistance

Plants differ in their nutritive value for herbivorous insects and have an impact on insect physiology and behaviour (Bartlet, 1995; Cole, 1996; Awmack *et al.*, 1997) and,

potentially, susceptibility to entomopathogenic fungi. Few studies have actually considered the role of the host plant on the susceptibility of insects to entomopathogenic fungi and most have focused on Hyphomycetes. In some studies fungal efficacy was synergized, and in others it was inhibited or unaffected (e.g. Hare and Andreadis, 1983; Ramoska and Todd, 1985; Inyang, 1997). In one study with *E. maimaiga*, time to kill was extended on plants less suitable for the host *L. dispar* (Hajek and St Leger, 1994). The effects of malnutrition or starvation, which might occur on unsuitable or resistant host plants, have also been examined. Starvation inhibited infection of the spotted alfalfa aphid *T. trifolii* f. *maculata* by *E. neoaphidis* (Milner and Soper, 1981) and *P. brassicae* by *Z. radicans* (Mietkiewski and van der Geest, 1986). Villacarlos *et al.* (1996) examined the effect of the host plant on *A. gossypii* susceptibility to *N. fresenii* and suggested that there is an impact of the host plant and that it differs with respect to the isolate. Recent work by Fuentes-Contreras *et al.* (1998) has shown that host-plant resistance can also have an impact on the interaction between the parasitoid *A. rhopalosiphi* and *E. neoaphidis* attacking *S. avenae*. In this study it extended the developmental time of the parasitoid but not of the fungus, thereby giving the fungus a competitive advantage. Integrated use of Entomophthorales and host-plant resistance will therefore require further research, particularly with the advent of new transgenic varieties of plants with resistance or partial resistance to pests (Schuler *et al.*, 1998).

Integrating Entomophthorales with other pathogens

The combined use of different pathogens with different biological attributes with respect to the biotic and abiotic environment could be extremely useful, not only in extending the spatial and temporal range over which a target pest can be controlled but also in targeting a number of pests on the same crop. Unfortunately, few studies have been made on interactions between any insect pathogens (Jaques and Morris, 1980). In the *E. maimaiga*/*L. dispar* system, a nuclear polyhedrosis virus (NPV) also causes epizootics and is well known to co-infect individual insects along with *E. maimaiga* (Hajek, 1997c). Co-infection studies suggest that these two important pathogens have minimal negative interactions; during simultaneous infections, *E. maimaiga* kills insects and produces conidia, but, for some treatments when the NPV infected before the *E. maimaiga*, the NPV produced fewer propagules (Malakar *et al.*, 1999).

Autodissemination of *Z. radicans* has the potential to be integrated with the use of other microbial pathogens against the diamondback moth. Complementary studies using *Beauveria bassiana* in the trap suggest that the greatest potential to effect control is given by using both pathogens together in the trap (Pell and Furlong, 1998).

Conclusion

Entomophthorales have great potential in pest management and have been used in a number of successful biological control programmes, particularly in long-term stable systems. They have also been successful in novel approaches under development for use in IPM. Future exploitation lies in their use as components in IPM, and will rely on an increasing understanding of their population genetics, physiology and epizootiology in a multitrophic environment.

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References

Andreadis, T.G., and Weseloh, R.M. (1990) Discovery of *Entomophaga maimaiga* in North American gypsy moth, *Lymantria dispar*. *Proceedings of the National Academy of Sciences, USA* 87, 2461–2465.

Aruta, M.C., Carillo, L.R. and Gonzalez, M.S. (1974) Determinacion para Chile de hongos entomopatogenos del genero *Entomophthora*. *Agro Sur* 2, 62–70.

Awmack, C.S., Harrington, R. and Leather, S.R. (1997) Host plant effects on the performance of the aphid *Aulocorthum solani* (Kalt.) (Homoptera: Aphididae) at ambient and elevated CO_2. *Global Change Biology* 3, 545–549.

Balazy, S. (1993) *Flora of Poland. Fungi (Mycota)* Vol. 24. *Entomophthorales*. Institute of Botany, Krakow, 356 pp.

Bartlet, E. (1995) Chemical cues to host-plant selection by insect pests of oilseed rape. *Agricultural Zoology Reviews* 7, 89–116.

Beauvais, A., Latgé, J.-P., Vey, A. and Prevost, M.-C. (1989) The role of surface components of the entomopathogenic fungus *Entomophaga aulicae* in the cellular immune response of *Galleria mellonella*. *Journal of General Microbiology* 135, 489–498.

Bellini, R., Mullens, B.A. and Jespersen, J.B. (1992) Infectivity of two members of the *Entomophthora muscae* complex (Zygomycetes: Entomophthorales) for *Musca domestica* (Dipt.: Muscidae). *Entomophaga* 37, 11–19.

Ben-Ze'ev, I. and Jaques, R.P. (1990) Entomopathogenic fungi in insects in alfalfa fields in south-western Ontario. *Proceedings of the Entomological Society of Ontario* 121, 71–78.

Ben-Ze'ev, I. and Kenneth, R.G. (1982a) Features-criteria of taxonomic value in the Entomophthorales: I. A revision of the Batkoan classification. *Mycotaxon* 14, 393–455.

Ben-Ze'ev, I. and Kenneth, R.G. (1982b) Features-criteria of taxonomic value in the Entomophthorales: II. A revision of the genus *Erynia* Nowakowski 1881 (= *Zoophthora* Batko (1964). *Mycotaxon* 14, 456–475.

Ben-Ze'ev, I. and Uziel, A. (1979) *Moniellia costalis* (Fitch), a new host for *Zoophthora radicans* (Brefeld) Batko and *Entomophthora planchoniana* Cornu (Zygomycetes: Entomophthoraceae) in Israel. *Phytoparasitica* 7, 159–167.

Bidochka, M.J. and Hajek, A.E. (1996) Protoplast plasma membrane glycoproteins in two species of entomopathogenic fungi. *Mycological Research* 100, 1094–1098.

Bidochka, M.J., and Hajek, A.E. (1998) A nonpermissive entomophthoralean fungal infection increases activation of insect prophenoloxidase. *Journal of Invertebrate Pathology* 72, 231–238.

Bidochka, M.J., Walsh, S.R.A., Ramos, M.E., St Leger, R.J., Silver, J.C. and Roberts, D.W. (1996) Fate of biological control introductions: monitoring an Australian fungal pathogen of grasshoppers in North America. *Proceedings of the National Academy of Sciences USA* 93, 918–921.

Bitton, S., Kenneth, R.G. and Ben-Ze'ev, I. (1979) Zygospore overwintering and sporulative

germination in *Triplosporium fresenii* (Entomophthoraceae) attacking *Aphis spiraecola* on citrus in Israel. *Journal of Invertebrate Pathology* 34, 295–302.

Boykin, L.S., Campbell, W.V. and Beute, M.K. (1984) Effects of pesticides on *Neozygites floridana* (Entomophthorales: Entomophthoraceae) and arthropod predators attacking the twospotted spider mite (Acari: Tetranychidae) in North Carolina peanut fields. *Journal of Economic Entomology* 77, 969–975.

Brandenburg, R.L. and Kennedy, G.G. (1981) Overwintering of the pathogen *Entomophthora floridana* and its host, the twospotted spider mite. *Journal of Economic Entomology* 74, 428–431.

Brandenburg, R.L. and Kennedy, G.G. (1983) Interactive effects of selected pesticides on the two-spotted spider mite and its fungal pathogen, *Neozygites floridana*. *Entomologia Experimentalis et Applicata* 34, 240–244.

Brefeld, O. (1870) Entwicklungsgeschichte der *Empusa muscae* und *Empusa radicans. Botanische Zeitschrift* 28, 161–166, 179–186.

Brobyn, P.J. and Wilding, N. (1977) Invasive and develomental processes of *Entomophthora* species infecting aphids. *Transactions of the British Mycological Society* 69, 349–366.

Brobyn, P.J., Wilding, N. and Clark, S.J. (1985) The persistence of infectivity of conidia of the aphid pathogen *Erynia neoaphidis* on leaves in the field. *Annals of Applied Biology* 107, 365–376.

Brobyn, P.J., Wilding, N. and Clark, S.J. (1987) Laboratory observations on the effect of humidity on the persistence of infectivity of conidia of the aphid pathogen *Erynia neoaphidis*. *Annals of Applied Biology* 110, 579–584.

Brobyn, P.J., Clark, S.J. and Wilding, N. (1988) The effect of fungus infection of *Metopolophium dirhodum* (Homoptera: Aphididae) on the oviposition behaviour of the aphid parasitoid *Aphidius rhopalosiphi* (Hymenoptera: Aphidiidae). *Entomophaga* 33, 333–338.

Brown, G.C. (1987) A modelling approach to epizootiological dynamics of insect host–pathogen interactions and an implementation example. In: Shoemaker, C.A. and Brown, G.C. (eds) *Insect Pest Management Modelling*. John Wiley & Sons, New York.

Brown, G.C. and Nordin, G.L. (1982) An epizootic model of an insect–fungal pathogen system. *Bulletin of Mathematical Biology* 44, 731–740.

Brown, G.C. and Nordin, G.L. (1986) Evaluation of an early harvest approach for induction of *Erynia* epizootics in alfalfa weevil populations. *Journal of the Kansas Entomological Society* 59, 446–453.

Brown, G.C., Prochaska, G.L., Hildebrand, D.F., Nordin, G.L. and Jackson, D.M. (1995) Green leaf volatiles inhibit conidial germination of the entomopathogen *Pandora neoaphidis* (Entomophthorales: Entomophthoraceae). *Environmental Entomology* 24, 1637–1643.

Butt, T.M. and Humber, R.A. (1989) An immunofluorescence study of mitosis in a mite-pathogen, *Neozygites* sp. (Zygomycetes: Entomophthorales). *Protoplasma* 151, 115–123.

Butt, T.M., Beckett, A. and Wilding, N. (1981) Protoplasts in the *in vivo* lifecycle of *Erynia neoaphidis*. *Journal of General Microbiology* 127, 417–421.

Butt, T.M., Wraight, S.P., Galaini-Wraight, S., Humber, R.A., Roberts, D.W. and Soper, R. S. (1988) Humoral encapsulation of the fungus *Erynia radicans* (Entomophthorales) by the potato leafhopper *Empoasca fabae* (Hemiptera: Cicadellidae). *Journal of Invertebrate Pathology* 52, 49–56.

Butt, T.M., Beckett, A. and Wilding, N. (1990) A histological study of the invasive and developmental process of the aphid pathogen *Erynia neoaphidis* (Zygomycetes: Entomophthorales) in the pea aphid *Acyrthosiphon pisum*. *Canadian Journal of Botany* 68, 2153–2163.

Byford, W.J. and Reeve, G.L. (1969) *Entomophthora* species attacking aphids in England, 1962–1966. *Transactions of the British Mycological Society* 52, 342–346.

Carner, G.R. and Canerday, T.D. (1968) Field and laboratory investigations with *Entomophthora fresenii*, a pathogen of *Tetranychus* spp. *Journal of Economic Entomology* 61, 956–959.

Carruthers, R. I. and Haynes, D.L. (1985) Laboratory transmission *in vivo* of *Entomophthora*

muscae (Entomophthorales: Entomophthoraceae) in the onion fly, *Delia antiqua* (Diptera: Anthomyiidae). *Journal of Invertebrate Pathology* 45, 282–287.

Carruthers, R.I. and Onsager, J.A. (1993) Perspective on the use of exotic natural enemies for biological control of pest grasshoppers. *Environmental Entomology* 22, 885–903.

Carruthers, R.I. and Soper, R.S. (1987) Fungal diseases. In: Fuxa, J.R. and Tanada, Y. (eds) *Epizootiology of Insect Diseases.* Wiley Intersciences, New York, pp. 357–416.

Carruthers, R.I., Haynes, D.L. and MacLeod, D.M. (1985) *Entomophthora muscae* (Entomophthorales: Entomophthoraceae) in the onion fly, *Delia antiqua* (Diptera: Anthomyiidae). *Journal of Invertebrate Pathology* 45, 81–93.

Carruthers, R.I., Feng, Z., Ramos, M.E. and Soper, R.S. (1988) The effect of solar radiation on the survival of *Entomophaga grylli* conidia. *Journal of Invertebrate Pathology* 52, 154–162.

Carruthers, R.I., Humber, R.A. and Ramos, M.E. (1989) *Biological Control of Rangeland Grasshoppers Using a Fungal Pathogen from Australia: a Proposed Field Release of an Exotic Fungal Pathogen.* USDA-APHIS Report, 23 pp.

Carruthers, R.I., Sawyer, A.L. and Hural, K. (1991) Use of fungal pathogens as biological control agents of insect pests. In: Rice, B.J. (ed.) *Symposia Proceedings of the National Academy of Sciences*. National Academy of Sciences Press, Washington, DC, pp. 336–372.

Carruthers, R.I., Larkin, T.S., Firstencel, H. and Feng, Z. (1992) Influence of thermal ecology on the mycosis of a rangeland grasshopper. *Ecology* 73, 190–204.

Carruthers, R.I., Ramos, M.E., Larkin, T.S., Hostetter, D.L. and Soper, R.S. (1997) The *Entomophaga grylli* (Fresenius) Batko species complex: its biology, ecology and use for biological control of pest grasshoppers. *Memoirs of the Entomological Society of Canada* 171, 329–353.

Chatigny, M.A., Dimmick, R.L. and Harrington, J.B. (1979) Principles of deposition of microbiological particles. In: Edmonds, R.L. (ed.) *Aerobiology – the Ecological Systems Approach*. Dowden, Hutchinson and Ross, Stroudsberg, Pennsylvania, pp. 111–150.

Cohn, F. (1855) *Empusa muscae* und die Krankheit der Stubenfliegen. *Hedwigia* 1, 57–61.

Cole, R.A. (1996) Comparison of feeding behaviour and development of two brassica pests, *Brevicoryne brassicae* and *Myzus persicae* on wild and cultivated *Brassica* species and an analysis of the glucosinolate and amino acid profiles. *Breeding Resistance in Insects and Mites. IOBC Bulletin* 19, 56–61.

Dean, G.J. and Wilding, N. (1971) *Entomophthora* infecting the cereal aphids, *Metopolophium dirhodum* and *Sitobion avenae. Journal of Invertebrate Pathology* 18, 169–176.

Dean, G.J. and Wilding, N. (1973) Infection of cereal aphids by the fungus *Entomophthora*. *Annals of Applied Biology* 74, 133–138.

Dedryver, C.A. (1978) Facteurs de limitation des populations d'*Aphis fabae* dans l'ouest de la France. III. Répartition et incidence des différentes espèces d'*Entomophthora* dans les populations. *Entomophaga* 23, 137–151.

Dedryver, C.A. (1979) Déclenchement en serre d'une épizootie à *Entomophthora fresenii* Nowak. sur *Aphis fabae* Scop. Par introduction d'inoculum et régulation de l'humidité relative. *Entomophaga* 24, 443–453.

Delalibera, I. Jr (1996) Producao '*in vitro*' de *Neozygites* sp. (Zygomycetes: Entomophthorales) patogeno de *Mononychellus tanajoa*. *Proceedings Simposio Controle Biologico, Foz do Iguacu, Brasil*, p. 171.

Delalibera, I. Jr, Sosa Gomez, D.R., de Moraes, G.J. de Alencar, J.A. and Farias Araujo, W. (1992) Infection of *Mononychellus tanajoa* (Acari: Tetranychidae) by the fungus *Neozygites* sp. (Entomophthorales) in northeastern Brazil. *Florida Entomologist* 75, 145–147.

Dent, D. (1995) *Integrated Pest Management.* Chapman & Hall, London, 356 pp.

van Driesche, R.G. and Bellows, T.S. (1996) *Biological Control.* Chapman & Hall, New York, 539 pp.

Dromph, K.M., Pell, J.K. and Eilenberg, J. (1996) Infection of *Sitobion avenae* with *Erynia neoaphidis*. In: *Proceedings of the III International Colloquium on* Bacillus thuringiensis *and the XXIX Annual Meeting of the Society for Invertebrate Pathology, Cordoba, Spain, September*, p. 22 (abstract).

Dromph, K., Pell, J.K. and Eilenberg, J. (1997) Sporulation of *Erynia neoaphidis* from alate *Sitobion avenae*. In: *Insect Pathogens and Insect Parasitic Nematodes. IOBC Bulletin* 21, 91–94.

Dromph, K.M., Eilenberg, J. and Esbjerg, P. (2000) Natural occurrence of entomophthoralean fungi on collembolans. *IOBC/WPRS Bulletin* 23, 147–149.

Dwyer, G., Elkinton, J.S. and Hajek, A.E. (1998) Spatial scale and the spread of a fungal pathogen of gypsy moth. *American Naturalist* 152, 485–494.

Eilenberg, J. (1985) Relationship between the carrot fly (*Psila rosae* F.) and its fungal pathogens from Entomophthorales, particularly *Entomophthora muscae* (C.) Fres. PhD thesis, Department of Zoology, Royal Veterinary and Agricultural University, Copenhagen, Denmark, 109 pp.

Eilenberg, J. (1987a) The culture of *Entomophthora muscae* (C.) Fres. in the carrot fly (*Psila rosae* F.) and the effect of temperature on the pathology of the fungus. *Entomophaga* 32, 425–435.

Eilenberg, J. (1987b) Abnormal egg-laying behaviour of female carrot flies (*Psila rosae*) induced by the fungus *Entomophthora muscae*. *Entomologia Experimentalis et Applicata* 52, 17–24.

Eilenberg, J. (1988) Occurrence of fungi from Entomophthorales in a population of carrot flies (*Psila rosae* F.). Results 1985 and 1986. *IOBC/WPRS Bulletin* 11, 53–59.

Eilenberg, J. (2000) Entomophthorales on Diptera. In: Papp, L. and Darvas, B. (eds) *Manual of Palearctic Diptera*, Vol. 1, *General and Applied Dipterology*. Science Herald, Budapest, pp. 519–533.

Eilenberg, J. and Michelsen, V. (1999) Natural host range and prevalence of the genus *Strongwellsea* (Zygomycota: Entomophthorales) in Denmark. *Journal of Invertebrate Pathology* 73, 189–198.

Eilenberg, J. and Philipsen, H. (1988) The occurrence of *Entomophthorales* on the carrot fly (Psila rosae F.) in the field during two successive seasons. *Entomophaga* 33, 135–144.

Eilenberg, J., Bresciani, J. and Martin, J. (1987) *Entomophthora* species with *E. muscae* like primary spores on two new insect orders, Coleoptera and Hymenoptera. *Nordic Journal of Botany* 7, 577–584.

Eilenberg, J., Bresciani, J. and Latgé, J.P. (1990) Primary spore and resting spore formation *in vitro* of *Entomophthora schizophorae* and *E. muscae*, both members of the *E. muscae*-complex. *Cryptogamic Botany* 1, 365–371.

Eilenberg, J., Wilding, N. and Bresciani, J. (1992) Isolation *in vitro* of *Strongwellsea castrans* (Fungi: Entomophthorales) a pathogen of adult cabbage root flies, *Delia radicum* (Dipt.: Anthomyiidae). *Entomophaga* 37, 65–77.

Elkinton, J.S., Hajek, A.E., Boettner, G.H. and Simons, E.E. (1991) Distribution and apparent spread of *Entomophaga maimaiga* (Zygomycetes: Entomophthorales) in gypsy moth (Lepidoptera: Lymantriidae) populations in North America. *Environmental Entomology* 20, 1601–1605.

Elliot, S.L., de Moraes, G.J., Delalibera I. Jr, da Silva, C.A.D., Tamai, M.A. and Mumford, J.D. (2000) Potential of the mite-pathogenic fungus *Neozygites floridana* (Entomophthorales: Neozygitaceae) for control of the cassava green mite *Mononychellus tanajoa* (Acari: Tetranychidae). *Bulletin of Entomological Research* 90, 191–200.

van Emden, H.F. (1989) *New Studies in Biology: Pest Control.* Edward Arnold, London, 117 pp.

Evans, H.C. (1989) Mycopathogens of insects of epigeal and aerial habitats. In: Wilding, N., Collins, N.M., Hammond, P.M. and Webber, J.F. (eds) *Insect–Fungus Interactions.* Academic Press, London, pp. 205–238.

Feng, M.-G., Johnson, J.B. and Halbert, S.E. (1991) Natural control of cereal aphids (Homoptera: Aphididae) by entomopathogenic fungi (Zygomycetes: Entomophthorales) and parasitoids (Hymenoptera: Braconidae and Encyrtidae) on irrigated spring wheat in southwestern Idaho. *Environmental Entomology* 20, 1699–1710.

Feng, M.-G., Nowierski, R.M., Klein, R.E., Scharen, A.L. and Sands, D.C. (1992) Spherical hyphal bodies of *Pandora neoaphidis* (Remaudière and Hennebert) Humber (Zygomycetes: Entomophthorales) on *Acyrthosiphon pisum* Harris (Homoptera: Aphididae): a potential overwintering form. *Pan-Pacific Entomologist* 68, 100–104.

Firstencel, H., Butt, T.M. and Carruthers, R.I. (1990) A fluorescence microscopy method for determining the viability of entomophthoralean fungal spores. *Journal of Invertebrate Pathology* 55, 258–264.

Freimoser, F.M., Grundchober, A., Aebi, M. and Tuor, U. (2000) *In vitro* cultivation of the entomopathogenic fungus *Entomophthora thripidum*: isolation, growth requirements and sporulation. *Mycologia* 92, 208–215.

Fresenius G. (1858) Ueber die Pilzgattung *Entomophthora*. *Abhandlungender Senckenbergischen Naturforschenden Gesellschaft* 2, 201–210.

Fuentes-Contreras, E., Pell, J.K. and Niemeyer, H.M. (1998) Influence of plant resistance at the third trophic level: interactions between parasitoids and entomopathogenic fungi of cereal aphids. *Oecologia* 117, 426–432.

Funk, C.J., Ramoska, W.A. and Bechtel, D.B. (1990) Light and electron microscopic studies of protoplast development during *Entomophaga grylli* pathotype 2 infections in *Melanoplus differentialis*. *Journal of Invertebrate Pathology* 55, 207–214.

Funk, C.J., Ramoska, W.A. and Bechtel, D.B. (1993) Histopathology of *Entomophaga grylli* pathotype 2 infections in *Melanoplus differentialis*. *Journal of Invertebrate Pathology* 61, 196–202.

Furlong, M.J. and Pell, J.K. (1996) Interactions between the fungal entomopathogen *Zoophthora radicans* Brefeld (Entomophthorales) and two hymenopteran parasitoids attacking the diamondback moth, *Plutella xylostella* L. *Journal of Invertebrate Pathology* 68, 15–21.

Furlong, M.J. and Pell, J.K. (1997) The influence of environmental factors on the persistence of *Zoophthora radicans* conidia. *Journal of Invertebrate Pathology* 69, 223–233.

Furlong, M.J. and Pell, J.K. (2000) Conflicts between a fungal entomopathogen, *Zoophthora radicans*, and two larval parasitoids of the diamondback moth. *Journal of Invertebrate Pathology* 76, 85–94.

Furlong, M.J., Pell, J.K., Ong, P.C. and Syed, A.R. (1995) Field and laboratory evaluation of a sex pheromone trap for the autodissemination of the fungal entomopathogen *Zoophthora radicans* (Entomophthorales) by the diamondback moth, *Plutella xylostella* (Lepidoptera: Yponomeutidae). *Bulletin of Entomological Research* 85, 331–337.

Furlong, M.J., Pell, J.K. and Reddy, G.V.P. (1997) Premortality effects of *Zoophthora radicans* (Zygomycetes: Entomophthorales) infection on larval feeding rate and adult fecundity in *Plutella xylostella* (Lepidoptera: Yponomeutidae). *Journal of Invertebrate Pathology* 70, 214–220.

Fuxa, J. (1998) Environmental manipulation for microbial control of insects. In: Barbosa, P. (ed.) *Conservation Biological Control*. Academic Press, London, pp. 255–268.

Galaini-Wraight, S., Wraight, S.P., Carruthers, R.I., Magalhaes, B.P. and Roberts, D.W. (1991) Description of a *Zoophthora radicans* (Zygomycetes: Entomophthoraceae) epizootic in a population of *Empoasca kraemeri* (Hemiptera: Cicadellidae) on beans in central Brazil. *Journal of Invertebrate Pathology* 58, 311–326.

Galaini-Wraight, S., Wraight, S.P., Carruthers, R.I. and Roberts, D.W. (1992) Temperature-dependent germination and host penetration of the entomophthoralean fungus *Zoophthora radicans* on the leafhopper *Empoasca kraemeri. Mycological Research* 96, 38–48.

Geden, C.J., Steinkraus, D.C. and Rutz, D.A. (1993) Evaluation of two methods for release of *Entomophthora muscae* (Entomophthorales: Entomophthoraceae) to infect house flies (Diptera: Muscidae) on dairy farms. *Environmental Entomology* 20, 1201–1208.

Giard, A. (1888) Note sur deux types remarquables d'Entomophthorées, *Empusa fresenii* Now. et *Basidiobolus ranarum* Eid. suivie de la description de quelques espèces nouvelles. *Compte Rendu des Seances de la Societe de Biologie Paris* 40, 783–787.

Glare, T.R. and Milner, R.J. (1991) Ecology of entomopathogenic fungi. In: Arora, D.K., Ajello, L. and Mukerji, K.G. (eds) *Handbook of Applied Mycology*, Vol. 2, *Humans, Animals, and Insects.* Marcel Dekker, New York, pp. 547–612.

Glare, T.R., Milner, R.J., Chilvers, G.A., Mahon, R.J. and Brown, W.V. (1987) Taxonomic implications of intraspecific variation amongst isolates of the aphid-pathogenic fungi

Zoophthora radicans Brefeld and *Z. phalloides* Batko (Zygomycetes: Entomophthorales). *Australian Journal of Botany* 35, 49–67.

Glare, T.R., Milner, R.J. and Chilvers, G.A. (1989a) Production, stabilisation and regeneration of protoplasts of *Zoophthora radicans* using a commercial enzyme. *Mycological Research* 92, 477–485.

Glare, T.R., Milner, R.J. and Chilvers, G.A. (1989b) Factors affecting the production of resting spores by *Zoophthora radicans* in the spotted alfalfa aphid *Therioaphis trifolii* f. *maculata. Canadian Journal of Botany* 67, 848–855.

Goettel, M.S. (1994) Host range and specificity in relation to safety of exotic fungi. In: *Proceedings of the VI International Colloquium on Invertebrate Pathology and Microbial Control. XXVII Annual Meeting of the Society for Invertebrate Pathology, Montpellier,* pp. 325–329.

Goettel, M.S., Poprawski, T.J., Vandenberg, J.D., Li, Z. and Roberts, D.W. (1990) Safety to nontarget invertebrates of fungal biocontrol agents. In: Laird, M., Lacey, L.A. and Davidson, E.W. (eds) *Safety of Microbial Insecticides.* CRC Press, Boca Raton, Florida, pp. 209–231.

Gray, S.N. and Markham, P. (1997) A model to explain the growth kinetics of the aphid-pathogenic fungus *Erynia neoaphidis* in liquid culture. *Mycological Research* 101, 1475–1483.

Gray, S.N., Robinson, P., Wilding, N. and Markham, P. (1990) Effect of oleic acid on vegetative growth of the aphid-pathogenic fungus *Erynia neoaphidis*. *FEMS Microbiology Letters* 68, 131–136.

Griggs, M.H., Vandenberg, J.D. and Sawyer, A.J. (1999) Effect of relative humidity on viability of primary conidia of *Zoophthora radicans. Journal of Invertebrate Pathology* 73, 315–320.

Grundschober, A., Tuor, U. and Aebi, M. (1998) *In vitro* cultivation and sporulation of *Neozygites parvispora* (Zygomycetes: Entomophthorales). *Systematic and Applied Microbiology* 21, 461–469.

Gustafsson, M. (1965a) On species in the genus *Entomophthora* Fres. in Sweden. I. Classification and distribution. *Lantbrukshögskolans Annaler* 31, 103–212.

Gustafsson, M. (1965b) On species of the genus *Entomophthora* Fres. in Sweden. II. Cultivation and physiology. *Lantbrukshögskolans Annaler* 31, 405–457.

Gustafsson, M. (1965c) On species of the genus *Entomophthora* Fres. in Sweden. III. Possibility of usage in biological control. *Lantbrukshögskolans Annaler* 35, 235–274.

Hajek, A.E. (1989) Food consumption of *Lymantria dispar* (Lepidoptera: Lymantriidae) larvae infected with *Entomophaga maimaiga* (Zygomycetes: Entomophthorales). *Environmental Entomology* 18, 723–727.

Hajek, A.E. (1997a) Ecology of terrestrial fungal entomopathogens. *Advances in Microbial Ecology* 15, 193–249.

Hajek, A.E. (1997b) *Entomophaga maimaiga* reproductive output is determined by the spore type initiating an infection. *Mycological Research* 101, 971–974.

Hajek, A.E. (1997c) Fungal and viral epizootics in gypsy moth (Lepidoptera: Lymantriidae) populations in central New York. *Biological Control* 10, 58–68.

Hajek, A.E. (1999) Pathology and epizootiology of the Lepidoptera-specific mycopathogen *Entomophaga maimaiga*. *Microbiology and Molecular Biology Reviews* 63, 814–835.

Hajek, A.E. and Humber, R.A. (1997) Formation and germination of *Entomophaga maimaiga* azygospores. *Canadian Journal of Botany* 75, 1739–1747.

Hajek, A.E. and Roberts, D.W. (1991) Pathogen reservoirs as a biological control resource: introduction of *Entomophaga maimaiga* to North American gypsy moth, *Lymantria dispar*, populations. *Biological Control* 1, 29–34.

Hajek, A.E. and St Leger, R.J. (1994) Interactions between fungal pathogens and insect hosts. *Annual Review of Entomology* 39, 293–322.

Hajek, A.E. and Shimazu, M. (1996) Types of spores produced by *Entomophaga maimaiga* infecting the gypsy moth *Lymantria dispar*. *Canadian Journal of Botany* 74, 708–715.

Hajek, A.E. and Webb, R.E. (1999) Inoculative augmentation of the fungal entomopathogen *Entomophaga maimaiga* as a homeowner tactic to control gypsy moth (Lepidoptera: Lymantriidae). *Biological Control* 14, 11–18.

Hajek, A.E. and Wheeler, M.M. (1994) Application of techniques for quantification of soil-borne entomophthoralean resting spores. *Journal of Invertebrate Pathology* 64, 71–73.

Hajek, A.E., Carruthers, R.I. and Soper, R.S. (1990a) Temperature and moisture relations of sporulation and germination by *Entomophaga maimaiga* (Zygomycetes: Entomophthoraceae), a fungal pathogen of *Lymantria dispar* (Lepidoptera: Lymantriidae). *Environmental Entomology* 19, 85–90.

Hajek, A.E., Humber, R.A., Elkinton, J.S., May, B., Walsh, S.R.A. and Silver, J.C. (1990b) Allozyme and RFLP analyses confirm *Entomophaga maimaiga* responsible for 1989 epizootics in North American gypsy moth populations. *Proceedings of the National Academy of Sciences USA* 87, 6979–6982.

Hajek, A.E., Larkin, T.S., Carruthers, R.I. and Soper, R.S. (1993) Modeling the dynamics of *Entomophaga maimaiga* (Zygomycetes: Entomophthorales) epizootics in gypsy moth (Lepidoptera: Lymantriidae) populations. *Environmental Entomology* 22, 1172–1187.

Hajek, A.E., Butler, L. and Wheeler, M.W. (1995a) Laboratory bioassays testing the host range of the gypsy moth fungal pathogen *Entomophaga maimaiga*. *Biological Control* 5, 530–544.

Hajek, A.E., Humber, R.A. and Elkinton, J.S. (1995b) Mysterious origin of *Entomophaga maimaiga* in North America. *American Entomologist* 41, 31–42.

Hajek, A.E., Butler, L., Walsh, S.R.A., Silver, J.C., Hain, F.P., Hastings, F.L., O'Dell, T.M. and Smitley, D.R. (1996a) Host range of the gypsy moth (Lepidoptera: Lymantriidae) pathogen *Entomophaga maimaiga* (Zygomycetes: Entomophthorales) in the field versus laboratory. *Environmental Entomology* 25, 709–721.

Hajek, A.E., Elkinton, J.S. and Witcosky, J.J. (1996b) Introduction and spread of the fungal pathogen *Entomophaga maimaiga* along the leading edge of gypsy moth spread. *Environmental Entomology* 25, 1235–1247.

Hajek, A.E., Walsh, S.R.A., Silver, J.C. and Strong, D.R. (1996c) A disjunct Californian strain of *Entomophaga aulicae* infecting *Orygia vetusta. Journal of Invertebrate Pathology* 68, 260–268.

Hajek, A.E., Bauer, L., Wheeler, M.M. and McManus, M.L. (1998a) Distribution of resting spores of the *Lymantria dispar* pathogen *Entomophaga maimaiga* in soil and on bark. *BioControl* 43, 189–200.

Hajek, A.E., Tatman, K.M., Wanner, P.H. and Wheeler, M.M. (1998b) Location and persistence of gypsy moth, *Lymantria dispar*, cadavers containing *Entomophaga maimaiga* azygospores. *Mycologia* 90, 754–760.

Hajek, A.E., Olsen, C.H., Elkinton, J.S. (1999) Dynamics of airborne conidia of the gypsy moth (Lepidoptera: Lymantriidae) fungal pathogen *Entomophaga maimaiga* (Zygomycetes: Entomophthorales). *Biological Control* 16, 111–117.

Hall, I.M. and Dunn, P.H. (1957) Entomogenous fungi on the spotted alfalfa aphid. *Hilgardia* 27, 159–181.

Hall, I.M. and Dunn, P.H. (1959) The effect of certain insecticides and fungicides on fungi pathogenic to the spotted alfalfa aphid. *Journal of Economic Entomology* 52, 28–29.

Harcourt, D.G., Guppy, J.C., MacLeod, D.M. and Tyrrell, D. (1974) The fungus *Entomophthora phytonomi* pathogenic to the alfalfa weevil, *Hypera postica. Canadian Entomologist* 106, 1295–1300.

Hare, J.D. and Andreadis, T.G. (1983) Variation in the susceptibility of *Leptinotarsa decemlineata* (Coleoptera: Chrysomelidae) when reared on different host plants to the fungal pathogen *Beauveria bassiana* in the field and laboratory. *Environmental Entomology* 12, 1892–1897.

Hatting, J.L., Humber, R.A., Poprawski, T.J. and Miller, R.M. (1999) A survey of fungal pathogens of aphids from South Africa with special reference to cereal aphids. *Biological Control* 16, 1–12.

Hemmati, F. (1999) Aerial dispersal of the entomopathogenic fungus *Erynia neoaphidis*. PhD thesis, University of Reading, UK, 186 pp.

Hemmati, F., Pell, J.K., McCartney, H.A., Clark, S.J. and Deadman, M.L. (2001a) Active discharge in the aphid pathogenic fungus *Erynia neoaphidis*. *Mycological Research* (in press).

Hemmati, F., Pell, J.K., McCartney, H.A. and Deadman, M.L. (2001b) Airborne concentrations of conidia of *Erynia neoaphidis* above cereal fields. *Mycological Research* (in press).

Hermann, D. (2000) *In vitro* effects of ten fungicides on the aphid pathogen *Erynia neoaphidis*. MSc thesis, Nottingham Trent University, UK.

Hodge, K.T., Sawyer, A.J. and Humber, R.A. (1995) RAPD-PCR for identification of *Zoophthora radicans* isolates in biological control of the potato leafhopper. *Journal of Invertebrate Pathology* 65, 1–9.

Hollingsworth, R.G., Steinkraus, D.C. and McNew, R.W. (1995) Sampling to predict fungal epizootics on cotton aphids (Homoptera: Aphididae). *Environmental Entomology* 24, 1414–1421.

Huang, Y., Zhen, B. and Li, Z. (1992) Natural and induced epizootics of *Erynia ithacensis* in mushroom hothouse populations of yellow-legged fungus gnats. *Journal of Invertebrate Pathology* 60, 254–258.

Humber, R.A. (1981) An alternative view of certain taxonomic criteria used in the Entomophthorales (Zygomycetes). *Mycotaxon* 13, 191–240.

Humber, R.A. (1989) Synopsis of a revised classification for the Entomophthorales (Zygomycotina). *Mycotaxon* 34, 441–460.

Humber, R.A. and Soper, R.S. (1981) Proposal to conserve the entomopathogenic fungal genus *Triplosporium* (Thaxter) Batko against *Neozygites* Witlaczil (Entomophthorales). *Taxon* 30, 353–357.

Inyang, E.N. (1997) Effect of the host plant on susceptibility of crucifer pests to the insect pathogenic fungus *Metarhizium anisopliae*. PhD thesis, University of London.

Jaques, R.P. and Morris, O.N. (1980) Compatibility of pathogens with other methods of pest control and with different crops. In: Burges, H.D. (ed.) *Microbial Control of Pests and Plant Diseases 1970–1980.* Academic Press, London.

Jensen, A.B. and Eilenberg, J. (2000) Genetic variation within the insect-pathogenic fungus genus *Entomophthora*, focusing on the *E. muscae* complex, using PCR-RFLP of the ITS II and the LSU rDNA. *Mycological Research* 105, 307–312.

Jensen, A.B., Gargas, A., Eilenberg, J. and Rosendahl, S. (1998) Relationships of the insect-pathogenic Order Entomophthorales (Zygomycota, Fungi) based on phyllogenetic analyses of the nuclear small subunit ribosomal DNA sequences (SSU rDNA). *Fungal Genetics and Biology* 24, 325–334.

Kalsbeek, V., Mullens, B.A. and Jespersen, J.B. (1999) Field studies of *Entomophthora* induced behavioural fever in *Musca domestica* in Denmark. In: *Proceedings of the XXXIII Annual Meeting of the Society for Invertebrate Pathology, Irvine, California, August*, p. 50.

Kanervo, V. (1946) Sporadic observations concerning diseases in certain species of insects 3. Diseases attacking *Plutella maculipennis* Curt. *Suomen Hyonteistieteellinen Aikakauskija* 12, 143–153.

Kanervo, V. (1949) On the epidemiology of the diamondback moth (*Plutella maculipennis* Curt.). *Suomen Hyonteistieteellinen Aikakauskija* 14, 99–105.

Keller, S. (1984) *Entomophthora muscae* als Artenkomplex. *Mitteilungen der Schweizerischen Entomologischen Gesellschaft* 57, 131–132.

Keller, S. (1987a) Die Bedeutung ökologischer ausgleichsflächen für den Pflanzenschutz. *Mitteilungen der Schweizerischen Landwirtschaft* 35, 56–65.

Keller, S. (1987b) Observations on the overwintering of *Entomophthora planchoniana. Journal of Invertebrate Pathology* 50, 333–335.

Keller, S. (1987c) Arthropod-pathogenic Entomophthorales of Switzerland. I. *Conidiobolus, Entomophaga* and *Entomophthora. Sydowia* 40, 122–167.

Keller, S. (1991) Arthropod-pathogenic Entomophthorales of Switzerland. II. *Erynia, Eryniopsis, Neozigites, Zoophthora* and *Tarichium. Sydowia* 43, 39–122.

Keller, S. (1997) The genus *Neozygites* (Zygomycetes, Entomophthorales) with special reference to species found in tropical regions. *Sydowia* 49, 118–146.

Keller, S. (1998) The role of Entomophthorales in a sustainable agriculture. In: *Insect Pathogens and Insect Parasitic Nematodes. IOBC Bulletin* 21, 13–16.

Keller, S. and Steenberg, T. (1997) *Neozygites sminthuri* sp. nov. (Zygomycetes: Entomophthorales) a pathogen of the springtail *Sminthurus viridis* L. (Collembola: Sminthuridae). *Sydowia* 49, 21–24.

Keller, S. and Suter, H. (1980) Epizootiologische Untersuchungen über das Entomophthora-Auftreten bei feldbaulich wichtigen Blattlausarten. *Acta Oecologica, Oecologica Applicata* 1, 63–81.

Keller, S. and Zimmerman, G. (1989) Mycopathogens of soil insects. In: Wilding, N., Collins, N.M., Hammond, P.M. and Webber, J.F. (eds) *Insect–Fungus Interactions.* Academic Press, London, pp. 240–270.

Keller, S., Kalsbeek, V. and Eilenberg, J. (1999) Redescription of *Entomophthora muscae* (Cohn) Fresenius. *Sydowia* 51, 197–209.

Kenneth, R. and Olmert, I. (1975) Entomopathogenic fungi and their insect hosts in Israel: additions. *Israel Journal of Entomology* 10, 105–112.

Kenneth, R., Wallis, G., Gerson, U. and Plaut, H.N. (1972) Observations and experiments on *Triplosporium floridanum* (Entomophthorales) attacking spider mites in Israel. *Journal of Invertebrate Pathology* 19, 366–369.

King, D.S. (1977) Systematics of *Conidiobolus* (Entomophthorales) using numerical taxonomy. III. Descriptions of recognised species. *Canadian Journal of Botany* 55, 718–729.

Klingen, I., Meadow, R. and Eilenberg, J. (2000) Prevalence of fungal infections in adult *Delia radicum* and *Delia floralis* trapped on the edge of a cabbage field. *Entomologia Experimentalis et Applicata* 97, 265–274.

Klubertanz, T.H., Pedigo, L.P. and Carlson, R.E. (1991) Impact of fungal epizootics on the biology and management of the twospotted spider mite (Acari: Tetranychidae) in soybean. *Environmental Entomology* 20, 731–735.

Kobayashi, Y., Mogami, K. and Aoki, J. (1984) Ultrastructural studies on the hyphal growth of *Erynia neoaphidis* in the green peach aphid *Myzus persicae*. *Transactions of the Mycological Society of Japan* 25, 425–434.

Kogan, M., Turnipseed, S.G., Shepard, M., Oliveira, E.B. and Borgo, A. (1977) Pilot insect pest management program for soybean in southern Brazil. *Journal of Economic Entomology* 70, 659–663.

Kogan, M., Gerling, D. and Maddox, J.V. (1999) Enhancement of biological control in annual agricultural environments. In: Bellows, T.S. and Fisher, T.W. (eds) *Handbook of Biological Control Principles and Applications of Biological Control.* Academic Press, London, pp. 789–817.

Kogan, P.H. and Hajek, A.E. (2000) Formation of azygospores by the insect pathogenic fungus *Entomophaga maimaiga* in cell culture. *Journal of Invertebrate Pathology* 75, 193–201.

Koyama, R. (1954) Two epizootic diseases of the gypsy moth. *Shirin-Boeki* 27, 296–298.

Kramer, J.P. and Steinkraus, D.C. (1981) Culture of *Entomophthora muscae in vivo* and its infectivity for six species of muscoid flies. *Mycopathologia* 76, 139–143.

Kramer, J.P. and Steinkraus, D.C. (1993) Experimental introduction of the mycosis caused by *Entomophthora muscae* in a population of house flies (*Musca domestica*) in a poultry house. *Journal of the New York Entomological Society* 95, 114–117.

Krasnoff, S.B., Watson, D.W., Gibson, D.M. and Kwan, E.C. (1995) Behavioral effects of the entomopathogenic fungus, *Entomophthora muscae*, on its host *Musca domestica*: postural changes in dying hosts and gated pattern of mortality. *Journal of Insect Physiology* 41, 895–903.

Kuntz, W.A. (1925) Aphid diseases. In: *Florida Agricultural Experimental Station Report,* Gainesville, Florida, pp. 46R–49R.

Kuramoto, H. and Shimazu, M. (1997) Control of house fly populations by *Entomophthora muscae* (Zygomycotina: Entomophthorales) in a poultry house. *Applied Entomology and Zoology* 32, 325–331.

Lagnaoui, A. and Radcliffe, E.B. (1998) Potato fungicides interfere with entomopathogenic fungi impacting population dynamics of green peach aphid. *American Journal of Potato Research* 75, 19–25.

Lambiase, J.T. and Yendol, W.G. (1977) The fine structure of *Entomophthora apiculata* and its penetration of *Trichoplusia ni. Canadian Journal of Microbiology* 23, 452–464.

Latgé, J.-P. (1975a) Croissance et sporulation de 6 espèces d'Entomophthorales. I. Influence de la nutrition carbonée. *Entomophaga* 20, 201–207.

Latgé, J.-P. (1975b) Croissance et sporulation de 6 espèces d'Entomophthorales. II. Influence de diverses sources d'azote. *Mycopathologia* 57, 53–57.

Latgé, J.-P. (1980) Sporulation de *Entomophthora obscura* Hall & Dunn en culture liquide. *Canadian Journal of Microbiology* 26, 1038–1048.

Latgé, J.-P. (1981) Comparaison des exigences nutritionelles des Entomophthorales. *Annales de Microbiologie (Institut Pasteur)* 132B, 299–306.

Latgé, J.-P. and de Bievre, C. (1976) Influence des lipides et acides gras du jaune d'oeuf sur la croissance et la sporulation des Entomophthorales. *Annales de Microbiologie (Institut Pasteur)* 127A, 261–274.

Latgé, J.-P. and Perry, D.F. (1980) The utilization of an *Entomophthora obscura* resting spore preparation in biological control experiments against cereal aphids. *Bulletin SROP* 3, 19–25.

Latgé, J.-P. and Sanglier, J.J. (1985) Optimisation de la croissance et de la sporulation de *Conidiobolus obscurus* en mileu défini. *Canadian Journal of Botany* 63, 68–85.

Latgé, J.-P. Soper, R.S. and Madore, C.D. (1977) Media suitable for industrial production of *Entomophthora virulenta* zygospores. *Biotechnology and Bioengineering* 19, 1269–1284.

Latgé, J.-P., Remaudière, G., Soper, R.S., Madore, C.D. and Diaquin, M. (1978) Growth and sporulation of *Entomophthora virulenta* on semidefined media in liquid culture. *Journal of Invertebrate Pathology* 31, 225–233.

Latgé, J.-P., Silvie, P., Papierok, B., Remaudière, G., Dedryver, C.A. and Rabasse, J.M. (1983) Advantages and disadvantages of *Conidiobolus obscurus* and of *Erynia neoaphidis* in the biological control of aphids. In: Cavalloro, R. (ed.) *Aphid Antagonists.* A.A. Balkema, Rotterdam, the Netherlands, pp. 20–32.

Latgé, J.-P., Eilenberg, J., Beauvais, A. and Prevost, M.C. (1988) Morphology of *Entomophthora muscae* protoplasts grown *in vitro. Protoplasma* 146, 166–173.

Latteur, G. and Godefroid, J. (1983). Trial of field treatments against cereal aphids with mycelium of *Erynia neoaphidis* (Entomophthorales) produced *in vitro*. In: Cavalloro, R. (ed.) *Aphid Antagonists*, A.A. Balkema, Rotterdam, the Netherlands, pp. 2–10.

Leite, L.G. (1991) Estudo de alguns fatores que afetam a epizootia de *Zoophthora radicans* e utilização do fungo para o controle de *Empoasca* sp. MS thesis, Univ. São Paolo, Piracicaba, Brazil.

Leite, L.G., Alves, S.B., Wraight, S.P., Galaini-Wraight, S., Roberts, D.W. and Magalhães, B.P. (1996a) Conidiogênese de *Zoophthora radicans* (Brefeld) Batko sobre *Empoasca kraemeri* (Ross and Moore) à diferentes temperaturas. *Arq. Instituto Biológico, São Paulo* 63, 47–53.

Leite, L.G., Alves, S.B., Wraight, S.P., Galaini-Wraight, S. and Roberts, D.W. (1996b) Habilidade de infecçã de isolados de *Zoophthora radicans* sobre *Empoasca kraemeri. Scientia-Agricola* 53, 152–158.

Leite, L.G., Alves, S.B., Wraight, S.P., Galaini-Wraight, S. and Roberts, D.W. (1996c) Processos de germinação de isolados do fungo *Zoophthora radicans* sobre *Empoasca kraemeri. Scientia-Agricola* 53, 179–183.

Leite, L.G., Alves, S.B., Wraight, S.P., Galaini-Wraight, S. and Roberts, D.W. (1996d) Comportamento de isolados do fungo *Zoophthora radicans* sobre *Empoasca kraemeri. Ecosistema* 21, 16–22.

Leite, L.G., Smith, L., Moraes, G.J. and Roberts, D.W. (1996e) Production of hyphal bodies of the fungus *Neozygites* nr. *floridana 'in vitro'*. In: *Proc. Simposio Controle Biologico, Foz do Iguacu, Brazil*, p. 448.

Li, Z., Butt, T.M., Beckett, A. and Wilding, N. (1993) The structure of dry mycelia of the entomophthoralean fungi *Zoophthora radicans* and *Erynia neoaphidis* following different preparatory treatments. *Mycological Research* 97, 1315–1323.

Lizen, E., Latteur, G. and Oger, R. (1985) Sensibilité à infection par l'Entomophthorale *Erynia neoaphidis* Remaud. et Henn. du puceron *Acyrthosiphon pisum* (Harris) selon sa forme, son stade et son âge. *Parasitica* 41, 163–170.

Lockwood, J.A. (1993a) Environmental issues involved in biological control of rangeland grasshoppers with exotic agents. *Environmental Entomology* 22, 503–518.

Lockwood, J.A. (1993b) Benefits and costs of controlling rangeland grasshoppers with exotic organisms: search for a null hypothesis and regulatory compromise. *Environmental Entomology* 22, 904–914.

McCabe, D. and Soper, R.S. (1985) Preparation of an entomopathogenic fungal insect control agent. US patent document no. 4530834.

McCabe, D.E., Humber, R.A. and Soper, R.S. (1984) Observation and interpretation of nuclear reductions during maturation and germination of entomophthoralean resting spores. *Mycologia* 76, 1104–1107.

McGuire, M.R., Maddox, J.V. and Ambrust, E.J. (1987a) Host range studies of an *Erynia radicans* strain (Zygomycetes: Entomophthorales) isolated from *Empoasca fabae* (Homoptera Cicadellidae). *Journal of Invertebrate Pathology* 50, 75–77.

McGuire, M.R., Morris, M.J., Armbrust, E.J. and Maddox, J.V. (1987b) An epizootic caused by *Erynia radicans* (Zygomycetes: Entomophthoraceae) isolated from *Empoasca fabae* (Homoptera: Cicadellidae). *Journal of Invertebrate Pathology* 50, 78–80.

McGuire, M.R., Maddox, J.V. and Ambrust, E.J. (1987c) Effect of temperature on distribution and success of introduction of an *Empoasca fabae* (Homoptera: Cicadellidae) isolate of *Erynia radicans* (Zygomycetes: Entomophthoraceae). *Journal of Invertebrate Pathology* 50, 291–301.

MacLeod, D.M. (1963) *Entomophthorales infections.* In: Steinhaus, E.A. (ed.) *Insect Pathology: an Advanced Treatise,* Vol. 2. Academic Press, New York, pp. 189–232.

MacLeod, D.M. and Tyrrell, D. (1979) *Entomophthora crustosa* n. sp. as a pathogen of the forest tent caterpillar, *Malacosoma disstria* (Lepidoptera, Lasiocampidae). *Canadian Entomologist* 111, 1137–1144.

MacLeod, D.M., Müller-Kögler, E. and Wilding, N. (1976) *Entomophthora* species with *E. muscae*–like conidia. *Mycologia* 68, 1–29.

McLeod, P.J., Steinkraus, D.C., Correll, J.C. and Morelock, T.E. (1998) Prevalence of *Erynia neoaphidis* (Entomophthorales: Entomophthoraceae) infections of green peach aphid (Homoptera: Aphididae) on spinach in the Arkansas River Valley. *Environmental Entomologist* 27, 796–800.

Magalhaes, B.P., Lord, J.C., Wraight, S.P., Daoust, R.A. and Roberts, D.W. (1988) Pathogenicity of *Beauveria bassiana* and *Zoophthora radicans* to the coccinellid predators *Coleomegilla maculata* and *Eriopis connexa. Journal of Invertebrate Pathology* 52, 471–473.

Magalhaes, B.P., Butt, T.M., Humber, R.A., Shields, E.J. and Roberts, D.W. (1990) Formation of appressoria *in vitro* by the entomopathogenic fungus *Zoophthora radicans* (Zygomycetes: Entomophthorales). *Journal of Invertebrate Pathology* 55, 284–288.

Magalhaes, B.P., Humber, R.A., Shields, E.J. and Roberts, D.W. (1991a) Effects of environment and nutrition on conidium germination and appressorium formation by *Zoophthora radicans* (Zygomycetes: Entomophthorales): a pathogen of the potato leafhopper (Homoptera: Cicadellidae). *Environmental Entomology* 20, 1460–1468.

Magalhaes, B.P., Wayne, R., Humber, R.A., Shields, E.J. and Roberts, D.W. (1991b) Calcium-regulated appressorium formation of the entomopathogenic fungus *Zoophthora radicans. Protoplasma* 160, 77–88.

Majchrowicz, I. and Poprawski, T.J. (1993) Effects *in vitro* of nine fungicides on growth of entomopathogenic fungi. *Biocontrol Science and Technology* 3, 321–336.

Malakar, R., Elkinton, J.S., Hajek, A.E. and Burand, J.P. (1999) Within-host interactions of *Lymantria dispar* (Lepidoptera: Lymantriidae) nucleopolyhedrosis virus and *Entomophaga maimaiga* (Zygomycetes: Entomophthorales). *Journal of Invertebrate Pathology* 73, 91–100.

Mietkiewski, R.T. and van der Geest, L.P.S. (1986) Effect of starvation on mortality of *Pieris brassicae* L. larvae after infection with *Erynia radicans* (Brefeld). In: Samson, R.A., Vlak, J.M. and Peters, R. (eds) *Fundamental and Applied Aspects of Invertebrate Pathology: Proceedings of the IV International Colloquium on Invertebrate Pathology and XIXth Annual Meeting of the Society for Invertebrate Pathology. Society for Invertebrate Pathology*, Veldhoven, p. 234.

Mietkiewski, R.T., van der Geest, L.P.S. and Balazy, S. (1986) Preliminary notes on the pathogenicity of some *Erynia* strains (Mycophyta: Entomophthoraceae) towards larvae of *Pieris brassicae*. *Journal of Applied Entomology* 102, 499–504.

Mietkiewski, R., Balazy, S. and van der Geest, L.P.S. (1993) Observations on a mycosis of spider mites (Acari: Tetranychidae) caused by *Neozygites floridana* in Poland. *Journal of Invertebrate Pathology* 61, 317–319.

Miller, J.R. and Cowles, R.S. (1990) Stimulo-deterrent diversion: a concept and its possible application to onion maggot control. *Journal of Chemical Ecology* 16, 3197–3212.

Milne, R., Wright, T., Welton, M., Budau, C., Gringorten, L. and Tyrrell, D. (1994) Identification and partial purification of a cell-lytic factor from *Entomophaga aulicae*. *Journal of Invertebrate Pathology* 64, 253–259.

Milner, R.J. (1982) On the occurrence of pea aphids, *Acyrthosiphon pisum*, resistant to isolates of the fungal pathogen *Erynia neoaphidis*. *Entomologia Experimentalis et Applicata* 32, 23–27.

Milner, R.J. (1985a) Distribution in time and space of resistance to the pathogenic fungus *Erynia neoaphidis* in the pea aphid *Acyrthosiphon pisum*. *Entomologia Experimentalis et Applicata* 37, 235–240.

Milner, R.J. (1985b) Field tests of a strain of *Entomophaga grylli* from the USA for biocontrol of the Australian wingless grasshopper, *Phaulacridium vittatum*. In: Chapman, R.F. (ed.) *Proceedings of the 4th Australasian Conference on Grassland Invertebrate Ecology, Lincoln College, Canterbury.* Caxton Press, Canterbury, pp. 255–261.

Milner, R.J. (1997) Prospects for biopesticides for aphid control. *Entomophaga* 42, 227–239.

Milner, R.J. and Holdom, D.G. (1986) First record of *Neozygites fresenii* (Nowakowski) Batko, a fungal pathogen of aphids, in Australia. *Journal of the Australian Entomological Society* 25, 85–86.

Milner, R.J. and Lutton, G.G. (1983) Effect of temperature on *Zoophthora radicans* (Brefeld) Batko: an introduced microbial control agent of the spotted alfalfa aphid, *Therioaphis trifolii* (Monell) f. *maculata. Journal of the Australian Entomological Society* 22, 167–173.

Milner, R.J. and Mahon, R.J. (1985) Strain variation in *Zoophthora radicans*, a pathogen on a variety of insect hosts in Australia. *Journal of the Australian Entomological Society* 24, 195–198.

Milner, R.J. and Soper, R.S. (1981) Bioassay of *Entomophthora* against the spotted alfalfa aphid, *Therioaphis trifolii* f. *maculata. Journal of Invertebrate Pathology* 37, 168–173.

Milner, R.J. and Soper, R.S. (1983) The potential of *Entomophaga grylli* for biological control of wingless grasshoppers. In: *Proceedings of the Australian Entomological Society 14th Annual Meeting and Conference, Armidale*, p. 18.

Milner, R.J., Teakle, R.E., Lutton, G.G. and Dare, F.M. (1980) Pathogens of the blue green aphid *Acyrthosiphon kondoi* Shinji and other aphids in Australia. *Australian Journal of Botany* 28, 601–619.

Milner, R.J., Soper, R.S. and Lutton, G.G. (1982) Field release of an Israeli strain of the fungus *Zoophthora radicans* (Brefeld) Batko for biological control of *Therioaphis trifolii* (*Monell*) f. *maculata. Journal of the Australian Entomological Society* 21, 113–118.

Milner, R.J., Mahon, R.J. and Brown, W.V. (1983) A taxonomic study of *Erynia neoaphidis* Remaudière and Hennebert (Zygomycetes: Entomophthoraceae) and description of the new species, *Erynia kondoiensis. Australian Journal of Botany* 31, 173–188.

Milner, R.J., Holdom, D.G. and Glare, T.R. (1984a) Diurnal patterns of mortality in aphids infected by entomophthoralean fungi. *Entomologia Experimentalis et Applicata* 36, 37–42.

Milner, R.J., Lutton, G.G., Bourne, J., Bailey, P. and Swincer, D. (1984b) A laboratory study of the interaction between aphids, fungal pathogens and parasites. In: *Proceedings of the 4th Australian Applied Entomological Research Conference, Adelaide, Australia,* pp. 375–381.

Møller, A.P. (1993) A fungus infecting domestic flies manipulates sexual behaviour of its hosts. *Behavioral Ecology and Sociobiology* 33, 403–407.

Montserrat, M., Castañé, C. and Santamaria, S. (1998) *Neozygites parvispora* (Zygomycotina: Entomophthorales) causing an epizootic in *Frankliniella occidentalis* (Thysanoptera: Thripidae) on cucumber in Spain. *Journal of Invertebrate Pathology* 71, 165–168.

Morgan, L.W. (1994) Survival, germination responses and infectivity of conidia of *Erynia neoaphidis* (Zygomycetes: Entomophthorales). PhD thesis, University of Cardiff, 192 pp.

Morgan, L.W., Boddy, L., Clark, S.J. and Wilding, N. (1995) Influence of temperature on germination of primary and secondary conidia of *Erynia neoaphidis* (Zygomycetes: Entomophthorales). *Journal of Invertebrate Pathology* 65, 132–138.

Mullens, B.A. (1989) Cross-transmission of *Entomophthora muscae* (Zygomycetes: Entomophthoraceae) among naturally infected muscoid fly (Diptera: Muscidae) hosts. *Journal of Invertebrate Pathology* 53, 272–275.

Mullens, B.A. and Rodriguez, J.L. (1985) Dynamics of *Entomophthora muscae* (Entomophthorales: Entomophthoraceae) conidial discharge from *Musca domestica* (Diptera: Muscidae) cadavers. *Environmental Entomology* 14, 317–322.

Mullens, B.A., Rodriguez, J.L. and Meyer, J.A. (1987) An epizoological study of *Entomophthora muscae* in muscoid fly populations on Southern California poultry facilities, with emphasis on *Musca domestica*. *Hilgardia* 55, 1–41.

Nanne, H.W. and Radcliffe, E.B. (1971) Green peach aphid populations on potatoes enhanced by fungicides. *Journal of Economic Entomology* 64, 1569–1570.

Nielsen, C., Hajek, A.E., Humber, R.A. and Eilenberg, J. (1998) Soil – a natural source of entomophthoralean fungi infecting aphids. In: *Insect Pathogens and Insect Parasitic Nematodes, IOBC Bulletin* 21, 45–48.

Nielsen, C., Eilenberg, J., Harding, S., Oddsdottir, E. and Haldórsson, G. (2000) Entomophthoralean fungi infecting the green spruce aphid (*Elatobium abietinum*) in the North-western part of Europe. *IOBC/WPRS Bulletin* 23, 131–134.

Nielsen, C., Eilenberg, J. and Dromph, K.M. (2001) *Entomophthorales on Cereal Aphids: Characterisation, Growth, Virulence, Epizootiology and Potential for Microbial Control.* Ministry of the Environment, Copenhagen, 58 pp.

Nolan, R.A. (1985) Protoplasts from Entomophthorales. In: Peberdy, J.F. and Ferenczy, L. (eds) *Fungal Protoplasts: Applications in Biochemistry and Genetics*. Marcel Dekker, New York, pp. 87–112.

Nolan, R.A. (1988) A simplified, defined medium for growth of *Entomophaga aulicae* protoplasts. *Canadian Journal of Microbiology* 34, 45–51.

Nolan, R.A. (1990) Enhanced hyphal body production by *Entomophaga aulicae* protoplasts in the presence of a neutral and a positively charged surface under mass fermentation conditions. *Canadian Journal of Botany* 68, 2708–2713.

Nolan, R.A. (1991) Influence of a negatively charged surface (Teflon disk) on *Entomophaga aulicae* protoplast morphogenesis under mass fermentation conditions. *Canadian Journal of Botany* 69, 2578–2581.

Nolan, R.A. (1993a) An inexpensive medium for mass fermentation production of *Entomophaga aulicae* hyphal bodies competent to form conidia. *Canadian Journal of Microbiology* 39, 588–593.

Nolan, R.A. (1993b) Physiological studies with the fungus *Entomophaga aulicae* during morphogenesis in three different media under fermentation conditions. *Canadian Journal of Microbiology* 39, 701–708.

Nolan, R.A. (1998) Growth media for entomophthoralean hyphal bodies. US Patent no. 5,728,572.

Nolan, R.A. and Dunphy, G.B. (1979) Effects of hormones on *Entomophthora egressa* morphogenesis. *Journal of Invertebrate Pathology* 33, 242–248.

Nolan, R.A., Dunphy, G.B. and MacLeod, D.M. (1976) *In vitro* germination of *Entomophthora egressa* resting spores. *Canadian Journal of Botany* 54, 1131–1134.

Nordin, G.L. (1984) Enhancement strategies for entomogenous fungi in integrated pest management. In: *Proceedings of the China National Academy of Science/United States National Academy of Science Joint Symposium on Biological Control of Insects, Beijing, China.* Science Press, Beijing, pp. 122–141.

Nowakowski, L. (1883) Entomophthoreae. Przyczynek do znajmosci pasorzytnych grzybków sprawiajacych pomór owadów. *Pamietn. Akad. Umiejetn. Krakowie, Wydz. Mat.-Przyr.* 8, 153–183.

Ooi, P.A.C. (1981) Microbial control of the diamondback moth in Cameron Highlands, Malaysia. *Malaysian Applied Biology* 10, 49–56.

Otvos, I.S., MacLeod, D.M. and Tyrrell, D. (1973) Two species of *Entomophthora* pathogenic to the eastern hemlock looper (Lepidoptera: Geometridae) in Newfoundland. *Canadian Entomologist* 105, 1435–1441.

Papierok, B. (1982) Entomophthorales: virulence and bioassay design. In: *Proceedings of the 3rd International Colloquium on Invertebrate Pathology, Brighton, UK,* pp. 176–181.

Papierok, B., Valadáo, B., Tôrres, L. and Arnault, M. (1984) Contribution to the study of the specificity of the entomopathogenic fungus *Zoophthora radicans* (Zygomycetes: Entomophthorales). *Entomophaga* 29, 109–119.

Pell, J.K. and Wilding, N. (1992) The survival of *Zoophthora radicans* Brefeld (Zygomycetes: Entomophthorales) isolates as hyphal bodies in mummified larvae of *Plutella xylostella* L. (Lepidoptera: Yponomeutidae). *Entomophaga* 37, 649–654.

Pell, J.K. and Wilding, N. (1994) Preliminary caged field trial using the fungal pathogen, *Zoophthora radicans* Brefeld (Zygomycetes: Entomophthorales) against the diamondback moth, *Plutella xylostella* L. (Lepidoptera: Yponomeutidae) in the UK. *Biocontrol Science and Technology* 4, 71–75.

Pell, J.K., Macaulay, E.D.M. and Wilding, N. (1993a) A pheromone trap for dispersal of the pathogen, *Zoophthora radicans* Brefeld (Zygomycetes: Entomophthorales) amongst populations of the diamondback moth, *Plutella xylostella* L. (Lepidoptera: Yponomeutidae). *Biocontrol Science and Technology* 3, 315–320.

Pell, J.K., Wilding, N., Player, A.L. and Clark, S.J. (1993b) Selection of an isolate of *Zoophthora radicans* (Zygomycetes: Entomophthorales) for biocontrol of the diamondback moth *Plutella xylostella* (Lepidoptera: Yponomeutidae). *Journal of Invertebrate Pathology* 61, 75–80.

Pell, J.K., Furlong, M.J., Reddy, G.V.P., Poppy, G.M. and Roy, H.E. (1996) Dispersal of entomopathogenic fungi by manipulation of insect behaviour. In: *Proceedings of the XXIX Annual Meeting of the Society for Invertebrate Pathology and III International Colloquium on* Bacillus thuringiensis, *Cordoba, Spain, September*, p. 61 (abstract).

Pell, J.K., Pluke, R., Clark, S.J., Kenward, M.G. and Alderson, P.G. (1997a) Interactions between two aphid natural enemies, the entomopathogenic fungus, *Erynia neoaphidis* and the predatory beetle, *Coccinella septempunctata. Journal of Invertebrate Pathology* 69, 261–268.

Pell, J.K., Tydeman, C. and McCartney, H.A. (1997b) Impact of rainfall on the persistence and transmission of *Erynia neoaphidis*. *Insect Pathogens and Insect Parasitic Nematodes*, *IOBC Bulletin* 21, 49.

Pell, J.K. Barker, A.D.P., Clark, S.J., Wilding, N. and Alderson, P.G. (1998) Use of a novel sporulation monitor to quantify the effects of formulation and storage on conidia pro-

duction by dried mycelia of the entomopathogenic fungus *Zoophthora radicans*. *Biocontrol Science and Technology* 8, 13–21.

Perry, D.F. and Fleming, R.A. (1989) The timing of *Erynia radicans* resting spore germination in relation to mycosis of *Choristoneura fumiferana. Canadian Journal of Botany* 67, 1657–1663.

Perry, D.F. and Latgé, J.-P. (1980) Chemically defined media for growth and sporulation of *Entomophthora virulenta. Journal of Invertebrate Pathology* 35, 43–48.

Perry, D.F. and Regnière, J. (1986) The role of fungal pathogens in spruce budworm population dynamics: frequency and temporal relationships. In: Samson, R.A., Vlak, J.M. and Peters, D. (eds) *Fundamental and Applied Aspects of Invertebrate Pathology: 4th International Colloquium on Invertebrate Pathology, Wageningen, the Netherlands,* pp. 167–170.

Perry, D.F., Tyrrell, D. and Delyzer, A.J. (1982) The mode of germination of *Zoophthora radicans* zygospores. *Mycologia* 74, 549–554.

Pickering, J., Dutcher, J.D. and Ekbom, B.S. (1989) An epizootic caused by *Erynia neoaphidis* and *E. radicans* (Zygomycetes: Entomophthoraceae) on *Acyrthosiphon pisum* (Homoptera: Aphidae) on legumes under overhead irrigation. *Journal of Applied Entomology* 107, 331–333.

Pickett, J.A., Wadhams, L.J. and Woodcock, C.M. (1991) New approaches to the development of semiochemicals for insect control. In: *Proceedings of the Conference on Insect Chemical Ecology, Tábor.* Academia, Prague, and SPB Academic Publishers, The Hague, pp. 333–345.

Pickett, J.A., Butt, T.M., Doughty, K.J., Wallsgrove, R.M. and Williams, I.H. (1995) Minimising pesticide input in oilseed rape by exploiting natural regulatory processes. In: *Proceedings of the Ninth International Rapeseed Congress: Rapeseed Today and Tomorrow, Cambridge, UK,* pp. 565–571.

Pickford, R. and Riegert, P.W. (1964) The fungus disease caused by *Entomophthora grylli* Fres., and its effects on grasshopper populations in Saskatchewan in 1963. *The Canadian Entomologist* 96, 1158–1166.

Poprawski, T.J. and Majchrowicz, I. (1995) Effects of herbicides on *in vitro* vegetative growth and sporulation of entomopathogenic fungi. *Crop Protection* 14, 81–87.

Poprawski, T.J. and Wraight, S.P. (1998) Fungal pathogens of Russian wheat aphid (Homoptera: Aphididae). In: Quisenberry, S.S. and Peairs, F.B. (eds) *Response Model for an Introduced Pest – Russian Wheat Aphid.* Entomological Society of America, Lanham, Maryland, pp. 209–233.

Poprawski, T.J., Mercadier, G. and Wraight, S.P. (1992) Interactions between *Diuraphis noxia, Zoophthora radicans* and *Aphelinus asychis* – preliminary results of laboratory studies. In: Morrison, W.P. (compiler) *Proceedings of the Fifth Russian Wheat Aphid Workshop, 26–28th January, 1992, Fort Worth, Texas.* Great Plains Agricultural Council Publication 142, pp. 180–188.

Powell, W., Wilding, N., Brobyn, P.J. and Clark, S.J. (1986a) Interference between parasitoids (Hymenoptera: Aphidiidae) and fungi (Entomophthorales) attacking cereal aphids. *Entomophaga* 31, 293–302.

Powell, W., Dean, G.J. and Wilding, N. (1986b) The influence of weeds on aphid-specific natural enemies in winter wheat. *Crop Protection* 5, 182–189.

Ramaseshiah, G. (1968) *Entomophthora fresenii*, parasitic on aphids in India. *Journal of Invertebrate Pathology* 10, 439–441.

Ramaseshiah, G. (1971) Occurrence of an *Entomophthora* on tetranychid mites in India. *Journal of Invertebrate Pathology* 18, 421–424.

Ramoska, W.A. and Todd, T. (1985) Variation in efficacy and viability of *Beauveria bassiana* in the chinch bug (Hemiptera: Lygaeidae) as a result of feeding activity on selected host plants. *Environmental Entomology* 14, 146–148.

Ramoska, W.A., Hajek, A.E., Ramos, M.E. and Soper, R.S. (1988) Infection of grasshoppers by members of the *Entomophaga grylli* species complex. *Journal of Invertebrate Pathology* 52, 309–313.

Reddy, G.V.P., Furlong, M.J., Poppy, G.M. and Pell, J.K. (1998) *Zoophthora radicans* infection inhibits the response to and the production of sex pheromone in the diamondback moth. *Journal of Invertebrate Pathology* 72, 167–169.

Remaudière, G. (1977) Sur quelques Aphidoidea de la Polynésie Française. *Bulletin de Société Entmologique France* 82, 151–155.

Remaudière, G. (1983) Lutte biologique contre les pucerons des céréales au moyen des champignons Entomophthorales. In: Cavalloro, R. and Piavaux, A. (eds) *Comm. Eur. Comm. Prog. Integr. Biol. Control: Progress Report 1979–1981,* pp. 227–246.

Remaudière, G. and Keller, S. (1980) Révision systématique des genres d'Entomophthoraceae potentialité entomopathogène. *Mycotaxon* 11, 323–338.

Riethmacher, G.W. and Kranz, J. (1994) Development of disease incidence of Entomophthoraceae in field populations of *Plutella xylostella* in the Philippines. *Zeitschrift für Pflanzenkrakheiten-und-Pflanzenschutz* 101, 357–367.

van Roermund, H.J.W., Perry, D.F. and Tyrrell, D. (1984) Influence of temperature, light, nutrients and pH in determination of the mode of conidial germination in *Zoophthora radicans. Transactions of the British Mycological Society* 82, 31–38.

Rohel, E., Couteaudier, Y., Papierok, B., Cavelier, N. and Dedryver, C.A. (1997) Ribosomal internal transcribed spacer size variation correlated with RAPD-PCR pattern polymorphisms in the entomopathogenic fungus *Erynia neoaphidis* and some closely related species. *Mycological Research* 101, 573–579.

Roy, H.E. (1997) Interactions between aphid predators and the entomopathogenic fungus *Erynia neoaphidis*. PhD thesis, University of Nottingham, 206 pp.

Roy, H.E. and Pell, J.K. (2000) Interactions between entomopathogenic fungi and other natural enemies: implications for biological control. *Biocontrol Science and Technology* 10, 737–752.

Roy, H.E., Pell, J.K., Clark, S.J. and Alderson, P.G. (1998) Implications of predator foraging on aphid pathogen dynamics. *Journal of Invertebrate Pathology* 71, 236–247.

Roy, H.E., Pell, J.K. and Alderson, P.G. (1999) Effects of fungal infection on the alarm response of pea aphids. *Journal of Invertebrate Pathology* 74, 69–75.

Samson, R.A., Ramakers, P.M.J. and Oswald, T. (1979) *Entomophthora thripidum*, a new fungal pathogen of *Thrips tabaci*. *Canadian Journal of Botany* 57, 1317–1323.

Sawyer, A.J., Griggs, M.H. and Wayne, R. (1994) Dimensions, density and settling velocity of entomophthoralean conidia: implications for aerial dissemination of spores. *Journal of Invertebrate Pathology* 63, 43–55.

Sawyer, A.J., Ramos, M.E., Poprawski, T.J., Soper, R.S. and Carruthers, R.I. (1997) Seasonal patterns of cadaver persistence and sporulation by the fungal pathogen *Entomophaga grylli* (Fresenius) Batko (Entomophthoraceae) infecting *Camnula pellucida* (Scudder) (Orthoptera: Acrididae). *Memoirs of the Entomological Society of Canada* 171, 355–374.

Sawyer, W.H. Jr (1929) Observations on some entomogenous members of the Entomophthoraceae in artificial culture. *American Journal of Botany* 16, 87–121.

Schmitz, V., Dedryver, C.A. and Pierre, J.S. (1993) Influence of an *Erynia neoaphidis* infection on the relative rate of increase of the cereal aphid *Sitobion avenae*. *Journal of Invertebrate Pathology* 61, 62–68.

Schofield, G. (1998) Low temperature biology of the aphid natural enemies *Erynia neoaphidis* and *Diaeretiella rapae*. PhD thesis, University of Birmingham, 144 pp.

Schuler, T.H., Poppy, G.M., Kerry, B.R. and Denholm, I. (1998) Insect-resistant transgenic plants. *Trends in Biotechnology* 16, 168–175.

Schweizer, G. (1948) Uber die Kultur von *Empusa muscae* Cohn und anderen Entomophthoraceen auf kalt Sterilisierten nahrboden. *Planta* 35, 132–176.

Shah, P.A., Aebi, M. and Tuor, U. (1998) Method to immobilize the aphid-pathogenic fungus *Erynia neoaphidis* in an alginate matrix for biocontrol. *Applied and Environmental Microbiology* 64, 4260–4263.

Shah, P.A., Aebi, M. and Tuor, U. (1999) Production factors involved in the formulation of *Erynia neoaphidis* as alginate granules. *Biocontrol Science and Technology* 9, 19–28.

Shah, P.A., Aebi, M. and Tuor, U. (2000) Infection of *Macrosiphum euphorbiae* with mycelial preparations of *Erynia neoaphidis* in a greenhouse trial. *Mycological Research* 104, 645–652.

Shimazu, M. (1979) Resting spore formation of *Entomophthora sphaerosperma* Fresenius (Entomophthorales: Entomophthoraceae) in the brown plant hopper *Niliparvata lugens* (Stal.) (Hemiptera: Delphacidae). *Applied Entomology and Zoology* 14, 383–388.

Shimazu, M. and Kuramoto, H. (1994) Introduction of *Entomophthora muscae* (Zygomycotina: Entomophthorales) to caged house fly populations. *Applied Entomology and Zoology* 29, 563–569.

Sierotzki, H., Camestral, F., Shah, P.A., Tuor, U. and Aebi, M. (2000) Biological characteristics of selected *Erynia neoaphidis* isolates. *Mycological Research* 104, 213–219.

Silvie, P. and Papierok, B. (1991) Les ennemis naturels d'insectes du cotonnier au Tchad: premières données sur les champignons de l'ordre des Entomophthorales. *Coton Fibres Tropicale* 46, 293–308.

Silvie, P., Dedryver, C.A. and Tanguy, S. (1990) Application expérimentale de mycélium d'*Erynia neoaphidis* (Zygomycetes: Entomophthorales) dans des populations de pucerons sur laitues en serre maraîchère: étude du suivi de l'inoculum par caractérisation enzymatique. *Entomophaga* 35, 375–384.

Six, D.L. and Mullens, B.A. (1996) Seasonal prevalence of *Entomophthora muscae* and introduction of *Entomophthora schizophorae* (Zygomycotina: Entomophthorales) in *Musca domestica* (Diptera: Muscidae) populations of Southern California dairies. *Biological Control* 6, 315–323.

Six, D.L. and Mullens, B.A. (1997) Distance of conidial discharge of *Entomophthora muscae* and *Entomophthora schizophorae. Journal of Invertebrate Pathology* 67, 253–258.

Smith, M.T. and Hardee, D.D. (1996) Influence of fungicides on development of an entomopathogenic fungus (Zygomycetes: Neozygitaceae) in the cotton aphid (Hemiptera: Aphididae). *Environmental Entomology* 25, 677–687.

Smith, R.F. and Reynolds, H.T. (1966) Principles, definitions and scope of integrated pest control. In: *Proceedings of FAO (United Nations Food and Agriculture Organisation) Symposium on Integrated Pest Control*, Vol. 1, FAO, Rome, pp. 11–17.

Smith, R.F., Apple, J.L. and Bottrell, D.G. (1976) The origins of integrated pest management concepts for agricultural crops. In: Apple, J.L. and Smith, R.F. (eds) *Integrated Pest Management*. Plenum Press, New York, pp. 1–16.

Smitley, D.R., Kennedy, G.G. and Brooks, W.M. (1986) Role of the entomogenous fungus, *Neozygites floridana*, in population declines of the twospotted spider mite, *Tetranychus urticae*, on field corn. *Entomologia Experimentalis et Applicata* 41, 255–264.

Smitley, D.R., Bauer, L.S., Hajek, A.E., Sapio, F.J. and Humber, R.A. (1995) Introduction and establishment of *Entomophaga maimaiga*, a fungal pathogen of gypsy moth (Lepidoptera: Lymantriidae) in Michigan. *Environmental Entomology* 24, 1685–1695.

Soper, R.S. and MacLeod, D.M. (1963) Spore morphology of *Entomophthora fresenii* Nowakowski. *Journal of Insect Pathology* 5, 478–482.

Soper, R.S., and MacLeod, D.M. (1981) *Descriptive Epizootiology of an Aphid Mycosis.* USDA, Technical Bulletin 1632, 17 pp.

Soper, R.S., Holbrook, F.R. and Gordon, C.C. (1974) Comparative pesticide effects on *Entomophthora* and the phytopathogen *Alternaria solanii*. *Environmental Entomology* 3, 560–562.

Soper, R.S., Holbrook, F.R., Majchrowicz, I. and Gordon, C.C. (1975) Production of *Entomophthora* resting spores for biological control of aphids. *University of Maine (Orono) Life Sciences and Agriculture Experimental Station Technical Bulletin* 76, 1–15.

Soper, R.S., Smith, L.F.R. and Delyzer, A.J. (1976) Epizootiology of *Massospora levispora* in an isolated population of *Okanagana rimosa. Annals of the Entomological Society of America* 69, 275–283.

Soper, R.S., Shimazu, M., Humber, R.A., Ramos, M.E. and Hajek, A.E. (1988) Isolation and characterization of *Entomophaga maimaiga* sp. nov., a fungal pathogen of gypsy moth, *Lymantria dispar*, from Japan. *Journal of Invertebrate Pathology* 51, 229–241.

Speare, A. T. (1922) *Natural Control of the Citrus Mealybug in Florida. USDA Bulletin* 1117.

Speare, A.T. and Colley, R.H. (1912) *The Artificial Use of the Brown-tail Fungus in Massachusetts with Practical Suggestions for Private Experiments, with a Brief Note on a Fungous Disease of Gypsy Caterpillar.* Wright & Potter, Boston, Massachusetts.

Steinkraus, D.C. and Boys, G.O. (1997) Update on prediction of epizootics with extension-based sampling service. In: *Proceedings Beltwide Cotton Conferences, January 6–10, 1997, New Orleans.* National Cotton Council of America, Memphis, Tennessee, pp. 1047–1048.

Steinkraus, D.C. and Rosenheim, J. (1995) Biological factors influencing the epizootiology of cotton aphid fungus. In: *Proceedings Beltwide Cotton Conference, 1995, January 4–7, San Antonio, Texas.* National Cotton Council of America, Memphis, Tennessee, pp. 887–889.

Steinkraus, D.C. and Slaymaker, P.H. (1994) Effect of temperature and humidity on formation, germination, and infectivity of conidia of *Neozygites fresenii* (Zygomycetes: Neozygitaceae) from *Aphis gossypii* (Hemiptera: Aphididae). *Journal of Invertebrate Pathology* 64, 130–137.

Steinkraus, D.C., Kring, T.J. and Tugwell, N.P. (1991) *Neozygites fresenii* in *Aphis gossypii* on cotton. *Southwestern Entomologist* 16, 118–122.

Steinkraus, D.C., Boys, G.O. and Slaymaker, P.H. (1993a) Culture, storage, and incubation period of *Neozygites fresenii* (Entomophthorales: Neozygitaceae), a pathogen of the cotton aphid. *Southwestern Entomologist* 18, 197–202.

Steinkraus, D.C., Geden, J.G. and Rutz, D.A. (1993b) Prevalence of *Entomophthora muscae* (Cohn) Fresenius (Zygomycetes: Entomophthoraceae) in house flies (Diptera: Muscidae) on dairy farms in New York and induction of epizootics. *Biological Control* 3, 93–100.

Steinkraus, D.C., Mueller, A.J. and Humber, R.A. (1993c) *Furia virescens* (Thaxter) Humber (Zygomycetes: Entomophthoraceae) infections in the armyworm, *Pseudaletia unipuncta* (Haworth) (Lepidoptera: Noctuidae) in Arkansas with notes on other natural enemies. *Journal of Entomological Science* 28, 376–386.

Steinkraus, D.C., Hollingsworth, R.G. and Slaymaker, P.H. (1995) Prevalence of *Neozygites fresenii* (Entomophthorales: Neozygitaceae) on cotton aphids (Homoptera: Aphididae) in Arkansas cotton. *Environmental Entomology* 24, 465–474.

Steinkraus, D.C., Boys, G.O., Hollingsworth, R.G., Bacheler, J.S., Durant, J.A., Freeman, B.L., Gaylor, M.J., Harris, F.A., Knutson, A., Lentz, G.L., Leonard, B.R., Luttrell, R., Parker, D., Powell, J.D., Ruberson, J.R. and Sorenson, C. (1996a) Multistate sampling for *Neozygites fresenii* in cotton. In: *Proceedings 1996 Beltwide Cotton Research Conferences, Nashville, Tennessee.* National Cotton Council of America, Memphis, Tennessee, pp. 735–738.

Steinkraus, D.C., Hollingsworth, R.G. and Boys, G.O. (1996b) Aerial spores of *Neozygites fresenii* (Entomophthorales: Neozygitaceae): density, periodicity, and potential role in cotton aphid (Hemiptera: Aphididae) epizootics. *Environmental Entomology* 25, 48–57.

Steinkraus, D.C., Boys, G.O., Bagwell, R.D., Johnson, D.R., Lorenz, G.M., Meyers, H., Layton, M.B. and O'Leary, P.F. (1998a) Expansion of extension-based aphid fungus sampling service to Louisiana and Mississippi. In: *1998 Beltwide Cotton Conferences, San Diego, California*. National Cotton Council of America, Memphis, Tennessee, pp. 1239–1242.

Steinkraus, D.C., Oliver, J.B. Humber, R.A. and Gaylor, M.J. (1998b) Mycosis of bandedwinged whitefly (*Trialeurodes abutilonea*) (Homoptera: Aleyrodidae) caused by *Orthomyces aleyrodis* gen. & sp. nov. (Entomophthorales: Entomophthoraceae). *Journal of Invertebrate Pathology* 72, 1–8.

Steinkraus, D.C., Howard, M.N., Hollingsworth, R.G. and Boys, G.O. (1999) Infection of sentinel cotton aphids (Homoptera: Aphididae) by aerial conidia of *Neozygites fresenii* (Entomophthorales: Neozygitaceae). *Biological Control* 14, 181–185.

Sterling, W. (1984) *Action and Inaction Levels in Pest Management*. Texas Agricultural Experimental Station Bulletin 1480, College Station, Texas.

Streett, D.A. and McGuire, M.R. (1990) Pathogenic diseases of grasshoppers. In: Chapman, R.F. and Joern, A. (eds) *Biology of Grasshoppers.* Wiley Interscience, New York, pp. 483–516.

Thaxter, R. (1888) The Entomophthoreae of the United States. *Memoirs of the Boston Society of Natural History* 4, 133–201.

Thoizon, G. (1970) Spécificité du parasitisme des aphides par les Entomophthorales. *Annales de Société de Entomologie Francais (N.S.)* 6, 517–562.

Thomsen, L. and Eilenberg, J. (2000) *Entomophthora muscae* resting spore formation and germination *in vivo* in *Delia radicum. Journal of Invertebrate Pathology* 76, 127–130.

Tomiyama, H. and Aoki, J. (1982) Infection of *Erynia blunckii* (Lak. Ex. Zimm.) Rem. et Henn. (Entomophthorales: Entomophthoraceae) in the diamondback moth *Plutella xylostella* L. (Lepidoptera: Yponomeutidae). *Applied Entomology and Zoology* 17, 375–384.

Tyrrell, D. (1970) Production of resting spores of some *Entomophthora* species on artificial media. *Bi-Monthly Research Notes, Department of Fisheries and Forestry, Ottawa, Canada* 26, 12–13.

Tyrrell, D. (1988) Survival of *Entomophaga aulicae* in dried insect larvae. *Journal of Invertebrate Pathology* 52, 187–188.

Tyrrell, D. (1990) Pathogenesis of *Entomophaga aulicae*. I. Disease symptoms and effect of infection on weight gain of infected *Choristoneura fumiferana* and *Malacosoma disstria* larvae. *Journal of Invertebrate Pathology* 56, 150–156.

Ullyett, G.C. and Schonken, D.B. (1940) *A Fungus Disease of* Plutella maculipennis *Curt. in South Africa with Notes on the Use of Entomogenous Fungi in Insect Control.* Union of South Africa, Department of Agriculture and Forestry Science Bulletin No. 218, Entomology Series No. 5.

Uziel, A. and Kenneth, R.G. (1986) *In vitro* resting spore production in *Erynia neoaphidis*. In: Samson, R.A., Vlak, J.M. and Peters, D. (eds) *Fundamental and Applied Aspects of Insect Pathology. Fourth International Colloquium on Invertebrae Pathology, Veldhoven,* p. 230.

Uziel, A. and Kenneth, R.G. (1991) Survival of primary conidia and capilliconidia at different humidities in *Erynia* (subgen. *Zoophthora*) spp. and in *Neozygites fresenii* (Zygomycetes: Entomophthorales), with special emphasis on *Erynia radicans*. *Journal of Invertebrate Pathology* 58, 118–126.

Uziel, A. and Shtienberg, D. (1993) Effect of meteorological variates on persistence of primary conidia and capilliconidia of *Erynia radicans* (Zygomycetes: Entomophthorales) under natural conditions. *Annals of Applied Biology* 122, 441–450.

Uziel, A., Kenneth, R.G. and Ben-Ze'ev, I. (1982) Spore types and other structures formed in adult housefly and medfly incubated with strains of *Erynia radicans*. In: *Third International Colloquium of Invertebrate Pathology, Brighton, England*, p. 191 (abstract).

Vandenberg, J.D. and Soper, R.S. (1978) Prevalence of Entomophthorales mycoses in populations of spruce budworm, *Choristoneura fumiferana. Environmental Entomology* 7, 847–853.

Villacarlos, L.T. and Keller, S. (1997) *Batkoa amrascae* Keller & Villacarlos, a new species of Entomophthorales (Zygomycetes) infecting the cotton leafhopper, *Amrasca biguttula* (Ishida) (Homoptera: Cicadellidae) in the Philippines. *Philippine Entomologist* 11, 81–86.

Villacarlos, L.T., Pell, J.K. and Steinkraus, D.S. (1996) Pathogenicity of *Neozygites fresenii* isolates from the Philippines and the United States. In: *Proceedings of the III International Colloquium on* Bacillus thuringiensis *and the XXIX Annual Meeting of the Society for Invertebrate Pathology, Cordoba, Spain, September*, p. 88 (abstract).

Walsh, S.R.A. (1996) Development of molecular markers for the detection and differentiation of *Entomophaga* strains pathogenic for insects. PhD dissertation, University of Toronto, Canada.

Watson, D.W. and Petersen, J.J. (1993a) Sexual activity of male *Musca domestica* (Diptera: Muscidae) infected with *Entomophthora muscae* (Entomophthoraceae: Entomophthorales). *Biological Control* 3, 22–26.

Watson, D.W. and Petersen, J.J. (1993b) Seasonal activity of *Entomophthora muscae* (Zygomycetes: Entomophthorales) in *Musca domestica* L. (Diptera: Muscidae) with reference to temperature and relative humidity. *Biological Control* 3, 182–190.

Watson, D.W., Mullens, B.A. and Petersen, J.J. (1993) Behavioral fever response of *Musca domestica* (Diptera: Muscidae) to infection of *Entomophthora muscae* (Zygomycetes: Entomophthorales). *Journal of Invertebrate Pathology* 61, 10–16.

Weseloh, R.M., and Andreadis, T.G. (1997) Persistence of resting spores of *Entomophaga*

maimaiga, a fungal pathogen of the gypsy moth, *Lymantria dispar*. *Journal of Invertebrate Pathology* 69, 195–196.

Wilding, N. (1969) Effect of humidity on the sporulation of *Entomophthora aphidis* and *E. thaxteriana*. *Transactions of the British Mycological Society* 53, 126–130.

Wilding, N. (1970a) *Entomophthora* conidia in the air-spora. *Journal of General Microbiology* 62, 149–157.

Wilding, N. (1970b) The effect of temperature on the infectivity and incubation periods of the fungi *Entomophthora aphidis* and *E. thaxteriana* for the pea aphid *Acyrthosiphon pisum*. In: *Proceedings of the 4th International Colloquium of Insect Pathology, Maryland, USA,* pp. 84–88.

Wilding, N. (1973) The survival of *Entomophthora* spp. in mummified aphids at different temperatures and humidities. *Journal of Invertebrate Pathology* 21, 309–311.

Wilding, N. (1976) Determination of the infectivity of *Entomophthora* spp. In: *Proceedings of the 1st International Colloquium of Invertebrate Pathology, Kingston, Ontario, Canada*, pp. 296–300.

Wilding, N. (1981) The effect of introducing aphid-pathogenic Entomophthoraceae into field populations of *Aphis fabae. Annals of Applied Biology* 99, 11–23.

Wilding, N. (1982a) Entomophthorales: field use and effectiveness. *Proceedings of the 3rd International Colloquium on Invertebrate Pathology, Brighton, UK*, pp. 170–175.

Wilding, N. (1982b) The effect of fungicides on development of *Entomophthora aphidis. Transactions of the British Mycological Society* 75, 297–302.

Wilding, N. and Brady, B.L. (1984) *Descriptions of Pathogenic Fungi and Bacteria,* Set 82, Nos 812, 814, 815, 817, 820. CMI, Kew, UK.

Wilding, N. and Brobyn, P.J. (1980) Effects of fungicides on the development of *Entomophthora aphidis*. *Transactions of the British Mycological Society* 75, 279–302.

Wilding, N. and Lauckner, F.B. (1974) *Entomophthora* infecting wheat bulb fly at Rothamsted, Hertfordshire, 1967–1971. *Annals of Applied Biology* 76, 161–170.

Wilding, N. and Perry, J.N. (1980) Studies on *Entomophthora* in populations of *Aphis fabae* on field beans. *Annals of Applied Biology* 94, 367–378.

Wilding, N., Latteur, G. and Dedryver, C.A. (1986a) Evaluation of *Entomophthorales* for aphid control: laboratory and field data. In: Samson, R.A., Vlak, J.M. and Peters, D. (eds) *Fundamental and Applied Aspects of Invertebrate Pathology: 4th International Colloquium on Invertebrate Pathology* Wageningen, the Netherlands, pp. 159–162.

Wilding, N., Mardell, S.K. and Brobyn, P.J. (1986b) Introducing *Erynia neoaphidis* into a field population of *Aphis fabae*: form of the inoculum and effect of irrigation. *Annals of Applied Biology* 108, 373–385.

Wilding, N., Mardell, S.K., Brobyn, P.J., Wratten, S.D. and Lomas, J. (1990) The effect of introducing the aphid-pathogenic fungus *Erynia neoaphidis* into populations of cereal aphids. *Annals of Applied Biology* 117, 683–691.

Witlaczil, E. (1885) *Neozygites aphidis*, eine neue Gregarine. *Archiven für Mikrobiologie und Anatomie* 24, 599–603.

Wraight, S.P. and Roberts, D.W. (1987) Insect control efforts with fungi. *Developments in Industrial Microbiology* 28, 77–87.

Wraight, S.P., Galaini-Wraight, S., Carruthers, R.I. and Roberts, D.W. (1986) Field transmission of *Erynia radicans* to *Empoasca* leafhoppers in alfalfa following application of a dry mycelial preparation. In: Samson, R.A., Vlak, J.M. and Peters, D. (eds) *Fundamental and Applied Aspects of Invertebrate Pathology: 4th International Colloquium of Invertebrate Pathology, Veldhoven*, p. 233.

Wraight, S.P., Butt, T.M., Galaini-Wraight, S., Allee, L.L., Soper, R.S. and Roberts, D.W. (1990) Germination and infection processes of the entomophthoralean fungus *Erynia radicans* on the potato leafhopper *Empoasca fabae. Journal of Invertebrate Pathology* 56, 157–174.

Yamamoto, M. and Aoki, J. (1983) Periodicity of conidial discharge of *Erynia radicans. Transactions of the Mycological Society of Japan* 24, 487–496.

Yaninek, J.S., Saizonou, S., Onzo, A., Zannou, I. and Gnanvossou, D. (1996) Seasonal and habitat variability in the fungal pathogens, *Neozygites* cf. *floridana* and *Hirsutella thompsonii*, associated with cassava mites in Benin, West Africa. *Biocontrol Science and Technology* 6, 23–33.

Yeo, H. (2000) Biorational selection of mycoinsecticides for aphid management. PhD thesis, University of Nottingham, 295pp.

5 Exploitation of the Nematophagous Fungal *Verticillium chlamydosporium* Goddard for the Biological Control of Root-knot Nematodes (*Meloidogyne* spp.)

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Introduction

The effective natural control of specific nematode pests in intensive agricultural systems has been well documented and the causal microbial parasites and pathogens have often been identified (Kerry, 1987; Stirling, 1991; Dickson *et al.*, 1994). The recognition of suppressive soils in which biotic factors prevent nematodes multiplying on susceptible crops has demonstrated that the biological control of nematodes has potential as a management strategy. Also, the environmental and health concerns over the use of some nematocides has led to increased interest in the development of strategies that integrate several control methods and reduce dependence on the use of chemicals. However, despite much research, the development of biological control agents (BCAs) for nematode control has proved difficult and although some commercial products have been developed, none is in widespread use. Research has concentrated on the use of nematode-trapping fungi for the control of cyst and root-knot nematodes, as these are the most economically important nematode pest species. However, these fungi proved difficult to manipulate and it was often not possible to ensure that they produced traps when the infective nematode juveniles were migrating towards roots. Also, large amounts of inoculum were required and, since the fungus selected, *Arthrobotrys irregularis*, did not produce a resting structure, fresh inoculum had to be transported in refrigerated trucks (Cayrol, 1983). As a consequence, field-scale applications were difficult and the control obtained was inconsistent. Most success in the biological control of sedentary nematodes, such as cyst and root-knot nematodes, has come from the use of bacteria and fungi that parasitize female nematodes, reducing fecundity and increasing egg mortality. Interest in trapping fungi has been restored in recent years as selected isolates of *Duddingtonia flagrans* have significantly reduced the numbers of nematodes in the faeces of infected cattle, sheep and other domestic her-

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bivores and reduced the burdens of infective larvae in the sward in small-scale, repeated field tests (Grønvold *et al.*, 1993).

The development of *Verticillium chlamydosporium*, a facultative parasite and rhizosphere-colonizing fungus, as a potential BCA against root-knot nematode species, is reviewed in this chapter. Attention is paid to the practical considerations in the deployment of such agents, especially where the factors affecting consistent and commercial control levels are relevant to the use of other fungal BCAs.

The Root-knot Nematode Problem

Plant-parasitic nematodes cause yield losses of approximately $100 billion (thousand million) worldwide each year (Sasser and Freckman, 1987) and, of this damage, 70% is considered to be due to root-knot nematodes. These nematodes have a worldwide distribution but they are more abundant in warm temperate and tropical soils, where *Meloidogyne incognita, Meloidogyne javanica* and *Meloidogyne arenaria* are responsible for most crop damage. Crop rotation with non-hosts or resistant cultivars remains the main management strategy to regulate populations of these pests, but success is often limited because of the wide host ranges of most root-knot nematode species and the frequent occurrence of infestations composed of more than one species. Nematocides are widely used and substantial yield increases may be obtained from crops grown in treated soil but, because of the rate of development and the fecundity of root-knot nematodes, populations are rarely controlled by these chemicals. The limitations of current nematode sampling and extraction methods mean that infestations that are undetectable at planting time can increase to cause significant damage to susceptible crops, such as tomato and cucurbits, within a growing season. Hence, it is difficult to plan control strategies for these nematode pests. The general soil sterilant, methyl bromide, is extensively used in southern Europe and elsewhere in intensive vegetable production systems to control root-knot nematode pests. However, methyl bromide will be banned in several countries by 2005 because it depletes the ozone layer. In developing agricultural systems, where there may be no suitable resistant cultivars and the use of nematocides is either too expensive or inappropriate, yield losses in excess of 50% are common (Luc *et al.*, 1990). Alternative control strategies, such as solarization, flooding, disease-free planting material and soil amendments, have been used with some success (Whitehead, 1997). However, it is generally recognized that several measures are needed to control these pests in integrated management strategies.

Biological control may provide an additional method for the management of these pests. The bacterium, *Pasteuria penetrans*, has been associated with soils that suppress the multiplication of *M. arenaria* and other root-knot species (Chen and Dickson, 1998) but difficulties in the mass production of this parasite have limited its use. Two species of fungi, *Paecilomyces lilacinus* and *V. chlamydosporium*, which attack nematode eggs, have been extensively studied for the control of several *Meloidogyne* species. Despite some early concern about human health risks associated with *P. lilacinus*, this fungus has been widely tested in the field and commercial products are available; presumably those isolates that have been collected from nematodes do not present a significant health risk. Inoculum produced on various grains has required large application rates and given variable results, and there is a need for improved formulations. At IACR-Rothamsted, research has concentrated on the use of *V. chlamydosporium* as a potential fungal BCA. The fungus produces a resistant resting structure that is easily handled,

effective application rates are smaller than those for *P. lilacinus* (Kerry, 1998) and consistent control of root-knot nematodes has been obtained in small-scale plot trials.

Taxonomy, Distribution and Host Range of *Verticillium chlamydosporium*

The deuteromycete *V. chlamydosporium* is a facultative endoparasite recorded from the eggs of cyst and root-knot nematodes throughout the world. It has also been recorded from the eggs of snails and from fungal hyphae. When tested as a potential BCA for some soil-borne fungal pathogens, it did not appear to have detrimental effects on mycorrhizae (Rao *et al.*, 1997). The rhizospheres of several plant species may be colonised by *V. chlamydosporium* but growth in soil is limited compared with growth in the rhizosphere, except in organic soils.

Several species of *Verticillium* are endoparasites of nematodes (Gams, 1988). Of those that colonize nematode eggs, *V. chlamydosporium* is the most widely reported, but it is part of a complex of several closely related species, which include *V. chlamydosporium* var. *chlamydosporium, V. chlamydosporium* var. *catenulatum, Verticillium suchlasporium* var. *suchlasporium, V. suchlasporium* var. *catenatum* and *Verticillium psalliotae.* The teleomorph of *V. chlamydosporium* is considered to belong to the *Cordiceps* but it has not been described (H. Evans, CABI Biosciences, personal communication). In its imperfect state, it produces more thick-walled resting spores or dictyochlamydospores on short pedicels than the other species and varieties. Parasitism of nematode eggs may enable the fungus to produce more chlamydospores and increase its long-term survival in soil (Kerry and Crump, 1998). The ability to produce chlamydospores and other characteristics relating to their potential as BCAs (see below) differs markedly between different isolates of the same fungal species. The importance of this variation in the regulation of nematode populations and the spatial and temporal dynamics of individual isolates is unknown. Conidia are produced in heads on simple phialides and may be important for the spread of the fungus in soil (de Leij *et al.*, 1993b).

Quantification of *Verticillium chlamydosporium* in Soil and on Roots

Understanding the quantitative relationships between hosts and their natural enemies is essential for the effective deployment of BCAs (Waage and Greathead, 1988). Few attempts have been made to model the mathematical relationships between fungi and their nematode hosts, even though those described for *Hirsutella rhossiliensis* (Jaffee *et al.*, 1992) largely conform to the dynamics reported by Anderson and May (1981). The dynamics of nematode population decline as a result of the build-up of nematophagous fungi and bacteria indicates that natural enemy populations are generally slow to establish in soil (Kerry and Crump, 1998) and may be significantly affected by nematode species (Atkinson and Dürschner-Pelz, 1995). To estimate changes in the density of *V. chlamydosporium* and to understand its epidemiology, techniques for the physical extraction of chlamydospores from soil (Crump and Kerry, 1981) and for estimating the total number of propagules in soil or on roots (Kerry *et al.*, 1993) have been developed. However, these methods only enable relative changes in the abundance of the fungus to be estimated and these may not relate to its activity. For example, changes from vegetative growth to sporulation, which greatly affect

the numbers of nematode eggs parasitized, may not be detected using the dilution-plate procedure on the semi-selective medium of Kerry *et al.* (1993). The medium does not allow the differentiation of colonies derived from different types of propagule (hyphal fragment, conidium or chlamydospore). Also, physical methods that only extract chlamydospores underestimate the abundance of the fungus; the relationship between numbers of chlamydospores and the total number of colony-forming units differs between soils of different texture and between cropping histories in the same soil (Kerry and Crump, 1998). Despite these limitations, significant differences in the abundance of *V. chlamydosporium* in cyst nematode-suppressive and non-suppressive soils have been demonstrated, and soil texture, nematode density and plant species have been identified as key factors affecting densities of the fungus in the rhizosphere. Molecular diagnostic tools based on polymerase chain reaction (PCR) techniques enable isolates of *V. chlamydosporium* to be distinguished and allow the spread of the fungus to be monitored after its release in soil (Arora *et al.*, 1996; Hirsch *et al.*, 2000). Immunological and molecular methods using monoclonal antibodies and the *gfp* reporter gene are under development to visualize the fungus in the rhizosphere, which would greatly facilitate studies on its ecology and interactions with nematode hosts.

Tritrophic Interactions

The tritrophic interaction (Fig. 5.1) between root-knot nematodes, *V. chlamydosporium* and the host plant has been studied extensively. As *V. chlamydosporium* is a facultative parasite, it has a saprophytic phase that is much affected by the plant, and the interactions in the rhizosphere are complex (Fig. 5.2). The role of root exudates in the

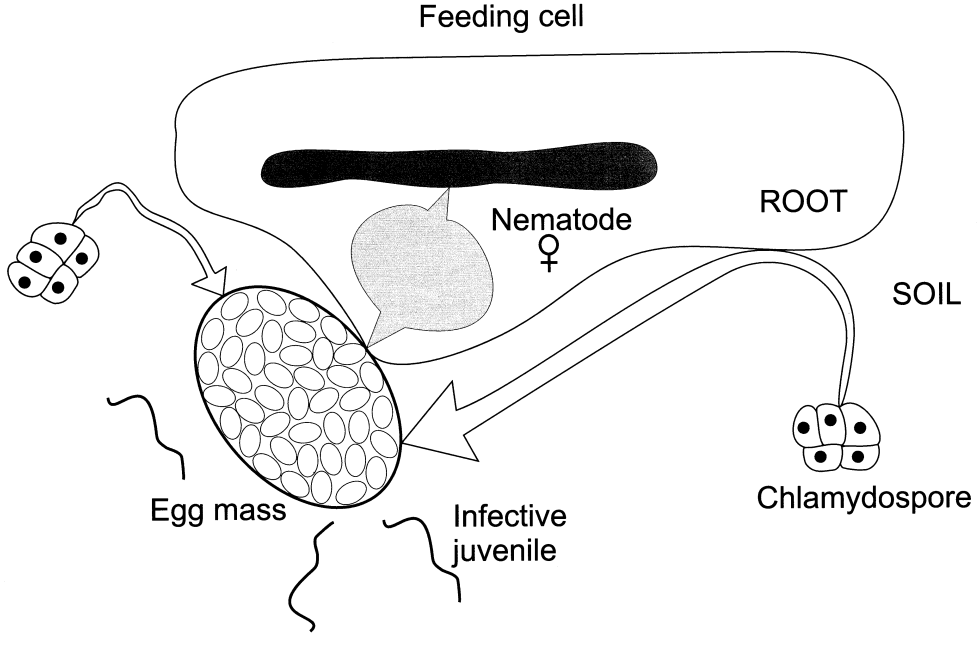

Fig. 5.1. Interaction between *Verticillium chlamydosporium* and root-knot nematodes in the rhizosphere.

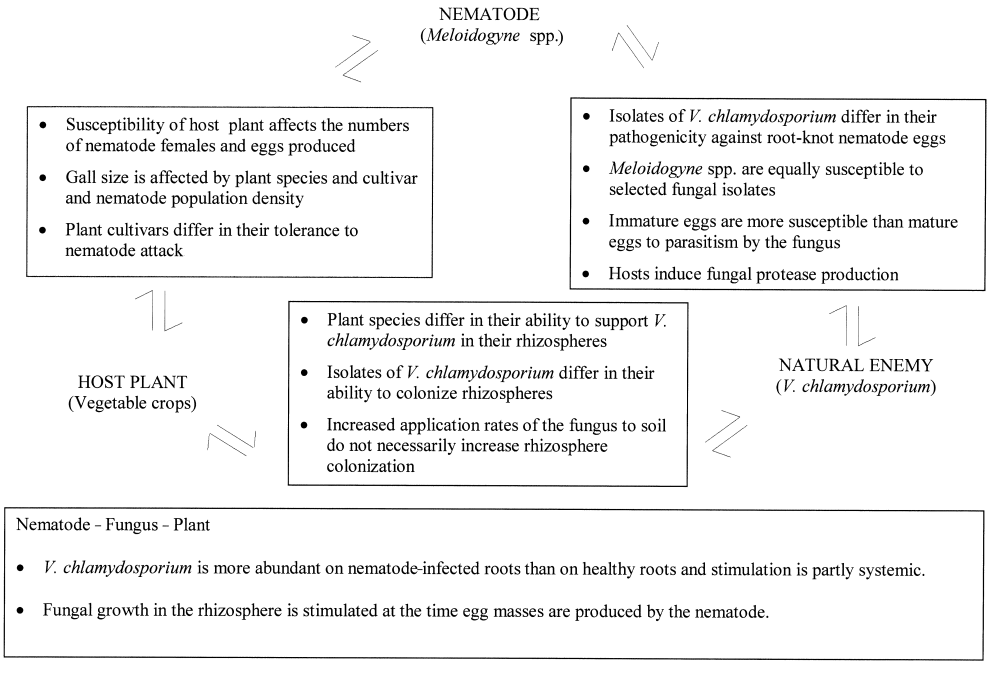

Fig. 5.2. A summary of the key factors in the tritrophic interactions between *Verticillium chlamydosporium,* root-knot nematodes (*Meloidogyne* spp.) and vegetable crops.

growth of the fungus and in its switch from the saprophytic to the parasitic state is not known, although, *in vitro*, excess carbon inhibits the production of key enzymes involved in the infection process (Segers *et al.*, 1999). The efficiency of the fungus as a BCA is affected by the susceptibility of the host plant, which influences the number of nematodes that invade the roots, the numbers becoming female and the size of the egg masses produced. Although much is known about the relationship between specific root-knot nematode pest densities and yield loss, these relationships have rarely been considered in terms of their effects on the efficiency of fungal BCAs. At large nematode densities on the roots of susceptible crops, a significant proportion of the egg masses remain within the gall and isolated from *V. chlamydosporium*, which is confined to the rhizosphere. Reductions in the populations of root-knot nematodes are likely to be greatest on poor hosts for the nematode, which support abundant growth of the fungus in their rhizospheres (Bourne *et al.*, 1996).

Extensive growth of *V. chlamydosporium* in the rhizosphere is essential for nematode control (de Leij and Kerry, 1991), and isolates of the fungus that proliferate only in the soil may have no significant effect on nematode multiplication. Plant species differ in their ability to support fungal growth (Table 5.1) and the fungus is more abundant on roots infected by nematodes compared with those that are healthy. This stimulation of fungal growth on nematode-infected roots occurs after *c.* 5 weeks of crop growth (when egg masses appear) and may result from the direct colonization of the egg mass or from the release of nutrients into the rhizosphere (Bourne *et al.*, 1996). However, part of the effect of the nematode is systemic and fungal growth is stimulated by nematodes separated from the fungus in split-root experiments (J.M. Bourne, personal communication). The density of *V. chlamydosporium* in the rhizosphere of

Table 5.1. Status of different plant species for compatibility with *Verticillium chlamydosporium* as estimated by their ability to support the growth of the fungus in their rhizospheres after 7 weeks.

Plant status		Plant species	Fungal density in rhizosphere (cfu cm^{-2} root)
Good	Cabbage	*Brassica oleracea*	
	Crotalaria	*Crotalaria* sp.	
	Kale	*B. oleracea*	> 200
	Maize	*Zea mays*	
	Pigeon-pea	*Cajanus cajan*	
	Phaseolus	*Phaseolus vulgaris*	
	Potato	*Solanum tuberosum*	
	Tomato	*Lycopersicon esculentum*	
Moderate	Chilli	*Capsicum anuum*	
	Sweet potato	*Ipomoea batatas*	
	Cow-pea	*Vigna unguiculata*	200–100
	Millet	*Pennisetum* sp.	
	Tobacco	*Nicotiana tabacum*	
	Cotton	*Gossypium* sp.	
Poor	Aubergine	*Solanum melongena*	
	Okra	*Abelmoschus esculatus*	
	Soybean	*Glycine max*	
	Sorghum	*Sorghum* sp.	< 100
	Wheat	*Triticum vulgare*	

cfu, colony-forming units.

some plant species that are poor hosts for the fungus may not be significantly increased even if application rates of chlamydospores to soil are increased tenfold (Bourne and Kerry, 1999). Hence, the plant species must be carefully selected if the impact of the fungus is to be maximized.

V. chlamydosporium parasitizes eggs of root-knot nematodes, which are colonized from appressoria developed from undifferentiated hyphae on the eggshell. An infection peg that penetrates the eggshell develops from the appressorium and gives rise to a post-infection bulb, from which the mycelium radiates and colonizes the egg (Morgan-Jones *et al.*, 1983). Penetration is thought to be the result of physical pressure and enzymatic activity. A serine protease enzyme, a subtilisin (designated VCP1), has been extracted from defined liquid media used to culture the fungus, partially characterized and demonstrated to be a key enzyme in the infection process (Segers *et al.*, 1996). The enzyme removed the outer vitelline membrane of the eggshell and exposed the chitin layer of the root-knot nematode but not the potato cyst nematode eggs; VCP1 might be a host-range or virulence determinant. However, isolates of *V. chlamydosporium* that differed markedly in their production of the enzyme showed little difference in their ability to parasitize eggs in simple tests on agar. A similar enzyme was originally identified in the closely related *V. suchlasporium* (Lopez-Llorca, 1990) and others have been reported in other nematophagous fungi, *Arthrobotrys oligospora* and *P. lilacinus*, and in the entomopathogens *Verticillium lecanii* and *Metarhizium anisopliae* (see Segers *et al.*, 1999). Isolates of *V. chlamydosporium* differ in their virulence but different species of root-knot nematodes are equally susceptible to a specific isolate of the fungus. Immature eggs are more readily infected than mature eggs containing

second-stage juveniles, and many escape infection at temperatures of about 30°C because the eggs mature and hatch before the egg mass is totally colonized by the fungus; mobile nematode stages are not parasitized.

Development of Biological Control Strategies

V. chlamydosporium has considerable potential as a BCA for root-knot nematodes, and applications of the fungus to soil have provided significant control in a range of experiments in glasshouses and small plots (de Leij *et al.*, 1993a). The fungus does not reduce the initial invasion of roots by infective second stage juveniles and the damage that they cause to plant growth. However, the parasitism of eggs may significantly reduce multiplication of the nematode and provide population control. Subsequent generations of root-knot nematodes should be smaller in soil treated with the fungus than in untreated soil but it may take more than one crop and fungal application to reduce nematode populations to non-damaging levels. To control crop damage in soils heavily infested with root-knot nematodes, other compatible control methods, such as chemical control, solarization and possibly the application of soil amendments, will be needed in addition to the fungus. The impact of *V. chlamydosporium* on nematode multiplication is maximized when the fungus is applied to soil: (i) around plants that support extensive fungal growth in their rhizospheres, produce only small galls in response to nematode infection and are relatively poor hosts for the nematode; and (ii) at temperatures < 30°C.

Isolates of *V. chlamydosporium*, even those collected from the same soil, differ greatly and must be carefully selected for introduction into soil as potential BCAs. This is a stepwise process (Table 5.2), which begins with simple *in vitro* tests in the laboratory, as it is impractical to screen large numbers of isolates in pot tests in the glasshouse. Such tests enable many isolates to be eliminated before more time-consuming (and expensive) screens are conducted in nematode-infested soils (Kerry, 1998). Testing the efficacy of selected isolates in a range of conditions in trials in the glasshouse is essential, as isolates that perform well in laboratory tests may not be effective in soil. As the scale of testing increases, more fungal inoculum will be required and so it is essential that the fungus be characterized in terms of its optimal growth conditions. *V. chlamydosporium* grows well on many media, including waste materials, over a considerable pH range (Kerry *et al.*, 1986), but the production of conidia is temperature-dependent (de Leij *et al.*, 1992a) and few chlamydospores develop in liquid fermentation (Kerry *et al.*, 1986). Isolates of *V. chlamydosporium* initially collected from infected nematode eggs or cultured from chlamydospores extracted from nematode-infested soils are screened in the laboratory to assess their ability to colonize the rhizosphere of selected plant species, to produce chlamydospores and to kill nematode eggs. These three criteria are essential for the success of *V. chlamydosporium* as a BCA, as only those isolates that grow in the rhizosphere and rapidly colonize nematode eggs are capable of controlling root-knot nematode populations. Chlamydospores may enable the fungus to be readily formulated, as they are robust and, when added to soil in aqueous suspensions without additional nutrients, the fungus is able to colonize the rhizosphere. However, isolates differ markedly in their ability to produce these spores on artificial media and again routine screening is necessary. Hyphae and conidia of the fungus produced by liquid fermentation remained viable in formulated granules for up to 12 months at 25°C (Stirling *et al.*, 1998).

Table 5.2. Steps in the selection of isolates of *Verticillium chlamydosporium* to assess their potential as biological control agents.

Purpose	Procedure
1. Collection of isolates	Isolate from nematode eggs in targeted surveys in known suppressive soils or intensively cropped soils with long history of nematode infestation Pure cultures of individual isolates stored after freeze-drying
2. Initial laboratory screen to assess biological control potential	Isolates selected for their ability to colonize plant rhizospheres, to produce chlamydospores and to parasitize nematode eggs in simple *in vitro* tests
3. Determine growth requirements to maximize *in vitro* chlamydospore production	Growth and development of selected isolates evaluated on different media in different conditions to optimize chlamydospore production in sterile conditions
4. Evaluate efficacy of selected isolates in pot tests in glasshouse to determine key factors limiting control	Isolates compared for their efficacy on different host plants, at different nematode densities, fungal application rates and soil conditions. All tests done in non-sterilized soil and impacts on non-target organisms measured
5. Evaluate efficacy of selected isolates in field trials	Mass-produced chlamydospore inoculum applied in integrated management strategies in commercial production systems. Spread of the fungus should be monitored after its release

The selection criteria and the use of arbitrary standards, based on experience during the screening process, supported the elimination of > 85% of the isolates of *V. chlamydosporium* before more expensive tests to assess the activity of the fungus in soil were started (Kerry, 1998). In limited tests on a few isolates, those that failed to meet the selection criteria in the laboratory screens did not provide adequate nematode control when tested in soil and so such screening procedures can save considerable time without the risk of discarding potentially useful agents. However, all isolates should be freeze-dried for long-term storage, both as a reference source and in case they need to be screened for other purposes, such as the production of enzymes or toxins.

Extensive glasshouse trials are required to determine the factors that limit the efficacy of the fungus, especially as root-knot nematodes frequently occur in mixed populations attacking a wide range of crops in different soils and the growth of *V. chlamydosporium* is affected by both soil type and plant species. Isolates were initially tested for their efficacy in the control of different densities of *M. incognita, M. javanica* and *M. arenaria* when applied to soil at different rates (de Leij *et al.*, 1992b). The ability to colonize the rhizospheres of different plant species susceptible or resistant to these nematodes and the effect of soil conditions such as temperature and texture were also assessed in controlled experiments. All pot tests should conform to the conditions described by Stirling (1991) and be done in non-sterilized soil with active microbial communities, which will compete with the introduced fungus; tests in sterilized soil frequently result in overestimates of an organism's ability to reduce nematode popula-

tions in field conditions. The population density of the fungus in the soil and rhizosphere should be estimated at the end of the test, in addition to estimates of nematode populations and fungal infection levels in treated and untreated soil. Too often in tests of other potential BCAs for nematodes, these procedures have not been followed and it has proved difficult to assess whether any nematode control was caused by the agent and, indeed, whether the organism even survived after its addition to soil. Results from a range of pot tests indicated that *V. chlamydosporium* was able significantly to reduce the populations of all root-knot nematodes tested after a single application of chlamydospores (5000 g^{-1} soil) to soils of different textures but not at large nematode densities or on highly susceptible crops. These limitations resulted from the effects of gall size on the efficiency of the fungus described above and highlighted the need to integrate the fungus with other control measures. *V. chlamydosporium* will require careful exploitation because its efficacy is markedly reduced if it is applied when nematode infestations are large and many egg masses remain embedded in galled roots. Applications of chlamydospores to soil in pots reduced the numbers of healthy eggs of *M. incognita* in the rhizosphere of kale, maize and tomato plants by 87% compared with the numbers produced in untreated soil. However, total nematode populations were reduced by only 54% because many nematodes survived within roots and were not infected by the fungus (Bourne *et al.*, 1996).

Current recommendations for the management of root-knot nematodes include the use of at least two poor hosts and a resistant cultivar between susceptible crops (Bridge, 1987). If the soil is heavily infested and resistant cultivars are not suitable or available, the cropping cycle must be extended. A strategy that combines crop rotation with poor host crops for the nematode and applications of selected isolates of *V. chlamydosporium* has been devised. The aim of the strategy is to use the fungus to enhance the ability of selected poor hosts in the cropping cycle to reduce nematode populations to non-damaging levels before the next susceptible crop is grown; it should be possible to reduce crop cycles in length without the need to apply nematocides.

V. chlamydosporium may have a significant effect in reducing root-knot nematodes on several crops, either alone or in combination with other control measures. However, most tests have been done in the glasshouse or in microplots infested in controlled conditions. In a recent experiment, microplots were planted with a susceptible tomato crop to build up an infestation of *M. incognita*; thereafter, four poor hosts for the nematode were grown before the next tomato crop (Table 5.3). The fungus was thoroughly mixed in the top 25 cm of soil (5000 chlamydospores g^{-1} soil) and one, two or three treatments, applied at different stages in the cropping cycle, were compared with no treatment to control the nematode. The fungus significantly reduced nematode populations on the next tomato crop in all plots. Surprisingly, the single application of the fungus to the bean crop had a greater effect on nematode densities than the multiple applications. Further work is required to determine if the timing of the application of the fungus within the crop cycle is important; the nematode was more abundant on the bean crop than on kale or cabbage. The fungus was recovered from the roots of treated plants throughout the 3-year experiment, even if it had been applied only once. These results and others in the literature suggest that *V. chlamydosporium* may have considerable potential as a BCA but much more extensive testing is necessary.

Table 5.3. The combined effects of *Verticillium chlamydosporium* and poor hosts for root-knot nematodes on the postharvest numbers of *Meloidogyne incognita* after a tomato crop in a 3-year cropping cycle.

Treatments: 3-year crop rotation

Tomato – Kale – Beans – Cabbage – Cabbage – Tomato
↑ ↑ ↑

Application of *V. chlamydosporium* at 5000 chlamydospores g^{-1} soil

Postharvest numbers of *M. incognita* g^{-1} root after the final tomato crop:

No. of applications of the fungus	Crop treated with fungus	Nematodes g^{-1} root	
0	–	2018	(2.854)
1	Beans	13	(0.566)
2	Kale + beans	215	(1.582)
3	Kale + beans + cabbage (1st crop)	420	(1.178)
	SE_{DIFF}		(0.506) ***

*** Significant at $P < 0.001$.

Future Research Priorities

It is essential that the research on the potential of *V. chlamydosporium* as a BCA moves from tests in controlled conditions to evaluations of its efficacy in the field. The practical exploitation of *V. chlamydosporium* partly depends on improved methods for the mass production of chlamydospores. At present, sufficient inoculum can be produced only for small-scale plot tests; solid media, such as sand:bran mixtures, typically produce 2.5×10^6 chlamydospores g^{-1} medium. Clearly, broadcast treatments at 5000 chlamydospores g^{-1} soil (standard application rate) are impractical on a commercial scale. Methods to increase production efficiency and to improve application techniques are essential to support the practical use of the fungus (Kerry, 1998). The development of integrated management strategies such as that outlined above should be compared with conventional methods in commercial production systems. Vegetable and horticultural crops are an important target to demonstrate the practicality of the biological control of nematode pests using *V. chlamydosporium* because of their high value, often small-scale production, dependence on nematocides and opportunities within current production systems for applying these agents. Also, many horticulturalists are familiar with the use of BCAs and depend on them for control of insect pests in glasshouses; they are therefore likely to be more receptive to a new biological product than an arable farmer. The compatibility of the fungus with agrochemicals used in commercial vegetable production must be established.

Although *V. chlamydosporium* is widespread in nematode-infested soils, it must be tested for effects on non-target organisms in order to prepare a proper risk assessment. Although it would be an advantage if isolates of *V. chlamydosporium* that parasitize nematodes also controlled fungal root pathogens, effects on beneficial fungi such as mycorrhizae or on nitrogen-fixing bacteria, other beneficial rhizosphere bacteria and the general nematode community could severely restrict their use. To date, there has

been no report of such detrimental effects on the microbial community in the rhizosphere from applications of the fungus but more extensive testing is essential. The application rates proposed for the control of root-knot nematodes are similar to the densities of the fungus found in naturally suppressive soils (Kerry *et al.*, 1993), so any effects of the fungus on soil communities might be most obvious in such soils and should be investigated.

Alongside such applied research, it is important that basic studies on the epidemiology and infection processes of the fungus are pursued. Understanding of the key factors affecting the dynamics of the fungus could lead to improvements in its deployment, and understanding of the molecular interactions during infection may lead to the identification of novel bioactive compounds and the development of bioassays to identify more effective isolates. Also, molecular markers for specific isolates would enable their activity to be monitored, and the role of such variation in the control of nematode populations could be elucidated. Information on the variation in *V. chlamydosporium* isolated from different countries is needed for the registration of the fungus as a BCA. In the longer term, the transformation of *V. chlamydosporium* for the incorporation of genes to enhance its performance could be considered. For example, the incorporation of a nematocidal gene expressed when the fungus colonizes the egg mass could increase the effectiveness of the fungus in soils at temperatures >30°C by preventing uncolonized eggs from hatching before they are parasitized. The research priorities for *V. chlamydosporium* have largely concentrated on its development as a BCA but fundamental studies on its tritrophic interactions could provide much information on signalling processes in the rhizosphere, which would have wider scientific relevance.

References

Anderson, R.M. and May, R.M. (1981) The population dynamics of microparasites and their invertebrate hosts. *Philosophical Transactions of the Royal Society, London, B* 291, 451–524.

Arora, D.K., Hirsch, P.R. and Kerry, B.R. (1996) PCR-based molecular discrimination of *Verticillium chlamydosporium* isolates. *Mycological Research* 100, 801–809.

Atkinson, H.J. and Dürschner-Pelz, U. (1995) Spore transmission and epidemiology of *Verticillium chlamydosporium*, an endozoic fungal parasite of nematodes in soil. *Journal of Invertebrate Pathology* 65, 237–242.

Bourne, J.M. and Kerry, B.R. (1999) Effect of the host plant on the efficacy of *Verticillium chlamydosporium* as a biological control agent of root-knot nematodes at different nematode densities and fungal application rates. *Soil Biology and Biochemistry* 31, 75–84.

Bourne, J.M., Kerry, B.R. and de Leij, F.A.A.M. (1996) The importance of the host plant in the interaction between root-knot nematodes (*Meloidogyne* spp.) and the nematophagous fungus, *Verticillium chlamydosporium* Goddard. *Biocontrol Science and Technology* 6, 539–548.

Bridge, J. (1987) Control strategies in subsistence agriculture. In: Brown, R.H. and Kerry, B.R. (eds) *Principles and Practice of Nematode Control in Crops*. Academic Press, Melbourne, pp. 389–420.

Cayrol, J.C. (1983) Lutte biologique contre les *Meloidogyne* au moyen d'*Arthrobotrys irregularis. Revue de Nématologie* 6, 265–273.

Chen, Z.X. and Dickson, D.W. (1998) Review of *Pasteuria penetrans*: biology, ecology and biological control potential. *Journal of Nematology* 30, 313–340.

Crump, D.H. and Kerry, B.R. (1981) A quantitative method for extracting resting spores of

two nematode parasitic fungi, *Nematophthora gynophila* and *Verticillium chlamydosporium* from soil. *Nematologica* 27, 330–339.

Dickson, D.W., Oostendorp, M., Giblin-Davis, R.M. and Mitchell, D.J. (1994) Control of plant-parasitic nematodes by biological antagonists. In: Rosen, D., Bennett, F.D. and Capinera, J.L. (eds) *Pest Management in the Subtropics Biological Control – a Florida Perspective*. Intercept, Andover, UK, pp. 575–601.

Gams, W. (1988) A contribution to the knowledge of nematophagous species of *Verticillium. Netherlands Journal of Plant Pathology* 94, 123–148.

Grønvold, J., Wolstrup, J., Larsen, M., Henrikson, S.A. and Nansen, P. (1993) Biological control of *Ostertagia ostertagi* by feeding selected nematode-trapping fungi to calves. *Journal of Helminthology* 67, 31–36.

Hirsch, P.R., Mauchline, T.H., Mendum, T.A. and Kerry, B.R. (2000) Detection of the nematophagous fungus *Verticillium chlamydosporium* in nematode-infested plant roots using PCR. *Mycological Research* 104, 435–439.

Jaffee, B.A., Phillips, R., Muldoon, A. and Mangel, M. (1992) Density-dependent host–pathogen dynamics in soil microcosms. *Ecology* 73, 495–506.

Kerry, B.R. (1987) Biological control. In: Brown, R.H. and Kerry, B.R. (eds) *Principles and Practice of Nematode Control in Crops*. Academic Press, New York, pp. 233–263.

Kerry, B.R. (1998) Progress towards biological control strategies for plant-parasitic nematodes. In: *Proceedings of the 1998 Brighton Conference – Pests and Diseases.* BCPC, Farnham, UK, pp. 739–746.

Kerry, B.R. and Crump, D.H. (1998) The dynamics of the decline of the cereal cyst nematode, *Heterodera avenae*, in four soils under intensive cereal production. *Fundamental and Applied Nematology* 21, 617–625.

Kerry, B.R., Irving, F. and Hornsey, J.C. (1986) Variation between strains of the nematophagous fungus, *Verticillium chlamydosporium* Goddard. I. Factors affecting growth *in vitro. Nematologica* 32, 461–473.

Kerry, B.R., Kirkwood, I.A., de Leij, F.A.A.M., Barba, J., Leijdens, M.B. and Brookes, P.C. (1993) Growth and survival of *Verticillium chlamydosporium* Goddard, a parasite of nematodes, in soil. *Biocontrol Science and Technology* 3, 355–365.

de Leij, F.A.A.M. and Kerry, B.R. (1991) The nematophagous fungus, *Verticillium chlamydosporium* Goddard, as a potential biological control agent for *Meloidogyne arenaria* (Neal) Chitwood. *Revue de Nématologie* 14, 157–164.

de Leij, F.A.A.M., Dennehy, J.A. and Kerry, B.R. (1992a) The effect of temperature and nematode species on interactions between the nematophagous fungus *Verticillium chlamydosporium* and root-knot nematodes (*Meloidogyne* spp.) *Nematologica* 38, 65–79.

de Leij, F.A.A.M., Kerry, B.R. and Dennehy, J.A. (1992b) The effect of fungal application rate and nematode density on the effectiveness of *Verticillium chlamydosporium* as a biological control agent for *Meloidogyne incognita. Nematologica* 38, 112–122.

de Leij, F.A.A.M., Kerry, B.R. and Dennehy, J.A. (1993a) *Verticillium chlamydosporium* as a biological control agent for *Meloidogyne incognita* and *M. hapla* in pot and micro-plot tests. *Nematologica* 39, 115–126.

de Leij, F.A.A.M., Dennehy, J.A. and Kerry, B.R. (1993b) The effect of watering on the distribution of *Verticillium chlamydosporium* in soil and the colonisation of egg masses of *Meloidogyne incognita* by the fungus. *Nematologica* 39, 250–265.

Lopez-Llorca, L.V. (1990) Purification and properties of extracellular proteases produced by the nematophagous fungus *Verticillium suchlasporium. Canadian Journal of Microbiology* 36, 530–537.

Luc, M., Sikora, R.A. and Bridge, J. (1990) *Plant Parasitic Nematodes in Subtropical and Tropical Agriculture.* CAB International, Wallingford, UK, 629 pp.

Morgan-Jones, G., White, J.F. and Rodriguez-Kabana, R. (1983) Phytonematode pathology: ultrastructural studies. I. Parasitism of *Meloidogyne arenaria* eggs by *Verticillium chlamydosporium. Nematropica* 13, 245–260.

Rao, M.S., Kerry, B.R., Gowen, S.R., Bourne, J.M. and Reddy, P.P. (1997) Management of *Meloidogyne incognita* in tomato nurseries by integration of *Glomus deserticola* with *Verticillium chlamydosporium. Zeitschrift für Pflanzenkrankheiten und Pflanzenschutz* 104, 419–422.

Sasser, J.N. and Freckman, D.W. (1987) A world perspective on nematology: the role of the Society. In: Veech, J.A. and Dickson, D.W. (eds) *Vistas on Nematology.* Society of Nematologists, Hyatsville, pp. 7–14.

Segers, R., Butt, T.M., Kerry, B.R., Beckett, A. and Peberdy, J.F. (1996) The role of the proteinase VCP1 produced by the nematophagous *Verticillium chlamydosporium* in the infection process of nematode eggs. *Mycological Research* 100, 421–428.

Segers, R., Butt, T.M., Carder, J.H., Keen, J.N., Kerry, B.R. and Peberdy, J.F. (1999) The subtilisins of fungal pathogens of insects, nematodes and plants: distribution and variation. *Mycological Research* 103, 395–402.

Stirling, G.R. (1991) *Biological Control of Plant Parasitic Nematodes.* CAB International, Wallingford, UK, 282 pp.

Stirling, G.R., Licastro, K.A., West, L.M. and Smith, L.J. (1998) Development of commercially acceptable formulations of the nematophagous fungus *Verticillium chlamydosporium. Biological Control* 11, 215–221.

Waage, J.K. and Greathead, D.J. (1988) Biological control: challenges and opportunities. *Philosophical Transactions of the Royal Society, London* 318, 111–128.

Whitehead, A.G. (1997) *Plant Nematode Control.* CAB International, Wallingford, UK, 384 pp.

6 Fungal Biocontrol Agents of Weeds

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Introduction

Weeds are an ever-present and increasingly significant constraint to agricultural production worldwide. Ironically, more 'advanced' and changing cultivation practices have led to weeds becoming more rather than less problematic, especially in the major cereal food crops. For example, in rice (*Oryza sativa* L.), labour shortages and rising costs have resulted in a widespread shift from the traditional transplanting culture to direct seeding, whilst the development of hardy upland rice varieties has meant that cultivation is expanding into drier ecosystems, where a radically different weed biota has to be overcome. Such factors, coupled with water shortages in irrigated rice systems, have resulted in increased weed pressures, particularly from grassy weeds (Baker and Terry, 1991). Similar problems are mirrored in other crop systems.

The overall impact of weeds on crop production can, at best, only be crudely calculated, with average losses varying from 10 to 20%. In the USA alone, it has been estimated that some US$15 billion (thousand million) are lost annually due to weeds (Bridges, 1994). In such highly developed, agriculturally mechanized countries, with extensive crop monocultures, production levels can only be maintained through the regular and wholesale application of pesticides, particularly of chemical herbicides, which account for almost 50% of the agrochemical market (Woodburn, 1995). However, the use of herbicides as a management tool is becoming more problematic due to the increasing occurrence of herbicide-resistant weed populations, especially in wheat (*Triticum aestivum* L.) and maize (*Zea mays* L.) (LeBaron and Gressel, 1982). Thus, new strategies for the long-term management of agricultural weeds need to be developed: or 'new tools for the toolkit', which at present comprises only chemical herbicides and cultural–mechanical options. In developing countries, however, the economics and logistics inherent in applying pesticides often dictate against their use, most notably in Africa, where manual weeding, predominantly by women, is the norm. In terms of human resources alone, which could be better invested in

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other more meaningful pursuits, such social costs are a continual hindrance to development.

As international travel and global trade increase, more plant species, with potentially weedy traits, are being moved around the world, giving rise to new, invasive weed problems, particularly in non-agricultural or natural ecosystems (Mack *et al.*, 2000). In these situations, alien or exotic weeds now contribute the major threat to biodiversity after habitat destruction (Bell, 1983; Heywood, 1989; Cronk and Fuller, 1995; Binggeli, 1996). Moreover, weed control is rarely practised, either because it is environmentally hazardous, as in the case of chemical herbicides, or prohibitively expensive, as with cultural–mechanical techniques, or simply impractical over extensively infested areas – although, more typically, it is a combination of all these factors. Thus, once again, alternative strategies need to be resourced and integrated into a management plan if the ongoing threat from invasive weeds is to be met.

In addition to weed infestations, crops are also subject to attacks from and are frequently devastated by fungal pathogens. In particular, exotic diseases have been and continue to be a significant threat to agriculture and have the potential to destabilize economies on a local as well as on a global scale, with serious socio-economic consequences (Large, 1940; Kingsolver *et al.*, 1983). Can this destructive power of plant pathogens be harnessed for humans' advantage and directed towards and integrated within an effective weed management strategy? The aim of the chapter is to try to answer this question by: monitoring the progress to date; analysing the problems involved; and assessing the long-term potential of biocontrol of weeds using fungal agents.

Fungal Biological Control Agents

Fungal biological control agents (BCAs) can be exploited for weed management using two seemingly distinct strategies: the inoculative or classical approach, and the inundative or mycoherbicidal approach. However, as will be shown, these need not necessarily be mutually exclusive.

The inoculative approach

Concepts

The inoculative or classical approach involves the use of host-specific or co-evolved fungal pathogens to control alien, highly aggressive and invasive weeds for which cultural and chemical methods have either failed or are inappropriate for economic or environmental reasons. The principles and protocols involved have been developed and employed by entomologists with considerable success since the early 1900s (Julien and Griffiths, 1998; McFadyen, 1998). The philosophy underpinning this approach is simple and is based on the assumption that, within natural or primary ecosystems, the component organisms are in a dynamic equilibrium, and that natural enemies play a pivotal role in the regulation of the plant or animal populations. When organisms are freed of these constraints, such as occurs after they are deliberately or accidentally introduced by humans into new or exotic ecosystems, then their fitness increases in relation to that of the indigenous flora or fauna. Thus, given favourable conditions for growth and reproduction, their populations can increase unchecked and, eventually, the alien

organism may reach pest status. Characteristically, many of the alien weeds which are now problematic in agricultural ecosystems are only minor weeds in their native ranges (Barreto and Evans, 1997). Those affecting natural ecosystems are rarely weeds in their centres of origin and may even be economically useful or ecologically important members of the indigenous flora (Barreto and Evans, 1988; Cronk and Fuller, 1995).

Progress

Despite the fact that insect natural enemies have been evaluated as BCAs of alien, invasive weeds and employed against them for more than a century, the exploitation of fungal BCAs has a relatively short history, dating back to only the 1970s. The first pioneering projects – one using a rust fungus (*Puccinia chondrillina* Bubak & Sydenham) against a weed (*Chondrilla juncea* L.) of Mediterranean origin in southeast Australia, the other involving the importation of a white smut (*Entyloma ageratinae* Barreto & Evans) into Hawaii for control of a rare Mexican plant (*Ageratina riparia* (Regel) K. & R.), which was invading upland pastures and natural forest ecosystems – were spectacularly successful (Fig. 6.1). In the case of *C. juncea* (skeleton weed),

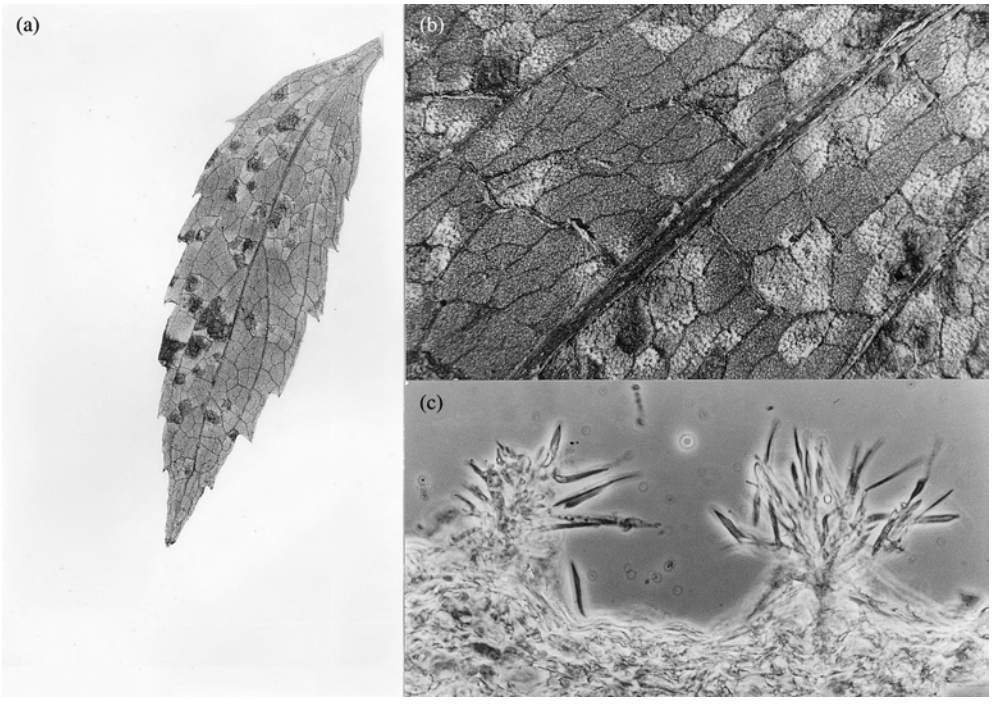

Fig. 6.1. White smut, *Entyloma ageratinae*, on mist-flower, *Ageratina riparia*. (a) Symptoms on mist-flower leaf (lower surface), collected in Veracruz, Mexico, showing pale green and necrotic lesions. (b) Close-up of above showing aggregated, white fructifications (caespituli). (c) Section through caespituli showing sporidial production; the spores are aseptate and thus distinguished from the cercosporoid group of fungi, in which the pathogen was initially classified following its release in Hawaii in 1975. The white smut achieved substantial to complete control of the invasive target weed and was later successfully introduced into South Africa in 1989 (Morris, 1991). It has recently been released in New Zealand (1998), where it has readily established and even exceeded its predicted impact on weed populations (Anon., 1999).

infestations in wheat were reduced by more than 99% to densities approaching those in the native range, and with benefits estimated at *c.* US$15 million per annum (Burdon *et al.*, 1981; Cullen and Hasan, 1988; Tisdell, 1990). For *A. riparia* (mist-flower) in Hawaii, agricultural land has been rehabilitated and natural ecosystems have been reclaimed and protected from further invasion (Trujillo, 1985; Davis *et al.*, 1992).

The question could be asked as to why the inoculative approach using fungal pathogens for biocontrol of weeds was not adopted earlier and, paradoxically, not rigorously pursued, since these initial successes, by biocontrol practitioners in general and plant pathologists in particular. There seems to be little doubt that this was and still is due mainly to concern about the safety aspects of transferring exotic plant pathogens between countries or geographic regions, especially by regulatory authorities. Indeed, many countries, including the UK, still have no specific protocol relating to the importation of fungal pathogens for biological control (Tatchell, 1996). The risks involved and the protocols developed to assess, minimize and predict these are dealt with in detail elsewhere in this book (see Goettel *et al.,* Chapter 13). Suffice it to say that extensive host-range testing and fundamental studies on the biology and ecology of the potential agents are essential prerequisites before any exotic fungal pathogen can be proposed for introduction into the target country (Adams, 1988; Weidemann and TeBeest, 1990; Watson, 1991; Weidemann, 1991). Such proposals are also subject to vetting by the in-country quarantine or legislative body, usually empowered with keeping out rather than bringing in exotic plant pathogens, before the selected agent can finally be approved for release.

Historically, Australia has played a key role in developing the protocols and in funding the majority of projects for the inoculative or classical use of fungal BCAs, and this recent history has been thoroughly reviewed (Wapshere, 1975, 1989; Hasan, 1980; Cullen and Hasan, 1988; McRae, 1988; Wapshere *et al.*, 1989; Evans and Ellison, 1990; Hasan and Ayres, 1990; TeBeest *et al.*, 1992; TeBeest, 1993; Mortensen, 1997; McFadyen, 1988; Evans, 2000). These should be consulted for a more comprehensive coverage of the subject. This review will concentrate on the present history, as well as on the long-term future of the inoculative approach.

Thus far, over 20 exotic fungal pathogens have been deliberately imported and released as classical BCAs of weeds worldwide, nearly half of these within the last 4–5 years (Julien and Griffiths, 1998), and a number of others are waiting in the wings (Evans, 2000). Most are obligate biotrophs, with the majority pertaining to the rust fungi (*Uredinales*), chosen because of their proved host specificity, their ability to disperse rapidly and efficiently over vast areas and their destructive powers, as evidenced by their impacts on cultivated crops. Several others have been illegally introduced, most notably blackberry rust, *Phragmidium violaceum* (Schulz.) Wint., in Australia in 1984, probably by farmers frustrated by the slow progress of the government-sponsored efforts (Marks *et al.*, 1984; Field and Bruzzese, 1985). Although none of the subsequent introductions of exotic fungal BCAs have yet to achieve the spectacular successes of the first releases, there is increasing evidence that there may be extended lag phases before the pathogens 'kick in' and begin to have a significant impact on weed populations. For example, an Australian gall-forming rust fungus, *Uromycladium tepperianum* (Sacc.) McAlpine, was introduced into South Africa in 1987 as a potential BCA of Port Jackson willow, *Acacia saligna* (Lab.) Wend., one of many *Acacia* spp. invading natural ecosystems in that country. However, the initial results were disappointing (Morris, 1991), and it is only within the last few years that the rust has built up to epiphytotic levels, with as many as 1500 galls developing on each tree. The continual pressure on the

growing points effectively kills the trees and weed populations have been reduced by 90–95%, with a corresponding increase in the native vegetation (Morris, 1997, 1999).

Another rust BCA that is now beginning to have an impact on its target weed host is *Maravalia cryptostegiae* (Cummins) Ono (Fig. 6.2), closely related to coffee-leaf rust, which was released in Queensland, Australia, in 1994, in an attempt to halt the spread of rubber-vine weed, *Cryptostegia grandiflora* Roxb. ex R. Br., originally imported from Madagascar as an ornamental (Evans, 1993; Evans and Tomley, 1994). This asclepiadaceous woody climber has been described as the single biggest threat to biodiversity in tropical Australia (McFadyen and Harvey, 1990). Because of the vast areas involved and their remoteness, helicopters have been used to disperse rust inoculum

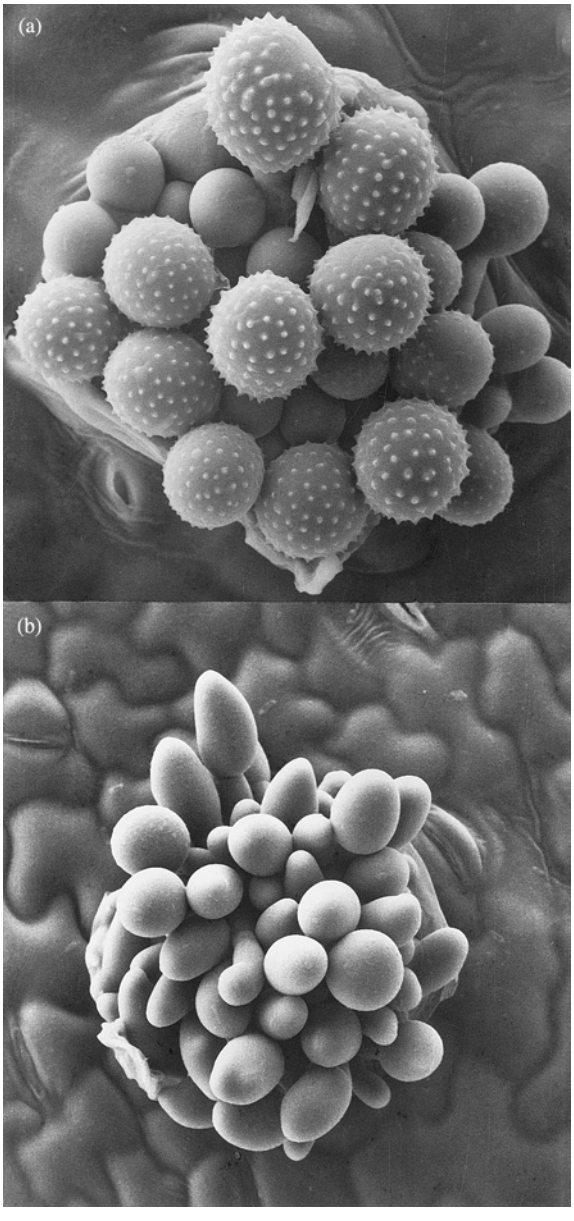

Fig. 6.2. The rust, *Maravalia cryptostegiae*, a biological control agent of rubber-vine in Australia. (a) SEM of uredinioid teliospores bursting through the lower leaf surface of *Cryptostegia grandiflora*. These spores are multifunctional fulfilling dispersal, survival and sexual roles (Evans, 1993). (b) SEM of thin-walled, non-resting telioid teliospores produced during sustained periods of high humidity but which appear to be non-functional in the partially expanded life cycle (Evans, 1993). The rust was released in Queensland in 1994 and rapidly caused severe and repeated defoliation of rubber-vine weed infestations (Tomley and Hardwick, 1996), which has been sustained leading to the resurgence of the native vegetation (A.J. Tomley, personal communication).

– initially, crudely, in the form of infected tissues, latterly by spraying spore suspensions with motorized mist-blowers into the forest canopies. Thus, the inoculative approach is being 'fast-tracked' by an inundative application strategy. A similar combination of strategies is also being exploited in the Northern Territory of Australia to combat the invasive, woody weed *Mimosa pigra* L. (or giant sensitive plant), which currently occupies large tracts of floodplains in this region and poses a potential threat to its prestigious national parks. In addition to a suite or guild of insect BCAs, two fungal pathogens have been introduced from Mexico (Fig. 6.3). The monotypic rust, *Diabole cubensis* (Arth. & John.) Arth., which causes defoliation, is mainly active during the dry season, whilst the hemibiotrophic ascomycete, *Sphaerulina mimosae-pigrae* Evans & Carrion (anamorph: *Phloeospora mimosae-pigrae* (Evans & Carrion), is a wet-season pathogen causing petiole and stem cankers (Evans *et al.*, 1995). It is anticipated that the two fungal BCAs will complement each other to exert pressure on the impenetrable weed thickets over the whole year. As an added bonus, it was later discovered that the anamorph is capable of yeast-like growth *in vitro*, producing a succession of viable and infective conidia by budding. These are now being formulated in methyl cellulose, and applied as a mycoherbicide with a knapsack sprayer (Forno *et al.*, 1996). Once again, these two seemingly distinct approaches to weed management have led to a common implementation strategy.

From an analysis of all the classical weed biocontrol projects which have reached this implementation phase, it becomes clear that, for certain invasive weeds, there is a 'silver bullet' solution. Notably, skeleton weed in Australia, mist-flower in Hawaii and now Port Jackson willow in South Africa have all been brought under complete control: and a single, classically released fungal BCA has been the principal cause of their demise. There is recent evidence to indicate that the same may apply for the rubber-vine weed in Queensland (A.J. Tomley, personal communication). Some outstanding examples can also be drawn from the entomological literature on the classical biocontrol of weeds (McFadyen, 1998). For other invasive weeds, however, there is no such simple and elegant remedy, and a guild of natural enemies (both insect and fungi) may be required to achieve the desired level of control, often integrated with more traditional management practices. Such will probably be the case with the giant sensitive plant in the Northern Territory and also certainly the same situation exists with parthenium weed (*Parthenium hysterophorus* L.) in Queensland, Australia. This composite weed of neotropical origin, which was accidentally introduced into both Australia and India within the last 30–40 years, is now the number one terrestrial weed target in the latter country, as well as in Queensland, where it not only constitutes an agricultural problem, invading both crops and grazing land, but also poses a serious health hazard due to its many allergenic properties (Evans, 1997). Biocontrol has been a major component of its management strategy in Australia, where, in addition to eight insect BCAs, two rust fungi from its Mexican native range have recently been introduced. The weed appears to have a significantly greater geographic range than any of its natural enemies, thus complicating the control strategy and necessitating a multi-release inoculative approach. One of these rust species, *Puccinia abrupta* Diet. & Holw. var. *partheniicola* (Jackson) Parmelee, has a predominantly subtropical, semi-arid distribution in Mexico, whilst the other (*Puccinia melampodii* Diet. & Holw.) is mainly restricted to the humid-tropical zones. Therefore, as the weed continues to invade other ecosystems in eastern Australia, spreading from its semi-arid rangeland foothold, a range of ecologically adapted BCAs will need to be deployed.

An analysis of the current work in exploring for and the exploitation of fungal

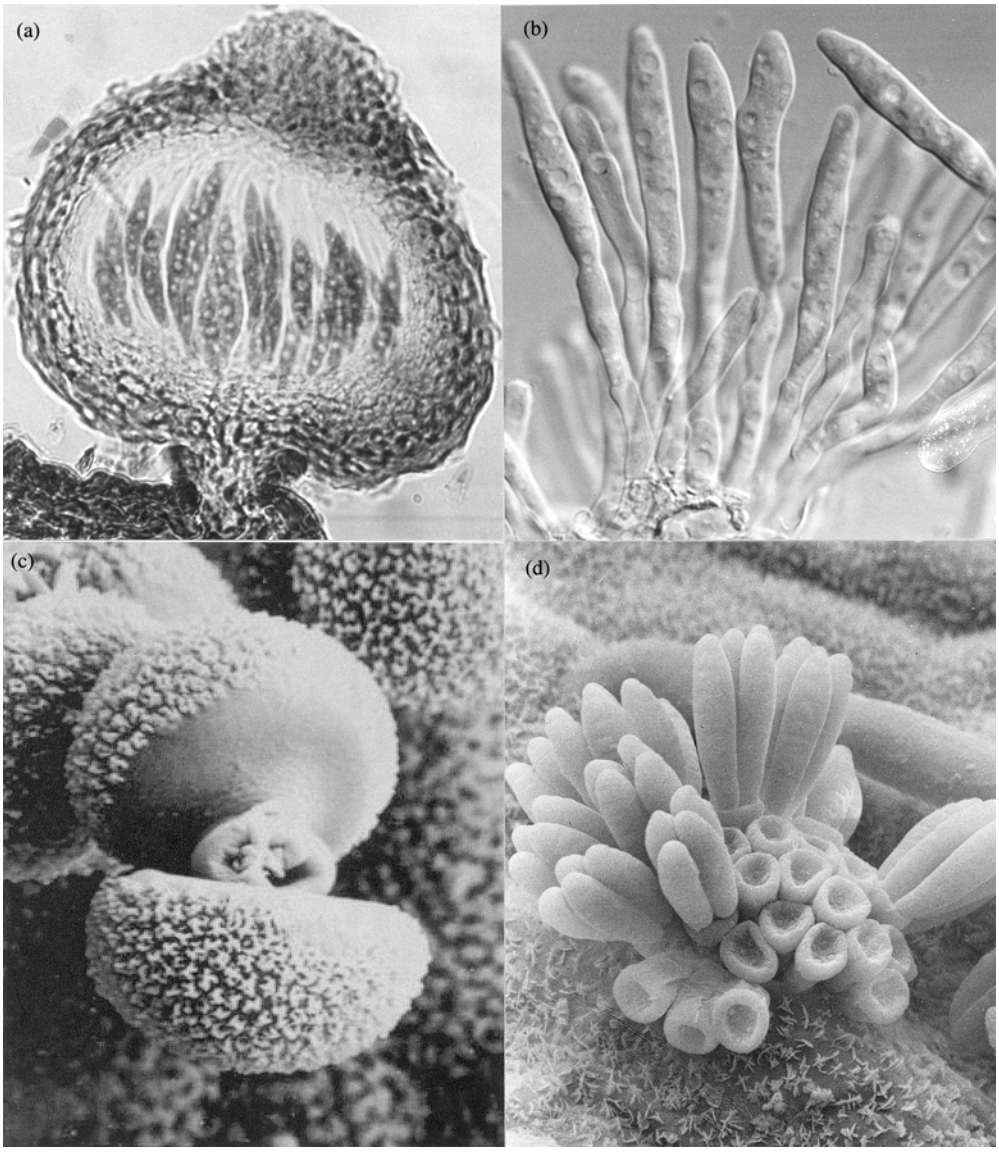

Fig. 6.3. Potential fungal biocontrol agents of *Mimosa pigra*. *Sphaerulina mimosae-pigrae* (a) and its anamorph *Phloeospora mimosae-pigrae* (b), released in the Northern Territory in 1994; both stages can be produced on still-green host tissues prior to necrosis and thus the pathogen can be classified as a hemibiotroph. (c) *Diabole cubensis*, a monotypic rust showing the highly distinctive unicellular, paired teliospores, which was introduced into Australia in 1996. (d) Hypophyllous fructification (basidioma) of *Microstroma ruizii-belinii* Evans & Carrion (*Exobasidiales*). Relatively little is known about the biology and taxonomic affinities of these basidiomycetes; hence it has not been evaluated further as a BCA. Note the production of four basidiospores from each basidial cup in the basidioma, and four sterigmatal scars at the base of empty cups.

pathogens for classical biocontrol of weeds shows that most is being directed towards subtropical and tropical ecosystems. Evans (1987) discussed the possibilities of applying the inoculative approach to a number of invasive tropical weeds; amongst them, *Lantana camara* L., *Rottboellia cochinchinensis* (Lour.) Clayton and *Mikania micrantha* H.B.K. were targeted. Since then, funding has been realized to explore the potential of exotic fungal pathogens for their control (Evans and Ellison, 1990; Evans, 1995a). In view of the fact that all these projects are now nearing completion, it is considered relevant here to highlight the problems involved and to detail the progress to date.

LANTANA CAMARA. Over the past century, this neotropical verbenaceous shrub has become a serious pan-tropical invasive weed of both agricultural and conservation land in Africa, Asia, Australasia and Oceania and is of particular ecological importance in small island ecosystems (Holm *et al.*, 1977; Cronk and Fuller, 1995). In Australia, for example, almost 4 million ha are now infested by the weed, with an estimated Aus$17 million being spent annually in Queensland state alone on clearing operations (Tomley and Evans, 1995). This weed occupies a seminal position in biocontrol history since this was one of the first pests to be targeted for the inoculative approach, dating back to the 1890s (Funasaki *et al.*, 1989). Of the many agents released over this period, all, until very recently, have been insect natural enemies (Julien and Griffiths, 1998). However, McFadyen (1998) has concluded that the biocontrol programmes in most of the target areas, perhaps with the exception of Hawaii, should be viewed as failures.

The extreme plasticity of *L. camara*, due to natural and human-mediated hybridization, makes this a difficult target, but two rusts, amongst other potential pathogens (Barreto *et al.*, 1995), are now showing promise since they both attack the major weed biotype in Australia. A strain of a leaf rust, *Prospodium tuberculatum* (Speg.) Arth. from Brazil, will probably be released in 2001, after its importation is approved by Australian Quarantine and Inspection Services. From greenhouse studies of its infection parameters and host range, it is expected to exert some pressure on the weed, at least in subtropical regions. Recently, an extremely virulent isolate of a microcyclic rust from the Peruvian Amazon, *Puccinia lantanae* Farlow, has been evaluated and, because it can attack both petioles and stems, as well as leaves, causing cankering and girdling, it is predicted that this rust strain could have a considerable impact on weed populations in tropical Australia. The importance of strain selection cannot be over-stressed, and this Amazonian isolate is the only one of many observed in the field in South and Central America that consistently invades and kills stem tissue.

ROTTBOELLIA COCHINCHINENSIS. *R. cochinchinensis* or itch grass is a plant of Afro-Asian origin which is now a major invasive weed in Latin America, especially in graminaceous crops (Evans, 1991; Ellison and Evans, 1995). The initial strategy was to develop a mycoherbicide based on a co-evolved necrotrophic fungal pathogen rather than a biotroph, this being the first deliberate attempt to combine both inoculative and inundative approaches. However, despite the fact that the *Colletotrichum* sp. selected and field-evaluated in Thailand proved to be undescribed and specific to the weed – too specific in fact, since it only infected a single biotype – it was adjudged to be politically sensitive to introduce an alien species of *Colletotrichum* into the New World. Two biotrophic fungi, the rust *Puccinia rottboelliae* P. & H. Sydow and the smut *Sporisorium ophiuri* (P. Henn.) Vanky, were considered to offer potential as inoculative agents. Following preliminary greenhouse evaluation, a strain of *S. ophiuri* from Madagascar was selected for further specificity screening. This head smut systemically

infects weed seedlings in the soil and completely replaces all the inflorescences. Since seeds are the only means of propagation and perennation and the seed bank is short-lived, the smut appears to have the potential to significantly affect weed populations (Smith *et al.*, 1997). A proposal and protocol for importing the smut into Costa Rica, based on Food and Agriculture Organisation (FAO) guidelines (FAO, 1996), have now been accepted by the quarantine authorities of that country (Reeder and Ellison, 1999), and it is expected that this BCA will be released within the year. This project is breaking new ground since it is the first time that a true smut has been exploited as a classical BCA, the first time that a grass weed has been targeted for the inoculative approach (Evans, 1991) and the first time that any exotic plant pathogen has been introduced into Central America for weed control. Once released, of course, the smut will eventually disperse to all the countries in the region where the weed is present, including the southern USA. Significantly, perhaps, international quarantine legislation does not cover such movement of fungal pathogens, and Costa Rica is under no legal obligation to inform its neighbours of the importation.

Mikania micrantha. This rampant climbing plant, one of several to share the epithet mile-a-minute weed, is a neotropical composite species with a native range extending from southern Brazil to Mexico. It has been a long-standing problem in plantation crops in Malaysia (Waterhouse, 1994) and northeast India (Parker, 1972), but is only a relatively recent arrival in the western Indian states, where it invades and smothers both natural and managed forests, as well as crops at the forest interface. In this biodiverse forest region, the weed has been the target of a classical biocontrol project over the last 4 years and an assessment of a microcyclic rust fungus, *Puccinia spegazzinii* de Toni, orginally highlighted as a potential inoculative BCA by Evans (1987) and Barreto and Evans (1995), has recently been completed. Rust strains were collected throughout Latin America, including the Caribbean. Eleven of these have now been screened against a comprehensive range of plant biotypes, which were collected and then characterized molecularly not only in India but in all the palaeotropic countries where it has become an invasive weed problem. A rust strain from Trinidad has proved to be highly pathogenic to all the major weed biotypes and is specific to *M. micrantha*, attacking not only the leaves but also the petioles and stems, leading to ring-barking and death. Other potential agents, identified during the initial field surveys (Barreto and Evans, 1995), also cause severe leaf damage but it is this ability of *P. spegazzinii* to kill the stems, and thus prevent further regrowth, which suggests that this pathogen will provide the 'silver bullet' solution. This will be an important test case for India since the inoculative approach has rarely been used as a weed management strategy. Thus, the in-country quarantine authorities will be on a learning curve regarding exotic plant pathogens, and great caution will need to be exercised in developing the protocol for importation.

Potential

There seems to be no doubt that the classical or inoculative approach can be an extremely effective, economic, sustainable and environmentally desirable strategy for the management of alien invasive weeds, many of which appear to be ideal targets. Indeed, with the burgeoning international trade in plants and plant produce, more exotic species, with actual or potential weedy traits, will be moved around the world, either deliberately for amenity purposes or accidentally as contaminants, and thus the

problem of invasives will only increase. Fragile island ecosystems are especially under threat but many other ecosystems, both natural and agricultural, can also be overrun by alien weeds (Cronk and Fuller, 1995). Of the relatively few fungal pathogens that have been exploited so far as classical BCAs of weeds, a significant proportion have shown or are showing great potential. Paradoxically, despite this obvious potential and the fact that significantly more insect natural enemies have been and are being considered as classical BCAs (McFadyen, 1998), there is still an illogical fear, or pathophobia (Freeman and Charudattan, 1985), surrounding the use of exotic plant pathogens for weed control. These ill-conceived and usually misinformed objections or reservations concerning the introduction of fungal BCAs invariably embrace the concepts of mutation and host-range extension, in spite of the rarity of the former and the certainty that the latter will be picked up in the centrifugal, phylogenetic host-range screens now routinely followed in pest-risk analysis (Wapshere, 1975). Indeed, these tests have since been shown to be too rigorous, with the possibility of rejection of potentially useful BCAs (Wapshere, 1989; Evans, 1995b, 1998). Moreover, one of the critical advantages of classical biocontrol over chemical pesticides is now considered to be its evolutionary stability. In a co-evolved association, the natural enemy adapts to genetic changes in the host but is genetically stable outside it. In contrast, chemical pesticides have been described as evolutionarily evanescent and hence the development of herbicide resistance in weeds is inevitable (Holt and Hochberg, 1997).

This inherent specificity of classical BCAs restricts their use to a single weed; in addition there is no saleable product and therefore there are no direct profits to an investor. Hence, the financing of inoculative biocontrol projects is and will continue to be problematic. Moreover, such projects can only be implemented as a matter of public interest since, once released, the exotic agents cannot be restricted to individual properties. This creates further problems if the alien weed is perceived as having some value, economic or otherwise, even if this is by only a tiny majority of the population in the target country. Thus, 'rule of law' legislation may need to be in place in order to avoid conflicts of interest (Harris, 1985). For example, one contentious dispute with bee-keepers in Australia delayed the implementation of a weed biocontrol project by up to 10 years, and resulted in the first specific Biological Control Act in 1984, which provided a legal basis for the introduction of weed BCAs (Cullen and Delfosse, 1985).

Whilst most northern-hemisphere countries have yet to approve or even to initiate the legislation for the introduction of exotic BCAs (Tatchell, 1996), particularly in Europe, where there are a number of well-documented, environmentally problematic alien weeds, the appropriate technologies and legislative issues have been developed, refined and promulgated for some considerable time by southern-hemisphere countries, such as Australia, New Zealand and South Africa. Although guidelines have been produced for the introduction of exotic BCAs of weeds in the USA (Klingman and Coulson, 1982), where half of the weeds and 13 of the top 15 weeds are alien species (Watson, 1991), very few fungal pathogens have been released so far.

It is not just a question of educating and lobbying individual countries, in order to change their pathophobic mentality, but also those international agencies concerned with protecting the environment, such as the World Wildlife Fund (WWF) and the International Union for the Conservation of Nature (IUCN), both of which maintain a policy whereby exotic organisms of any description are prohibited from being moved between countries or geographic regions. In effect, therefore, such bodies legislate

against or prevent the adoption of the inoculative biocontrol approach for invasive weeds.

In conclusion, massive infestations of alien weeds, especially over vast areas and/or in fragile or ecologically sensitive ecosystems, dictate that conventional control practices are impractical, uneconomic or environmentally undesirable. In such situations, classical biocontrol offers the only economically suitable method for the long-term management of these weeds. For other alien weeds, particularly those of agricultural importance, the solution lies in developing an integrated pest management strategy, but one in which the inoculative approach, or, indeed, the inundative approach, as will be discussed below, could underpin more traditional control measures.

The inundative approach

Concepts

In this approach, pathogens of weeds are typically used as an inundative inoculum to incite sufficient disease to provide weed control, typically on a seasonal basis, with a need for annual repeated applications

Progress

Augmentation of natural populations of indigenous fungal pathogens has had some commercial success. DeVine™, a liquid formulation of *Phytophthora palmivora* (Butler) Butler, is used for control of *Morrenia odorata* (H. & A.) Lindl. (strangler vine) in Florida citrus groves. DeVine was first registered in 1981 and continues to be available from Abbott Laboratories on a pre-order basis for a very restricted market (Charudattan, 1991). Collego™, a dry powdered formulation of *Colletotrichum gloeosporioides* (Penz.) Penz. & Sacc. f. sp. *aeschynomene*, is used for the control of *Aeschynomene virginica* (L.) B.S.P. (northern joint-vetch) in rice and soybeans in the southeastern USA. Collego was marketed from 1982 to 1992, with approximately 2500 ha treated annually (Templeton, 1992). Environmental Protection Agency (EPA) reregistration was not pursued until 1997 and the product is once again available, with approximately 5000 ha treated in 1998 (D. Johnson, personal communication). BioMal™ is a dry formulation of *C. gloeosporioides* f. sp. *malvae* and, although registered in Canada for the control of round-leaved mallow (*Malva pusilla* Sm.), it has not been marketed because of high production costs (K. Mortensen, personal communication). Another product, Lubao (*C. gloeosporioides* f. sp. *cuscutae*) has been used in China since 1966 for the control of dodder (*Cuscuta chinensis* Lam. and *Cuscuta australis* R. Br.) in soybeans (Gao and Gan, 1992), with inoculum being produced and distributed locally at the cottage-industry level. The present status of Lubao in China is unknown, but the lack of quality control has frequently resulted in the loss of culture virulence (R. Wang, personal communication).

Problems

To date, there have been no real commercial successes with mycoherbicides. Both DeVine and Collego have provided good to excellent weed control, but, since the market sizes are so small, profit margins are slight, if any. Commercial development mod-

els for mycoherbicides have been based on the premise that mycoherbicides had to be 'like a herbicide' as a 'stand-alone' product. A mycoherbicide must provide complete kill, have a long shelf-life and compete economically with chemical herbicides. Numerous linkages were forged between public institutions and private industry to facilitate mycoherbicide development, but for the most part these have failed, mainly due to the 'like a herbicide' paradigm. Most candidate mycoherbicides have failed due to biological, technological or commercial constraints impeding their commercial development (Auld and Morin, 1995). Extended dew requirements, low fecundity, low virulence, minimal shelf-life and restricted niche markets are common faults impeding commercial mycoherbicide development. Biological and technological constraints are being actively researched in a number of model systems, but scientists have limited opportunity to address commercial constraints other than choice of target and to shift away from the 'agrochemical industrial partner' development model.

Many pathogens with mycoherbicide potential have been discovered, but most lack sufficient aggressiveness to overcome weed defences and achieve adequate control (Gressel *et al.*, 1996). Improved understanding of mechanisms involved in determining virulence would assist in the selection of preferred isolates as biological weed control agents. Luo and TeBeest (1998) have developed an infection component analysis to aid in the assessment of best overall relative fitness of isolates of *C. gloeosporioides* f. sp. *aeschynomene*, which is applicable to most other mycoherbicide candidates.

Weed defences can be suppressed and mycoherbicide efficacy improved by chemical and biological synergy (Gressel *et al.*, 1996). The chemical synergist can be a compound that specifically overcomes one of the host responses to pathogen attack (Sharon *et al.*, 1992) or a chemical that otherwise weakens or wounds the weed, facilitating pathogenicity. Biological synergy is the use of other organisms to enhance dispersal or infection.

Fungi can be divided into those that readily sporulate in liquid culture and those that do not (Stowell, 1991). Commercial development requires low-cost production methods, and the use of liquid-culture fermentation is thought to be necessary to achieve this goal. Much of the success of Collego was due to the mass production of spores and preservation by drying being achieved in commercial-scale facilities. However, this has been elusive not only with other *Colletotrichum* species but with other fungal genera. Submerged liquid production systems are preferred for mass production of mycoherbicides (Churchill, 1982; Stowell, 1991), but many fungi being evaluated as prospective mycoherbicides do not sporulate in liquid culture. Nevertheless, they are generally very adaptable to solid-substrate fermentation on agriculturally based products and this method has been used to produce inoculum of several fungi currently being evaluated as mycoherbicides (Morin *et al.*, 1989; Watson *et al.*, 1997; Zhang and Watson, 1997).

Despite a wealth of research over the last four decades, which has resulted in a formidable list of candidates for further development, there has been relatively little progress in translating potential efficacy as a mycoherbicide into practical reality. The failure of candidate organisms to achieve product development status may be due to a number of reasons, several of which have already been touched upon. It is only too obvious from the literature that poor strain selection, strain instability and poor target selection are all important contributors to that failure. Far more often, however, it is apparent that poor or unreliable performance in field conditions is responsible for development being abandoned.

Inconsistent field performance may itself have several causes. Production difficulties can affect product reliability and thus the organism may deteriorate in storage,

poor timing of application may seriously reduce efficacy and environmental constraints may influence the outcome (Greaves *et al.*, 1998). Storage factors and environmental constraints are usually cited as the most common causes of unreliability and these are the two factors most likely to respond to improved formulation. Unfortunately, the majority of publications about mycoherbicides show a woeful ignorance of the need for and the provision of a suitable formulation. It seems to be accepted that a spore suspension in water containing a little surfactant (to ensure even suspension of the hydrophobic spores) is adequate. In many cases, it does appear to be sufficient, but only because the suspension contains a massive concentration of spores and is applied at high volume rates (up to 3000 l ha^{-1}). Such sprays run off the plant target area, distributing spores to all the most vulnerable sites, such as axillary meristems and stem bases. Subsequently, the plants are usually exposed to a long (up to 24 h) dew period, so ensuring that spore germination, infection of the plant and disease expression are all maximized.

While this procedure is academically satisfying, in that it provides positive results, it completely misrepresents the practical realities of applying such living products and is responsible for many organisms being wrongly identified as potential microbial herbicides and for much time being wasted in unproductive development research. The goal of developing robust and reliable products will only be achieved via selection of aggressive, specific and stable agents, properly formulated to withstand storage and environmental constraints and to allow for application at reasonable volumes using conventional spray equipment. It is not really possible or appropriate to totally separate application and formulation, as each interacts dynamically with the other. However, within the space constraints of this chapter, we shall focus on potential solutions to improve the efficiency of fungal BCAs exploited as mycoherbicides.

Solutions

APPLICATION FACTORS. Formulation must be integrated carefully with the application equipment and system to be used. Current trends in agriculture are to minimize the application volume used and to deliver it so as to minimize drift. This latter objective requires attention to be paid to the droplet spectrum generated by the sprayer so as to avoid producing small, drift-prone droplets. Droplet size can be markedly affected by formulation and, in turn, can affect the capacity of the droplet to carry spores. Many of the aspects relevant to these processes are described by Greaves *et al.* (1998) and are summarized as diagrams that relate formulation to the unit processes involved: droplet formation; application; impaction and retention on the target; and deposit formation, leading subsequently to infection and disease expression. It is worth emphasizing that retention and distribution are affected by the nature of the plant. Generally, a determinate growth habit allows better chances of successful control than an indeterminate habit, which favours survival from structures such as rhizomes. Similarly, plants with highly hydrophobic surfaces are less prone to infection from foliar applications. In this latter case, formulation can overcome the problem to a large degree. Although it is beyond the scope of this chapter to review spray application factors, it is important to reiterate that spray application volume and spray distribution on the target are critically important for the efficacy of mycoherbicides.

FORMULATION. Formulation is the combination of specific ingredients (additives and adjuvants) to provide a practical crop protection product. Additives are used to

maintain the long-term viability of the agent's propagules and to facilitate ease of handling, storage and application. Adjuvants are proprietary products added to improve spray delivery of the product or to enhance the pathological impact of the propagules.

To enable proper formulation of any mycoherbicide, it is essential to understand fully those factors which affect efficacy. Each factor may vary quantitatively and qualitatively for different agents and, therefore, may require different formulation inputs to allow for maximum effect. In practice, of course, it is unlikely that all formulation demands can be met in full and an informed compromise will be required. The following will deal with formulation requirements in relation to specific important functions that must be aided.

Distribution of inoculum on the target

This is probably the most important area where formulation can improve efficacy; however, despite its importance, this area has been greatly under-researched. Most plants can withstand a high degree of defoliation and, although this may initially check growth, subsequent growth can be accelerated, often producing multiple stems, so overcompensating for the previous check. Thus, the target plant may be even more vigorous after treatment than before. The most useful target sites are the meristems, from which regrowth occurs after defoliation, and, preferably, the stem below the lowest meristems, at the cotyledonary node. Girdling of the stem with disease lesions at this level cannot be overcome by the host and death is inevitable and quick. Unfortunately, these preferred target sites are often shielded from spray by the foliage of the target weed and of the neighbouring crop. This shielding effect is, of course, readily overcome in laboratory experiments by the routine use of high application volumes that lead to mass flow of the applied suspension of spores across plant surfaces, so redistributing the spores to the favoured sites. In practical field situations, however, this is more difficult to achieve. Addition of powerful surfactants can aid movement of deposited spray, especially down steeply angled surfaces, such as stems. In this case, the difficulty is in stopping the movement at the desired point. As the meristems are in the angle between stem and leaf petiole, there is a good chance that spray will be trapped and retained, although there is less chance of this happening on the open stem. Perhaps, if spores are trapped at the intersection of stem and soil surface, they may cause sufficient infection to girdle the stem. Whilst this specific phenomenon has not been investigated, it has been noted that the majority of foliar pathogens will not function effectively when applied as a soil drench.

It is beyond the scope of this short review to deal with the subject in detail. Nevertheless, one possibility for improving the situation is to manipulate the spraying system in order to maximize deposition on the preferred target sites on the weed. This may involve changing either the nozzle type, so as to alter the droplet size spectrum, or the nozzle position relative to the crop. The use of angled spray nozzles, for example, may be advantageous. Preliminary results suggest that spray deposition on the lower stem of the target weed can be significantly increased using an 'Evenspray' nozzle angled at 45° and positioned 40 cm above the plant (J. Lawrie and M.P. Greaves, unpublished data). This seems to be a better option than using a drop-leg sprayer to apply a sticky formulation from close to the soil surface. Boyette *et al.* (1996) and Greaves *et al.* (1998) have recently reviewed formulation and application of mycoherbicides.

Whatever spraying system is adopted, there will still be a need for formulation to ensure good infection at the site of retention and deposit formation. Surfactants, used to aid the spread of spray deposit across the plant surface, also increase evaporation

and so reduce the persistence of the aqueous component of the spray. This will reduce the chances of germination of the spores and will need to be prevented by the use of an adjuvant such as a humectant. Alternatively, an oil-based formulation, in which water droplets become entrained (Boyette, 1994; Greaves *et al.*, 1998), or an invert emulsion (Quimby *et al.*, 1988; Amsellem *et al.*, 1990) can be used. These will be effective at moving across hydrophobic plant surfaces and are less volatile than water, so avoiding the clumping of spores that may occur when aqueous deposits dry out (Potyka, 1996).

Factors affecting efficacy

Mycoherbicides applied to the aerial parts of plants are severely affected by lack of free water to promote spore germination, growth and infection. Dew periods are generally short (> 6 h) and irregular in occurrence. Fungal pathogens on the other hand, usually require long periods of exposure to free water (> 12 h) relatively soon after application (Auld *et al.*, 1988; Say, 1990; Boyette, 1994; Auld and Morin, 1995). Timing the application to match the occurrence of rain or, especially, dew is not easy as forecasting these events is not a precise science and they may not occur when weed control is required. Although the formulation of herbicides has advanced our ability to significantly reduce the drying rates of deposits, it is not so easy to achieve when the deposit contains a living fungus. The adjuvants used to reduce drying may be directly toxic to the fungus or may induce osmotic shock (Potyka, 1996). Recent work (G. Dutton, J. Lawrie and M.P. Greaves, unpublished data) has shown that polymers such as polyvinyl alcohols or plant mucilloids can be effective in reducing dependency on dew and have no undesirable effects, at least on the fungi tested so far. It has to be stressed that non-toxicity to one fungus does not necessarily mean that all fungi will be equally unaffected. In general, it has to be assumed that each candidate mycoherbicide will require a custom-made formulation. Equally, it has to be recognized that, despite the recent advances in research on formulation in order to overcome dew dependency, additional research into this subject is still urgently required (Greaves *et al.*, 1998).

Toxicity and synergism from additives

As mentioned above, it is axiomatic that any formulation additive must be non-toxic to the candidate agent. Preferably, it should enhance action in a synergistic way. Unfortunately, the literature rarely presents records of additives that have been found to be toxic to a range of organisms, suggesting that such tests have not been done. Where such data are presented, generally, they are only relevant to spore germination. This reliance on germination data to assess 'toxicity' may be misleading. Inhibition of germination can be more than compensated for if appressorium formation is stimulated (Potyka, 1996), and infection may then be unimpaired or even enhanced. To be sure that the most appropriate formulation is achieved, it is necessary to test each proposed formulation component against the candidate organism in comprehensive laboratory tests of toxicity and synergism. The results of such laboratory tests should be confirmed, for short-listed components, in greenhouse tests, using the living plant target to assess infection efficacy.

The inherent specificity of mycoherbicides is highly desirable from the point of view of environmental safety, although recent trends show that this is not always necessary, as will be shown later. At the same time, it can be a disadvantage from a commercial aspect. Farmers rarely have a monospecies weed problem and therefore those

relatively rare niche markets where a monospecific product can be sold must be identified. Alternatively, the range of weeds controlled by the product can be increased. The obvious tactic of mixing spores of different fungi, each effective against a different weed has a clear limit. A minimum spore concentration in each spray droplet is required to achieve infection and, as spores have a fixed volume, only so many can be packed into each droplet. Thus, in practice, it is likely to be possible to mix no more than two or three fungi in one formulation. This assumes, of course, that all the fungi are compatible with the formulation components. Specificity may also be manipulated in one fungus by growing it on particular substrates (Boyette and Abbas, 1994). However, a more feasible way of extending the range of weeds infected is to adjust the chemical components of the formulation. Amsellem *et al.* (1991) have demonstrated such abolition of specificity using an invert emulsion. It would be reasonable to suppose that tank mixing with a low dose of chemical herbicide might easily achieve the same objective and, indeed, this has received significant attention. A second attractive benefit of this approach is the possibility of achieving significant reduction of herbicide input to the environment. Beneficial effects of mixing chemical herbicides and mycoherbicides have been reported to be both additive and synergistic (Scheepens, 1987; Wymore *et al.*, 1987; Wymore and Watson, 1989; Grant *et al.*, 1990; Gohbara and Yamaguchi, 1993). As pointed out by Hoagland (1996), iatrogenic disease, arising from the use of pesticides, is common and is a powerful argument for focusing significant effort in order to exploit the phenomenon in the context of mycoherbicides.

There is a wide range of adjuvants available to formulate mycoherbicides. The toxic and synergistic attributes of these are reviewed by Greaves *et al.* (1998) and need not be repeated here. However, it is worth emphasizing that the effects of each surfactant, humectant, thickener, skinning agent or adhesive can be different with each fungus requiring formulation. There is no alternative to the tedium of individually testing each adjuvant in both *in vitro* and *in vivo* tests. Failure to do so will inevitably lead to unreliable performance of the product in the field and to its withdrawal from commercial development.

Storage

Mycoherbicides are most commonly applied as a liquid containing suspended spores. Clearly, this is not the most appropriate form in which to store the product. Not only is it bulky and heavy but the presence of water will permit the spores to germinate, after which they will rapidly die if they do not contact their host weed. Consequently, the favoured initial formulation is a wettable powder comprising a hydrophilic carrier mixed with the hydrophobic spores. In this state, many spores have been found to survive for up to 2 years without unacceptable loss of viability and efficacy (Mortensen, 1988), although difficulties have been encountered with some fungi (Jackson *et al.*, 1996). The ability to survive long-term storage is highly desirable in a mycoherbicide since it allows the passage of the product through the production, packaging, distribution and sales process without loss of efficacy. However, at least one product (DeVine) is made to order and supplied to the grower for immediate use. It is unlikely that such a system will be used as a commercial norm and its economic value must be questionable.

Solid formulations

A wide range of solid formulations has been developed and several have been applied in practice. These range from products based on plant residues, such as wheat bran

(Morris, 1989) and sorghum straw (Ciotola *et al.*, 1995) to those based on agar (Scheepens, 1987, 1990). Although these formulations are effective, they are also bulky, often inconsistent in quality and expensive to apply. The first uniform, reliable, solid carriers were mineral-based granules (Walker, 1981), but subsequently alginate-based granules (Walker and Connick, 1983; Fravel *et al.*, 1985) have been favoured. Such granular formulations have a number of advantages over foliar-applied liquid formulations. Amongst these are: the ability to place them precisely in the top of the seed furrow, leaving weeds between the rows to be removed using mechanical methods; and the residual activity that they exhibit can enhance efficacy. Most recently, the development of 'Pesta' granules (Connick *et al.*, 1991), in which the fungal agent is encapsulated in a wheat gluten matrix, has shown some promising results. The nutrient content of these granules is said to aid the effect of the fungus. Unfortunately, it can also have undesirable results and 'Pesta' granules containing no fungus will sometimes impair control of *Amaranthus retroflexus* by promoting the growth of soil-borne fungi (J. Lawrie and M.P. Greaves, unpublished data). This growth, which is usually of rapidly growing fungi, such as *Penicillium* spp., can overgrow the agent included in the granule and prevent its infection of the target weed. On the other hand, it is certain that the soil environment is buffered against those environmental perturbations, especially desiccation, which can make the leaf surface such a hostile environment for foliar-applied fungi. There is a clear need for more research into granule development and performance so as to capitalize on the potential advantages and minimize the problems. The experience gained in the encapsulation in materials, such as gelatinized starch (Schisler *et al.*, 1996), may offer further opportunities for better development of mycoherbicides. Similarly, exploitation of BCAs encapsulated in granules of solid substrates, coated with oil and an absorbent, suggests that this may be a way forward. Quimby *et al.* (1994) patented this system for granules consisting of alginate, starch or wheat gluten with a coating of oil that forms an invert emulsion with water. The oil is absorbed into a material such as hydrated silica. On re-wetting, these granules retain water for as long as 12 h and so may preclude the need for dew to promote and sustain fungal growth on and infection of the host weed. Much of the work to develop granular or encapsulated formulations of mycoherbicides duplicates work that was done previously to develop formulations of entomopathogenic fungi. As yet, however, it has not matched the extent, depth or achievement of that work. Dialogue between these two aspects of the same problem should therefore be encouraged. At the same time, the needs for appropriate and properly understood spraying systems must be recognized and addressed. In this way it is possible that real progress in developing effective and reliable formulations of mycoherbicides will be achieved.

It is imperative that the predominantly piecemeal development that has characterized mycoherbicide formulation to the present is replaced with a more organized and sustained effort. Otherwise, the scene will continue to be dogged by formulations that function adequately within the confines of a defined experimental programme but which are of unreliable efficacy in practice.

Potential

The use of indigenous, naturally occurring weed pathogens, formulated and applied like chemical herbicides, has potential to reduce chemical inputs and to provide viable, economic and effective weed-control components within integrated weed management programmes, such as in rice and other tropical crops. Much of the research effort

elsewhere is shifting away from discovering new mycoherbicides to solving production, storage and efficacy problems of existing ones. There is clearly a need to better understand the biochemical and physiological aspects of pathogenesis by the selected fungal BCA so that weak links in host defence can be exploited. As detailed above, many mycoherbicides still need the augmentation of formulants, as well as chemical and/or biological synergists, to provide the lethality to weeds that the consumer desires. The key to the successful development of mycoherbicides, especially in developing countries, may lie in the involvement of 'small market' business enterprises or producer cooperatives, each supplying their immediate area, using local labour and thereby adding to the economic viability of rural areas.

Thus far, agrochemical companies or the 'industry partners' have not invested any serious money in mycoherbicide research and development and they have tended to 'sit on the fence', awaiting any significant advance or breakthrough from the many and diverse, small, public-funded projects (Charudattan, 1991). These have not been forthcoming and it is considered doubtful that a commercially viable mycoherbicide will ever be marketed against the relatively narrow range of priority weeds targeted by the industry. None the less, there are recent encouraging signs that mycoherbicides can satisfy an important niche market in situations where chemical herbicides are either ineffective or environmentally undesirable. Coincidentally, two of these products incorporate non-specific pathogens which have adapted to and are highly pathogenic against exotic, invasive, woody weeds. In South Africa, Stumpout™, based on an indigenous white rot fungus, *Cylindrobasidium laeve* (Pers.: Fr.) Chamois, and produced at the cottage-industry level, is currently sold and used as a mycoherbicide to prevent regrowth of Australian wattle species (Morris *et al.*, 1998). As well as invading native ecosystems, such as the fynbos, these alien trees (*Acacia mearnsii* De Wildemann and *Acacia pycnantha* Benth.) disrupt water flow and have a direct and indirect impact on diminishing water resources. Thus, this mycoherbicide is now playing an important role in an ambitious clearance programme (Moran *et al.*, 1999). Similarly, Biochon™ is also based on a white rot fungus, *Chondrostereum purpureum* (Pers. ex Fr.) Pouzar, and is marketed in the Netherlands by a small biocontrol company (Koppert Biological Systems) for suppression of an introduced North American tree species (*Prunus serotina* Ehr.), which is invading both natural forests and plantation tree crops (Ravensburg, 1998). Elegant modelling and epidemiological studies were undertaken, initially as part of a pest risk analysis, to assess the threat posed by this plurivorous pathogen to commercial fruit-tree crops (de Jong *et al.*, 1990, 1991). This has stimulated further work in Canada on the same BCA (Prasad, 1994), and a commercial product (Ecoclear™) has recently been registered in North America for use in conifer plantations and public rights of way (Shamoun and Hintz, 1998). The Canadian Forestry Service is under tremendous public pressure to minimize or eliminate completely the reliance on chemical pesticides in forest management and therefore it would seem that the increasing use of biological alternatives, such as mycoherbicides, is both necessary and inevitable. Indeed, in Ontario, a government initiative was proposed to reduce pesticide inputs by 50% by 2002 (Swanton *et al.*, 1993). If similar pressures are directed against pesticides in agricultural ecosystems, the agrochemical companies may need to revisit and reassess the inundative approach, using fungal BCAs, for the long-term and sustainable management of weeds.

References

Adams, E.B. (1988) Fungi in classical biocontrol of weeds. In: Burge, M.N. (ed.) *Fungi in Biological Control Systems.* Manchester University Press, Manchester, UK, pp. 111–124.

Amsellem, Z., Sharon, A., Gressel, J. and Quimby, P.C. (1990) Complete abolition of high inoculum threshold of two mycoherbicides (*Alternaria cassiae* and *A. crassa*) when applied in invert emulsion. *Phytopathology* 80, 925–929.

Amsellem, Z., Sharon, A. and Gressel, J. (1991) Abolition of selectivity of two mycoherbicidal organisms and enhanced virulence of avirulent fungi by an invert emulsion. *Phytopathology* 81, 985–988.

Anon. (1999) What's new in biological control of weeds? *Landcare Research Newsletter, New Zealand* 13, 4.

Auld, B.A. and Morin, L. (1995) Constraints in the development of bioherbicides. *Weed Technology* 9, 638–652.

Auld, B.A., McRae, C.F. and Say, M.M. (1988) Possible control of *Xanthium spinosum* by a fungus. *Agriculture, Ecosystems and Environment* 21, 219–223.

Baker, F.W.G. and Terry, P.J. (1991) (eds) *Tropical Grassy Weeds*. CAB International, Wallingford, UK, 203 pp.

Barreto, R.W. and Evans, H.C. (1988) Taxonomy of a fungus introduced into Hawaii for biological control of *Ageratina riparia* (Eupatorieae; Compositae), with observations on related weed pathogens. *Transactions of the British Mycological Society* 91, 81–97.

Barreto, R.W. and Evans, H.C. (1995) Mycobiota of the weed *Mikania micrantha* in southern Brazil with particular reference to fungal pathogens for biological control. *Mycological Research* 99, 343–352.

Barreto, R.W. and Evans, H.C. (1997) Role of fungal biocontrol of weeds in ecosystem sustainability. In: Palm, M.E. and Chapela, I.H. (eds) *Mycology in Sustainable Development*. Parkway, Boone, North Carolina, pp. 183–210.

Barreto, R.W., Evans, H.C. and Ellison, C.A. (1995) Mycobiota of the weed *Lantana camara* in Brazil, with particular reference to biological control. *Mycological Research* 99, 769–782.

Bell, A. (1983) Native plants facing extinction. *Ecos* 37, 21–25.

Binggeli, P. (1996) A taxonomic, biogeographical and ecological overview of invasive woody plants. *Journal of Vegetation* 7, 121–124.

Boyette, C.D. (1994) Unrefined corn oil improves the mycoherbicidal activity of *Colletotrichum truncatum* for hemp sesbania (*Sesbania exaltata*) control. *Weed Technology* 8, 526–529.

Boyette, C.D. and Abbas, H.K. (1994) Host range alteration of the bioherbicidal fungus *Alternaria crassa* with fruit pectin and plant filtrates. *Weed Science* 42, 487–491.

Boyette, C.D., Quimby, P.C., Caesar, A.J., Birdsall, J.L., Connick, W.J., Daigle, D.J., Jackson, M.A., Egley, G.H. and Abbas, H.K. (1996) Adjuvants, formulations and spraying systems for improvement of mycoherbicides. *Weed Technology* 10, 637–644.

Bridges, D.C. (1994) Impacts of weeds on human endeavors. *Weed Technology* 8, 392–395.

Burdon, J.J., Groves, R.H. and Cullen, J.M. (1981) The impact of biological control on the distribution and abundance of *Chondrilla juncea* in south-eastern Australia. *Journal of Applied Ecology* 8, 957–966.

Charudattan, R. (1991) The mycoherbicide approach with plant pathogens. In: TeBeest, D.O. (ed.) *Microbial Control of Weeds*. Chapman & Hall, New York, pp. 24–57.

Churchill, B.W. (1982) Mass production of micro-organisms for biological control. In: Charudattan, R. and Walker, H.L. (eds) *Biological Control of Weeds with Plant Pathogens.* John Wiley & Sons, New York, pp. 139–156.

Ciotola, M., Watson, A.K. and Hallett, S.G. (1995) Discovery of an isolate of *Fusarium oxysporum* with potential to control *Striga hermonthica* in Africa. *Weed Research* 35, 303–309.

Connick, W.J., Boyette, C.D. and McAlpine, J.R. (1991) Formulation of mycoherbicides using a pasta-like process. *Biological Control* 1, 99–100.

Cronk, Q.C.B. and Fuller, J.L. (1995) *Invasive Plants: the Threat to Natural Ecosystems Worldwide*. Chapman & Hall, London, 241 pp.

Cullen, J.M. and Delfosse, E.S. (1985) *Echium plantagineum*: catalyst for conflict and change in Australia. In: Delfosse, E.S. (ed.) *Proceedings of the Sixth International Symposium on Biological Control of Weeds.* Agriculture Canada, Ottawa, Canada, pp. 249–252.

Cullen, J.M. and Hasan, S. (1988) Pathogens for control of weeds. *Philosophical Transactions of the Royal Society of London* 318, 213–224.

Davis, C.J., Yoshioka, E.R. and Kageler, D. (1992) Biological control of lantana, prickly pear and Hamakoa pamakani: a review and update. In: Stone, C.P., Smith, C.W. and Tunison, J.T. (eds) *Alien Plant Invasions in Native Ecosystems of Hawaii.* University of Hawaii, Honolulu, Hawaii, pp. 411–431.

Ellison, C.A. and Evans, H.C. (1995) Present status of the biological control programme for the graminaceous weed *Rottboellia cochinchinensis*. In: Delfosse, E.S. and Scott, R.R. (eds) *Proceedings of the Eighth International Symposium on Biological Control of Weeds*. DSIR/CSIRO, Melbourne, Australia, pp. 493–500.

Evans, H.C. (1987) Fungal pathogens of some subtropical and tropical weeds and the possibilities for biological control. *Biocontrol News and Information* 8, 7–30.

Evans, H.C. (1991) Biological control of tropical grassy weeds. In: Baker, F.W.G. and Terry, P.J. (eds) *Tropical Grassy Weeds.* CAB International, Wallingford, UK, pp. 52–72.

Evans, H.C. (1993) Studies on the rust, *Maravalia cryptostegiae*, a potential biological control agent of rubber-vine weed, *Cryptostegia grandiflora* (Asclepiadaceae: Periplocoideae), in Australia. I: Life-cycle. *Mycopathologia* 124, 164–174.

Evans, H.C. (1995a) Fungi as biocontrol agents of weeds: a tropical perspective. *Canadian Journal of Botany* 73, 558–564.

Evans, H.C. (1995b) Pathogen–weed relationships: the practice and problems of host range screening. In: Delfosse, E.S. and Scott, R.R. (eds) *Proceedings of the Eighth International Symposium on Biological Control of Weeds.* DSIR/CSIRO, Melbourne, Australia, pp. 539–551.

Evans, H.C. (1997) *Parthenium hysterophorus*: a review of its weed status and the possibilities for biological control. *Biocontrol News and Information* 18, 89–98.

Evans, H.C. (1998) The safe use of fungi for biological control of weeds. *Phytoprotection* 79 (suppl.), 67–74.

Evans, H.C. (2000) Evaluating plant pathogens for biological control: an alternative view of pest risk assessment. *Australasian Plant Pathology* 29, 1–14.

Evans, H.C. and Ellison C.A. (1990) Classical biological control of weeds with micro-organisms: past, present, prospects. *Aspects of Applied Biology* 24, 39–49.

Evans, H.C. and Tomley, A.J. (1994) Studies on the rust, *Maravalia cryptostegiae*, a potential biological control agent of rubber-vine weed, *Cryptostegia grandiflora* (Asclepiadaceae: Periplocoideae), in Australia. III: Host range. *Mycopathologia* 26, 93–108.

Evans, H.C., Carrion, G. and Ruiz-Belin, F. (1995) Mycobiota of the giant sensitive plant, *Mimosa pigra sensu lato* in the Neotropics. *Mycological Research* 99, 420-428.

FAO (1996) *International Standards for Phytosanitary Measures.* I. *Import Regulations.* Secretariat of the International Plant Protection Convention, Rome, 21 pp.

Field, R.P. and Bruzzese, E. (1985) Biological control of blackberries: resolving a conflict in Australia. In: Delfosse, E.S. (ed.) *Proceedings of the Sixth International Symposium on Biological Control of Weeds.* Agriculture Canada, Ottawa, Canada, pp. 341–349.

Forno, W., Seier, M., Chakraborty, S., Weinert, M. and Hennecke, B. (1996) Release of the fungus, *Sphaerulina mimosae-pigrae* (*Phloeospora mimosae-pigrae*), in Australia for biological control of giant sensitive plant, *Mimosa pigra*. In: Moran, V.C. and Hoffmann, J.H. (eds) *Proceedings of the Ninth International Symposium on Biological Control of Weeds*. University of Cape Town, Cape Town, South Africa, p. 334.

Fravel, D.R., Marois, J.J., Lumsden, R.D. and Connick, W.J. (1985) Encapsulation of potential mycoherbicides in alginate. *Phytopathology* 75, 774–777.

Freeman, T.E. and Charudattan, R. (1985) Conflicts in the use of plant pathogens as biocontrol agents for weeds. In: Delfosse, E.S. (ed.) *Proceedings of the Sixth International Symposium on Biological Control of Weeds*. Agriculture Canada, Ottawa, Canada, pp. 351–357.

Funasaki, G.Y., Lai, P., Nakahara, L.M., Beardsley, J.W. and Ota, A.K. (1989) A review of biological control introductions in Hawaii: 1890–1985. *Proceedings of the Hawaiian Entomological Society* 28, 105–160.

Gao, Z.Y. and Gan, J.E. (1992) Biological control of dodder – a review on research progress of the bioherbicide 'Lubao No. 1'. *Chinese Journal of Biological Control* 8, 173–175.

Gohbara, M. and Yamaguchi, K. (1993) *Biological Agents for the Control of Paddy Weeds.* Extension Bulletin No. 379, Food and Fertilizer Technology Centre, Taipei City, Taiwan.

Grant, N.T., Prusinkiewicz, E., Mortensen, K. and Makowski, R.M.D. (1990) Herbicide interactions with *Colletotrichum gloeosporioides* f.sp. *malvae*, a bioherbicide for round-leaved mallow (*Malva pusilla*) control. *Weed Technology* 4, 716–723.

Greaves, M.P., Holloway, P.J. and Auld, B.A. (1998) Formulation of microbial herbicides. In: Burges, H.D. (ed.) *Formulation of Microbial Biopesticides, Beneficial Microorganisms, Nematodes and Seed Treatments*. Kluwer Academic Publishers, London, pp. 203–233.

Gressel, J., Amsellem, Z., Warshawski, A., Kampel, V. and Michaeli, D. (1996) Biocontrol of weeds: overcoming evolution for efficacy. *Journal of Environmental Science and Health* 31, 399–405.

Harris, P. (1985) Biocontrol of weeds: bureaucrats, botanists, beekeepers and other bottlenecks. In: Delfosse, E.S. (ed.) *Proceedings of the Sixth International Symposium on Biological Control of Weeds.* Agriculture Canada, Ottawa, Canada, pp. 3–13.

Hasan, S. (1980) Plant pathogens and biological control of weeds. *Review of Plant Pathology* 59, 349–356.

Hasan, S. and Ayres, P.E. (1990) The control of weeds through fungi: principles and prospects. *New Phytologist* 115, 201–222.

Heywood, V.H. (1989) Patterns, extents and modes of invasions by terrestrial plants. In: Drake, J.A., Moore, H.A., di Castri, F., Groves, R.H., Kruger, F.J., Rejmanek, M. and Wiliamson, M. (eds) *Biological Invasions: a Global Perspective.* John Wiley & Sons, Chichester, pp. 31–60.

Hoagland, R.E. (1996) Chemical interactions with bioherbicides to improve efficacy. *Weed Technology* 10, 651–674.

Holm, L.G., Plucknett, D.L., Pancho, J.V. and Herberger, J.P. (1977) *The World's Worst Weeds: Distribution and Biology.* University Press of Hawaii, Honolulu, Hawaii, 609 pp.

Holt, R.D. and Hochberg, M.E. (1997) When is biological control evolutionarily stable (or is it)? *Ecology* 78, 1673–1683.

Jackson, M.A., Shasa, B.S. and Schisler, D.A. (1996) Formulation of *Colletotrichum truncatum* microsclerotia for improved biocontrol of the weed hemp sesbania (*Sesbania exaltata*). *Biological Control* 7, 107–113.

de Jong, M.D., Scheepens, P.C. and Zadoks, J.C. (1990) Risk analysis for biological control: a Dutch case study in biocontrol of *Prunus serotina* by the fungus *Chondrostereum purpureum. Plant Disease* 74, 189–194.

de Jong, M.D., Wagenmakers, P.S. and Goudriaan, J. (1991) Modelling the escape of *Chondrostereum purpureum* spores from a larch forest with biological control of *Prunus serotina. Netherlands Journal of Plant Pathology* 97, 55–61.

Julien, M.H. and Griffiths, M.W. (eds) (1998) *Biological Control of Weeds: a World Catalogue of Agents and their Target Weeds,* 4th edn. CAB International, Wallingford, UK, 223 pp.

Kingsolver, C.H., Melching, J.S. and Bromfield, K.R. (1983) The threat of exotic plant pathogens to agriculture in the United States. *Plant Disease* 67, 595–600.

Klingman, D.L. and Coulson, J.R. (1982) Guidelines for introducing foreign organisms into the US for biological control of weeds. *Plant Disease* 66, 1205–1209.

Large, E.C. (1940) *The Advance of the Fungi.* Jonathan Cape, London, UK, 488 pp.

LeBaron, H.M. and Gressel, J. (eds) (1982) *Herbicide Resistance in Plants.* Wiley Interscience, New York.

Luo, Y. and TeBeest, D.O. (1998) Behavior of a wild-type and two mutant strains of *Colletotrichum gloeosporioides* f.sp. *aeschynomeme* on northern jointvetch in the field. *Plant Disease* 82, 374–379.

Mack, R.N., Simberloff, D., Lonsdale, W.M., Evans, H.C., Clout, M. and Bazzaz, F. (2000) Biotic invasions: causes, epidemiology, global consequences and control. *Issues in Ecology* 5, 1–20.

Marks, G.C., Pascoe, I.G. and Bruzzese, E. (1984) First record of *Phragmidium violaceum* on blackberry in Victoria. *Australasian Plant Pathology* 13, 12–13.

McFadyen, R.E.C. (1998) Biological control of weeds. *Annual Review of Entomology* 43, 369–393.

McFadyen, R.E. and Harvey, G.J. (1990) Distribution and control of rubber-vine, *Cryptostegia grandiflora*, a major weed in northern Queensland. *Plant Protection Quarterly* 5, 152–155.

McRae, C.F. (1988) Classical and inundative approaches to biological weed control compared. *Plant Protection Quarterly* 3, 124–127.

Moran, V.C., Hoffmann, J.H., Donnelly, D., Zimmermann, H.E. and van Wilgen, B. (1999) Biological control of alien invasive pine trees (*Pinus* species) in South Africa. In: Spencer, N. and Nowierski, R. (eds) *Abstracts of the Tenth International Symposium on Biological Control of Weeds.* University of Montana, Bozeman, p. 59.

Morin, L., Watson, A.K. and Reeleder, R.D. (1989) Production of conidia by *Phomopsis convolvulus. Canadian Journal of Microbiology* 36, 86–91.

Morris, M.J. (1989) A method for controlling *Hakea sericea* Schrad. seedlings using the fungus *Colletotrichum gloeosporioides* (Penz) Sacc. *Weed Research* 29, 449–454.

Morris, M.J. (1991) The use of plant pathogens for biological weed control in South Africa. *Agriculture, Ecosystems and Environment* 37, 239–255.

Morris, M.J. (1997) *Acacia saligna* in South Africa. *Biological Control* 10, 75–82.

Morris, M.J. (1999) The contribution of the gall-forming rust fungus *Uromycladium tepperianum* (Sacc.) McAlp. to the biological control of *Acacia saligna* (Labill.) Wendl. (Fabaceae) in South Africa. In: Okkers, T. and Hill, M.P. (eds) *Biocontrol of Weeds in South Africa* (1990-1998). African Entomology Memoir 1, Entomological Society of Southern Africa, Johannesburg, pp. 125–128.

Morris, M.J., Wood, A.R. and Breeyenden, A.D. (1998) Development and registration of a fungal inoculant to prevent re-growth of cut wattle tree stumps in South Africa. In: Burge, M.N. (ed.) *Abstracts of the Fourth International Bioherbicides Workshop.* University of Strathclyde, Glasgow, UK, p. 15.

Mortensen, K. (1988) The potential of an endemic fungus, *Colletotrichum gloeosporioides*, for biological control of round leaved mallow (*Malva pusilla*) and velvetleaf (*Abutilon theophrasti*). *Weed Science* 36, 473–478.

Mortensen, K. (1997) Biological control of weeds using microorganisms. In: Boland, G.J. and Kuykendall, L.D. (eds) *Plant–Microbe Interactions and Biological Control.* Marcel Dekker, New York, pp. 223–248.

Parker, C. (1972) The *Mikania* problem. *PANS* 18, 312–315.

Potyka, I. (1996) Emulsion formulation of microbial herbicides, PhD thesis, University of Bristol, UK.

Prasad, R. (1994) Influence of several pesticides and adjuvants on *Chondrostereum purpureum* – a bioherbicide agent for control of forest weeds. *Weed Technology* 8, 445–449.

Quimby, P.C., Fulgham, F.E., Boyette, C.D. and Connick, W.J. (1988) An invert emulsion replaces dew in biocontrol of sicklepod – a preliminary study. In: Hovde, D.A. and Beestman, G.B. (eds) *Pesticide Formulations and Application Systems,* Vol. 8. American Society for Testing and Materials, Philadelphia, Pennsylvania, pp. 264–270.

Quimby, P.C., Birdsall, J.L. and Caesar, A.J. (1994) Oil and absorbent coated granules containing encapsulated living organisms for controlling agricultural pests. US Patent No. 5 358 863.

Ravensburg, W.J. (1998) *Biochon® Effective Biological and Environmentally Friendly Product.* Koppert Biological Systems, The Hague, the Netherlands, 2 pp.

Reeder, R.H. and Ellison, C.A. (1999) Estado actual de la investigacion en control biologico clasico de la *Rottboellia cochinchinensis* con el carbon *Sporisorium ophiuri*: potencial y riesgos. In: Sanchez, V. (ed.) *Control Biológico de* Rottboellia cochinchinensis. Informe Técnica No. 308, CATIE, Turrialba, Costa Rica, pp. 101–135.

Say, M.M. (1990) The role of free water in the spread of *Colletotrichum orbiculare. Plant Protection Quarterly* 5, 171–172.

Scheepens, P.C. (1987) Joint action of *Cochliobolus lunatus* and atrazine on *Echinochloa crus-galli* (L.) Beauv. *Weed Research* 27, 43–47.

Scheepens, P.C. (1990) Control of *Prunus serotina* in forests with the endemic fungus *Chondrostereum purpureum.* In: Delfosse, E.S. (ed.) *Proceedings of the Seventh International Symposium on Biological Control of Weeds.* CSIRO, Melbourne, Australia, pp. 545–551.

Schisler, D.A., Jackson, M.A., McGuire, M.R. and Bothast, R.J. (1996) The use of pregelantinised starch and casamino acids to improve the efficacy of *Colletotrichum truncatum* conidia produced in different nutritional environments. In: Delfosse E.S. and Scott, R.R. (eds) *Proceedings of the Eighth International Symposium on Biological Control of Weeds.* DSIR/CSIRO, Melbourne, Australia, pp. 659–664.

Shamoun, S.F. and Hintz, W.E. (1998) Development of *Chondrostereum purpureum* as a biological control agent for red alder in utility rights-of-way. In: Wagner R.G. and Thompson, D.G. (eds) *Third International Conference on Forest Vegetation Management*. Ontario Ministry of Natural Resources, Toronto, Ontario, Canada, pp. 308–310.

Sharon, A., Amsellem, Z. and Gressel, J. (1992) Glyphosate suppression of an elicited defense response; increased susceptibility of *Cassia obtusifolia* to a mycoherbicide. *Plant Physiology* 98, 654–659.

Smith, M.C., Reeder, R.H. and Thomas, M. (1997) A model of the biological control of *Rottboellia cochinchinensis* with head smut *Sporisorium ophiuri. Journal of Applied Ecology* 34, 388–398.

Stowell, L.J. (1991) Submerged fermentation of biological herbicides. In: TeBeest, D.O. (ed.) *Microbial Control of Weeds*. Chapman & Hall, New York, pp. 225–261.

Swanton, C.J., Harker, K.N. and Anderson, R.L. (1993) Crop loss due to weeds in Canada. *Weed Technology* 7, 537–542.

Tatchell, G.M. (1996) Supporting the introduction of exotic biological control agents for UK horticulture. In: *Biological Control Introductions – Opportunities for Improved Crop Production.* BCPC Symposium Proceedings No. 67, BCPC, Farnham, UK, pp. 101–111.

TeBeest, D.O. (1993) Biological control of weeds with fungal plant pathogens. In: Jones, D.G. (ed.) *Exploitation of Microorganisms.* Chapman & Hall, London, pp. 1–17.

TeBeest, D.O., Yang, X.B. and Cisar, C.R. (1992) The status of biological control of weeds with fungal pathogens. *Annual Review of Phytopathology* 30, 637–657.

Templeton, G.E. (1992) Use of *Colletotrichum* strains as mycoherbicides. In: Bailey, J.A. and Jaeger, M.J. (eds) Colletotrichum: *Biology, Pathology, and Control.* CAB International, Wallingford, UK, pp. 358–380.

Tisdell, C.A. (1990) Economic impact of biological control of weeds and insects. In: Mackauer, M., Ehler, L.E. and Roland, J. (eds) *Critical Issues in Biological Control*. Intercept Press, Andover, Hants, UK, 301–316.

Tomley, A.J. and Evans, H.C. (1995) Some problem weeds in tropical and subtropical Australia and prospects for biological control using fungal pathogens. In: Delfosse, E.S. and Scott, R.R. (eds) *Proceedings of the Eighth International Symposium on Biological Control of Weeds.* DSIR/CSIRO, Melbourne, Australia, pp. 477–482.

Tomley, A.J. and Hardwick, G. (1996) Bulking up, field distribution and establishment of rubber vine rust, *Maravalia cryptostegiae* in far north Queensland. In: Shepherd, R.C.H. (ed.) *Proceedings of the Eleventh Australian Weed Conference*. Australian Weed Society, Melbourne, Australia, pp. 661–671.

Trujillo, E.E. (1985) Biological control of Hamakua pa-makani with *Cercosporella* sp. in Hawaii. In: Delfosse, E.S. (ed.) *Proceedings of the Sixth International Symposium on Biological Control of Weeds.* Agriculture Canada, Ottawa, Canada, pp. 661–671.

Walker, H.L. (1981) Granular formulation of *Alternaria macrospora* for the control of spurred anoda (*Anoda cristata*). *Weed Science* 29, 342–345.

Walker, H.L. and Connick, W.J. (1983) Sodium alginate for the production and formulation of mycoherbicides. *Weed Science* 31, 333–338.

Wapshere, A.J. (1975) A protocol for programmes for biological control of weeds. *PANS* 21, 295–303.

Wapshere, A.J. (1989) A testing sequence for reducing rejection of potential biological control agents for weeds. *Annals of Applied Biology* 114, 515–526.

Wapshere, A.J., Delfosse, E.S. and Cullen, J.M. (1989) Recent advances in biological control of weeds. *Crop Protection* 8, 227–250.

Waterhouse, D.F. (1994) *Biological Control of Weeds: Southeast Asian Prospects.* ACIAR, Canberra, Australia, 302 pp.

Watson, A.K. (1991) The classical approach with plant pathogens. In: TeBeest, D.O. (ed.) *Microbial Control of Weeds.* Chapman & Hall, New York, pp. 3–23.

Watson, A.K., Mabbayad, M.O., Zhang, W., Masangkay-Watson, R.F., De Luna Couture, L.Z., Yandoc, C.B. Paulitz, T.C. and Mortimer, A.M. (1997) Progress of a biological weed project in rice-based cropping systems. In: Rajan, A. (ed.) *Proceedings of the Sixteenth Asian-Pacific Weed Science Society Conference.* Malaysian Plant Protection Society, Kuala Lumpur, pp. 324–344.

Weidemann, G.J. (1991) Host-range testing: safety and science. In: TeBeest, D.O. (ed.) *Microbial Control of Weeds.* Chapman & Hall, New York, pp. 83–96.

Weidemann, G.J. and TeBeest, D.O. (1990) Biology of host range testing for biocontrol of weeds. *Weed Technology* 4, 465–470.

Woodburn, A.T. (1995) The market for agrochemicals present and future. In: *Proceedings of the Brighton Crop Protection Conference – Weeds*. British Crop Protection Council, Farnham, UK, pp. 121–128.

Wymore, L.A. and Watson, A.K. (1989) Interaction between a velvetleaf isolate of *Colletotrichum coccodes* and thiadiazuron for velvetleaf (*Abutilon theophrasti*) control in the field. *Weed Science* 37, 478–483.

Wymore, L.A., Watson, A.K. and Gottlieb, A.R. (1987) Interaction between *Colletotrichum coccodes* and thiadiazuron for velvetleaf (*Abutilon theophrasti*). *Weed Science* 35, 377–383.

Zhang, W.M. and Watson, A.K. (1997) Characterization of growth and conidia production of *Exerohilum monoceras* on different substrates. *Biocontrol Science and Technology* 7, 75–86.

7 Monitoring the Fate of Biocontrol Fungi

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Introduction

The release of any endemic, exotic or genetically modified microbe into the ecosystem generates some apprehension in the general public and the scientific community. This apprehension revolves around the conceivable negative effects on human health and ecosystem stability. We are caught in a dilemma where, on the one hand, we desire to manipulate certain parts of our natural or agricultural ecosystem through these microbial releases and, on the other, we wish to maintain ecosystem stability and to reduce risks to human health.

Microorganisms have been released regularly into the environment over the last 15 years and this has resulted in some successes. This includes the inoculation of *Rhizobium* spp. into the soil for enhancement of nitrogen fixation, the application of several *Pseudomonas* species for bioremediation and the inoculation of fungi for biological control of agricultural pests and noxious weeds (Julien, 1987). In 1987, the first environmental introduction of a lacZY-engineered strain of *Pseudomonas aureofaciens* (L11) was approved by the US Environmental Protection Agency (Kluepfel *et al.*, 1991). Although it may appear paradoxical, the production, release and study of a genetically modified organism may yet provide the best insight into aspects of microbial ecology since the modified strain can be tracked in the environment. L11 was tracked over three successive crop rotations and provided some valuable information on the population dynamics (i.e. persistence and fate) of bacteria in the rhizosphere (Kluepfel, 1993).

The release of biocontrol fungi is sometimes perceived as a threat to human welfare and environmental stability. The North American Microbial Biocontrol Working Group considered several possible environmental and human health effects associated with the release of microorganisms for pest control. These were: (i) the competitive displacement of non-target organisms; (ii) human allergens; (iii) toxicity to non-target organisms; and (iv) pathogenicity to non-target organisms (Cook *et al.*, 1996). Risks associated with the release of fungal biocontrol agents are discussed in more detail in Goettel and Jaronski (1997). In order to alleviate public and scientific concerns with fungal introduction, it is evident that the risk evaluation agency or individuals

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releasing a biocontrol agent must also have the means to track and identify it in the environment. To date, there is little information available on the fate of a single fungal clone released into the environment nor is there any information available on the transfer of genes from released fungi to other individuals in the population or to other species.

Biocontrol Fungi and the 'New' Green Revolution

There are several features of biocontrol fungi which garner public support. First and foremost, they may potentially displace or reduce the application of chemical products, many of which are toxic to humans and the environment. Biocontrol products currently hold less than 2% of the global pesticide market but this was not always the case. Biocontrol agents were used before chemical pesticides were developed. For example, the first recorded use of an insect-pathogenic fungus was the application of *Metarhizium anisopliae* to control the wheat cockchafer, *Anisoplia austriaca*, in Ukraine by Metschnikoff in 1879 (Ferron, 1978). *M. anisopliae* was also used to control the sugar-cane froghopper, *Aeneolamia saccharina*, in Trinidad at the turn of the century (Rorer, 1913). *Erynia radicans* was employed against aphids in apple orchards in Canada in the 1920s (Dustan, 1924).

At the onset of the 1950s, and the 'green revolution', the slogan 'better living through chemistry' shifted the pest control focus from a biocontrol approach to a chemical approach, which was easier, faster-acting and more effective. However, the benefits of chemical pesticide application are typically based only on direct crop returns. They do not include the indirect environmental, economic and human health costs associated with pesticides (Pimentel *et al.*, 1992). The application of a variety of chemicals over the last 40 years has left the agriculture and forestry industries with pesticide resistance in pests, environmental toxicity, fishery losses, groundwater and surface-water contamination, depletion of rhizosphere microorganisms and human health concerns.

In the 1980s the effort to return to biological control was met with some successes. Many examples are found for fungal biocontrol agents. A classical biocontrol approach was used in the application of *Zoophthora radicans* to control spotted alfalfa aphid in Australia. The fungus quickly spread from the point of introduction and was successful in control of the aphid (Milner *et al.*, 1982). In Europe, success in controlling the cockchafer, *Melolontha melolontha*, using *Beauveria brongniartii* was achieved (Keller, 1989). In Australia, *M. anisopliae* has been formulated to control a major scarab pest, *Aphodius tasmanae*, a pest of pasture grasses (Roberts and Hajek, 1992). *M. anisopliae* has also been used to control sugar-cane pests in Brazil for more than 15 years (Moscardi, 1989). In China, *Beauveria bassiana* has been applied on a large scale to control pine-moth larvae (Xu, 1988). Success has also been achieved with plant biocontrol fungi. Massion and Lindow (1986) have shown control of Canada thistle with *Puccinia obtegens*, yellow nutsedge with *Puccinia canaliculata* and Johnson grass with *Sphacelotheca holci*. Daniel *et al.* (1973) successfully controlled northern joint-vetch, *Aeschynomene virginia*, in a rice crop with *Colletotrichum gloeosporioides* f. sp. *aeschynomene*.

Biocontrol fungi provide an alternative to chemical pesticides but caution should be exercised in their release; do we really understand fungal epidemiology?

The application of biocontrol fungi has also met with many failures, though these are not widely reported in the scientific literature. As an example, much research had been done in England, Canada and Australia on the mycoparasite, *Coniothymium minitans* for biological control of the plant–pathogenic *Sclerotinia* species but each research group discontinued the project, based primarily on the poor performance of *C. minitans* in the field (Adams, 1990). Large quantities of the fungal propagules were required when applied directly to the soil, where the mycoparasites were met by microbial antagonists.

Measuring Fungal Biocontrol Success

One of the major setbacks with biocontrol has been its inconsistent record (compared with the relative consistency of chemical control). It has been almost impossible to identify reasons for the failures or, for that matter, the successes of fungal biological control introductions. This is partially due to an inadequate understanding of the epidemiology and ecology of these fungi in the field. Improving our understanding of disease epizootiology is critical to understanding the successes or failures of fungal biocontrol agents and also to the understanding of fungal microbial ecology. As an integral part of the measurement of success or failure of a biocontrol fungus, one has to ask the following questions. What was the fate of the released fungus? Was it really the released fungus that caused the desired effect? The answers can only be ascertained with an adequate understanding of the genetic structure of the endemic fungal population and a method of identifying and monitoring the persistence and fate of the introduced fungal clone.

> *The ability to monitor a biocontrol fungal release has three major uses; the first is the identification of the introduced agent in a biological impact assessment; secondly, it allows for a rational economic cost/benefit analysis of the fungal release; and, thirdly, it provides valuable information about fungal epidemiology.*

The following review consists of three major sections. First, I shall introduce the relevance of the need to identify the introduced fungal biocontrol agents as part of an environmental risk assessment. Secondly, I shall cursorily review molecular and non-molecular methods available for identifying the genetic variability in biocontrol fungi. Finally, I shall present a case-study of the release of an exotic fungal pathogen of insects into the prairie ecosystem.

Risk Assessment in Biocontrol Releases

Biological impact assessment is a process designed to evaluate the possible risks of releasing specific endemic or exotic organisms. The application or augmentation of biocontrol fungi is designed to have a specific effect on the ecosystem. It may even be argued that the release of a biocontrol fungus is meant to engender change in the targeted system. The risk evaluation asks whether the researcher can predictably meet the demands of agriculture and at the same time preserve the ecological integrity of the impact area in a sustainable manner.

Risk assessments have been conducted with a variety of fungal biocontrol agents of weeds. In 1975 the first permit for field testing of the fungal plant pathogen,

C. gloeosporioides (now registered as Collego), was issued as a biocontrol agent for weed control. This field release was issued only after a 6-year test period in a containment which showed that the field release would be safe (Templeton, 1975). Toxicity to mammals and survival of the fungus in the field were studied. The fate and survival of the fungus in non-target aquatic organisms have also been evaluated (Genthner *et al.*, 1993).

Public perception and scientific calculation of the potential impact of biocontrol releases motivate political conviction to fund assessment impact programmes.

An integral part of a biological impact assessment is that some type of marker be employed to monitor and detect the microorganism introduced into the environment. This microorganism should be monitored in the environment against a background of the microbial community into which it is introduced. In particular, it should be possible to distinguish the introduced microorganism from those related to it in the microbial community.

Methods of Identifying the Genetic Individual

The term genetic individual is used in plant biology (Harper, 1977) and more recently in fungi (Rayner, 1991) and is sometimes referred to as the genet. The genet includes all clonally reproducing individuals of a mitotic cell lineage that originates from an individual. Risk assessment studies require that the genet be tracked in the field against the background fungal population. Tracking the genet would also provide an idea of the efficacy of the biocontrol agent, patterns of cycling of the biocontrol agent in the environment and dispersal and persistence in the soil. Simply releasing a fungus into the environment and evaluating the desired impact lack the precision of identifying the genet in an epizootiological investigation.

Most of the fungal biocontrol releases to date compare the effects of a fungal release on the host insect or plant population at the release site with that of a control site. This is fraught with problems since the same species of biocontrol fungi may be found naturally at both the release and the control sites and it may be impossible to distinguish the resident population from the introduced genet. It would also be impossible to determine the synergistic effects of the resident fungal population with that of the introduced genet. For example, the insect-pathogenic fungi *M. anisopliae* or *B. bassiana*, or the plant-pathogenic fungi *Colletotrichum* spp. are ubiquitous (Sutton, 1992; Bidochka *et al.*, 1998). There must be some means by which the particular genet that has been chosen for the application can be positively identified and distinguished from the background population in the field.

After the release of a biocontrol fungus, the causal relationship between a specific biocontrol fungus and a specific disease will require genetic characterization of the endemic population and comparison with the introduced genet.

In the past, fungal taxonomists have relied on phenotypic markers, such as fungal and conidial colony characteristics, vegetative compatibility and mating type, to differentiate individuals. These markers may be useful in differentiating species or certain groups within species, but they generally lack the resolution to distinguish a genet within a population. Deficiencies in traditional microbial detection techniques have led to research into new methodologies and, in particular, molecular techniques. These

techniques have the sensitivity and selectivity required to track fungal genets released into the environment. Lessons in monitoring biocontrol fungi can probably best be learned from the vast number of techniques employed to track bacteria released into the environment (Prosser, 1994). The techniques fall into four major groups: morphological methods, immunological methods, nucleic acid probing and the introduction of molecular markers, or 'tagging'.

The major problem with utilizing DNA-based techniques for identifying the fungal genet is that they provide information on the presence and perhaps total cell concentrations but they do not provide information on the viability or activity of the introduced organisms. The introduction of molecular markers is designed to confer a distinctive genotype/phenotype on the transformed fungus, which enables tracking in the environment. The advantage is that this method provides information on the viability and activity of the introduced organism, but it also has its drawbacks. Special care must be taken to avoid introduction of the gene marker (tag) within any genomic DNA region that may cause genetic instability, impair fitness or affect, in any way, expression of metabolic regulatory genes. The expression of the marker system could impair the fitness of the organism if an undue metabolic burden is imposed by the expression and maintenance of the marker gene.

Several excellent reviews are available on methods of determining genetic variability in fungi, such as those by Bruns *et al.* (1991), Burdon (1993), Leslie (1993), McDermott and McDonald (1993) and Rosewich and McDonald (1994). There are many fungi that are being commercially exploited, as outlined in Table 7.1, and there will be a growing necessity to monitor these releases. Due to the large number of examples of biocontrol fungi, the following section will focus on methods used to identify genets in a plant biocontrol fungus, *C. gloeosporioides* (see also Manners and He, 1997), and several species of deuteromycetous (*M. anisopliae* and *B, bassiana*) and zygomycetous insect-pathogenic fungi. However, many of these techniques can be applied to just about any fungi mentioned in Table 7.1. The suitable utility (and associated problems) of applying techniques for monitoring in epizootiological studies is outlined in Table 7.2.

Morphological and biochemical markers and vegetative compatibility groups (VCGs)

Morphological markers are restricted to those pathogens that are easily cultured on artificial media. Many morphological and antibiotic-resistance variants have been identified in biocontrol fungi. These include colony variants in mycelial or conidial colour, growth rate, colony surface texture, topology and viscosity and resistance to antibiotics. Morphological variants and antibiotic resistance may occur as spontaneous mutants or may be generated through mutagenesis. For example, acriflavine-resistant mutants occur spontaneously in *M. anisopliae* at a mutation rate as high as 1×10^{-9} (Tinline and Noviello, 1971). The major problem associated with the use of morphological or antibiotic-resistance mutants is that they are unstable and may revert back to a wild type. Colony morphology also varies considerably in many fungal species and this morphology may not be stable upon subculturing since many fungi exhibit pleiomorphic deterioration of colony characteristics. Reasons for phenotypic instability in fungi include changeable chromosomal complements, transposons, cytoplasmically transmitted genetic elements (e.g. cytoplasmic RNAs and invertrons) and DNA

Table 7.1. A selection of fungal species and how they are being commercially exploited.

Fungal species	Biological activity	Application
Ampelomyces quisqualis	Biological fungicide	Germinating spores suppress the development of powdery mildews on a variety of crops (e.g cucumbers, apples, grapes, ornamentals, strawberries and tomatoes) by hyperparasitism
Beauveria bassiana	Biological insecticide	Invades insect body and kills the host insect. Target pests include European corn-borer, Asiatic corn-borer, whiteflies, thrips, aphids and mealy bugs. Target crops include maize, vegetables and ornamentals
Beauveria brongniartii	Biological insecticide	Target pests include white grubs and cockchafers infesting sugar cane and barley
Candida oleophila	Biological fungicide	Prevents postharvest diseases in citrus fruits by producing secondary metabolites inhibitory to storage-disease fungi
Chondostereum purpureum	Biological herbicide	A wood-rot fungus that invades cut stumps on target trees such as American black cherry, yellow birch and poplar
Colletotrichum gloeosporioides f. sp. *aechynomene*	Biological herbicide	Plant pathogen with specificity to the weed, northern joint-vetch
Coniothyrium minitans	Biological fungicide	A non-pathogenic fungus that grows on leaf surfaces and prevents the invasion of plant pathogens such as *Sclerotinia*
Endothia parasitica	Biological fungicide	A non-pathogenic strain of this fungus that grows on potential infection sites on trees and prevents the invasion of pathogens
Fusarium oxysporum	Biological fungicide	A non-pathogenic strain of this fungus that protects crops from pathogenic strains of *Fusarium* by competing for sites at the root-infection sites
Gliocladium catenulatum	Biological fungicide	Produces secondary metabolites inhibitory to some plant pathogens, such as *Pythium* spp. and *Rhizoctonia* spp.
Metarhizium anisopliae	Biological insecticide	Insect pathogen used on a variety of crops, including greenhouse vegetables and ornamentals
Myrothecium verrucaria	Biological nematocide	Fungal mycelia invade plant-parasitic nematodes present in the soil
Paecilomyces fumosoroseus	Biological insecticide	For the control of whitefly, aphids, thrips and spider mites
Phlebiopsis gigantea	Biological fungicide	Competes for entry sites and prevents establishment of the rot fungus, *Heterobasidum annosum*, on pine and spruce stumps
Phytophthora palmivora	Biological herbicide	A specific pathogen of the roots of the strangler vine or milkweed vine

Table 7.1. continued

Fungal species	Biological activity	Application
Pythium oligandrum	Biological fungicide	Outcompetes pathogenic soil fungi in greenhouse crops, outdoor vegetables and cereal crops
Trichoderma harzianum	Biological fungicide	Competes for soil nutrients with plant-pathogenic fungi such as *Botrytis* and *Sclerotinia*
Verticillium lecanii	Biological insectide	Used primarily for the control of whitefly and aphids in greenhouses

Table 7.2. Methods used to identify population level variability in fungi and their adequacy or potential for monitoring introduced clones into the ecosystem. The level of natural variation observed in a population is dependent on fungal species and certain demes. The relative qualitative assessment of low to high variation is a general estimate based on studies to date.

	Level of natural variation observed		
Method	Low	High	Adequacy as a marker system
Colony morphology	------>		Unstable, subject to reversion
Derived mutants, e.g. antibiotic resistance, conditional mutants	-->		Unstable, subject to reversion. May have reduced fitness (e.g. a conditional mutant), which may be desirable. Potential for antibiotic resistance transfer into natural populations
Karyotype analysis	------>		Technique is lengthy (up to 3 days for a CHEF)
Allozymes	-------->		Easy to score and identify if introduced clone has unique alleles. Fungus must be easy to culture since a relatively large amount of material is required
dsRNA	---------->		dsRNA isolation can be difficult. Fidelity is suspect. Transmission of dsRNA to other isolates possible
Transposable elements	-------(?)		Identification difficult. Few examples
RAPD	---------->		Technique is relatively easy. Small quantities of DNA required. Fungus does not need to be cultured. Potential problems with reproducibility
RFLP	---------->		Technique involves relatively large quantities of DNA. Appropriate probe required
Directed PCR	---------->		Technique is relatively easy. Small quantities of DNA required. Pathogen does not require culturing. Some difficulty in screening for variability at the population level
DNA probes	----------(?)		Same as directed PCR
VCG	-------->		Vegetative compatability studies in some fungi requires the production of complementation mutants and a large number of comparisons
Introduced molecular markers	NA		Unique in the population. Ease of transformation of many fungi. Stability of markers still questionable. Effects on fungal fitness questionable

CHEF, clamped homogeneous electrical field; dsRNA, double-stranded RNA; NA, not applicable; RAPD, random amplification of polymorphic DNA; RFLP, restriction fragment length polymorphism; PCR, polymerase chain reaction; VCG, vegetative compatability groups.

and RNA polymerases that may act as mitochondrial retroposons (Kistler and Miao, 1992).

Two populations of *C. gloeosporioides* from citrus differed in mycelial colour, growth rate, serine esterase profiles, restriction fragment length polymorphism (RFLP) of ribosomal DNA (rDNA) and Southern hybridization of a cutinase gene (Liyanage *et al.*, 1992, 1993). Variations in colony parameters were observed, such as patterns of concentric conidial production in response to light and darkness, perithecia and conidial production, conidial size and appressorium production (Menezes and Hanlin, 1996). Kuramae-Izioka *et al.* (1997) showed variation in *Colletotrichum* spp. (*Colletotrichum acutatum* vs. *C. gloeosporioides*) based on mycelial growth, benomyl resistance, pathogenicity and random amplification of polymorphic DNA (RAPD) patterns.

Another approach to identifying plants or insects infected by a certain fungus would be to utilize differences in sterol and fatty acid profiles of fungi (Mueller *et al.*, 1994). Although this approach would be a good initial diagnosis of fungal identification, its utility in identifying a fungal genet within a species has yet to be shown.

Conditional lethal mutants (heat-sensitive mutants) have been generated using ultraviolet (UV) mutagenesis in *B. bassiana* (Hegedus and Khachatourians, 1994). Heat-sensitive mutants grew at 20°C but not 30°C. Not only could such mutants be used to monitor pathogenicity development in an infected insect but they could also be used to limit the stability of and/or detect in the environment a conditional lethal strain. The use of such strains could be of some utility in situations, such as inundative applications, where cycling of the pathogen in the ecosystem is undesirable.

VCGs have been frequently used in plant pathology to evaluate fungal population structure. However, many fungi are anamorphic (asexual), precluding any studies of sexual compatibility. One of the methods used to investigate compatibility reactions, occuring either through heterokaryosis or parasexual recombination, is to select chlorate-resistant mutants and then test for complementary nitrate reductase, *nia* and *cnx*, mutants. Chlorate conversion to toxic chlorite is presumably catalysed through one of the nitrate-reducing enzymes. Thus, chlorate resistance may be due to a mutation at one of those loci. Couteaudier and Viaud (1997) analysed VCGs in *B. bassiana* using this method and observed that the VCGs correlated to RFLP patterns using a telomeric probe. VCGs could limit gene flow, resulting in demic subdivisions in the fungal population. Similarly, isolates within each biotype of *C. gloeosporioides* that infects legumes (*Stylosanthes* spp.; biotypes A and B) were vegetatively compatible but it was not possible to form heterokaryons between biotypes (Masel *et al.*, 1996). Selectable markers (hygromycin and phleomycin resistance) carried on vectors containing telomeric sequences from *Fusarium oxysporum* were used to transform isolates of the biotypes (Poplawski *et al.*, 1997). These were then allowed to contact each other, which resulted in a double-antibiotic-resistant progeny, but these were slow-growing.

VCGs may be considered good markers for monitoring biocontrol fungi if the introduced biotype has a distinctive VCG that is incompatible with individuals in the endemic population.

Immunological markers

Immunological markers have been primarily used to distinguish species differences in fungi. Immunological markers and, in particular, enzyme-linked immunosorbent assays (ELISA) have been reported for the detection of many plant-pathogenic fungi (Schotts

et al., 1994). Serological tests have also been used to identify *B. bassiana, M. anisopliae* and species of the Entomophthorales (Fargues *et al.*, 1981; Shimizu and Aizawa, 1988; Guy and Rath, 1990). A potential problem in employing ELISA is the ability of the polyclonal antibody to react with genetically similar species. For example, Hajek *et al.* (1991a) developed an ELISA with polyclonal antibodies to the plasma membrane of *Entomophaga maimaiga*, a pathogen of gypsy-moth larvae, *Lymantria dispar*. However, a closely related fungus, *Entomophaga aulicae*, showed cross-reactivity with the antisera.

Allozymes, karyotypes and molecular markers

Allozymes

Electrophoretic separation of allozymes was one of the first techniques used to screen for genetic variability in fungal populations. Conventionally, what is required is between 8 and 20 allozymes, depending on the degree of polymorphism and the number of alleles at each locus. Evaluation of 120 isolates of *M. anisopliae* showed that they fell into 48 distinctive genotypic classes. A large amount of variability was found for *M. anisopliae*, with less than 20% similarity among some classes (St Leger *et al.*, 1992a). Similar results were found for *B. bassiana* (St Leger *et al.*, 1992b). Allozyme analysis has also been applied in an attempt to distinguish pathotypes of *B. brongniartii*. Isolates showed high intraspecific variation and little association was observed between genotypic class and virulence to European cockchafer (*M. melolontha*) larvae (Reineke and Zebitz, 1996).

If the fungal strain that is being released into the field is distinctive at several loci it is sufficient to use allozyme analysis for identifying the genet. Allozymic variation has been used to monitor an entomophthoralean fungal genet in several studies. For example, Silvie *et al.* (1990) used allozyme analysis to show the fate and survival of a strain of *Pandora neoaphidis* released to control greenhouse aphids. The released strain was eventually displaced by endemic strains. Milner and Mahon (1985) showed that allozyme analysis could be used to distinguish an Israeli isolate of *Z. radicans* from isolates endemic to Australia. The Israeli isolate was released and provided control in populations of spotted alfalfa aphids.

Karyotype analysis

One approach to identifying the genet has been to look at chromosomal length polymorphism (CLP), using pulsed-field gel electrophoresis (PFGE) for identifying fungal karyotypes. The most commonly employed PFGE technique is clamped homogeneous electrical field (CHEF). This technique has been used in several insect-pathogenic and plant-pathogenic fungi and the results show fungal genome sizes between 30 and 45 Mb and the number of chromosomes varies between five and nine. The chromosomes range in size from 0.2 to 10 Mb. Natural populations of fungi have a high degree of CLP (Mills and McCluskey, 1990), particularly the small, 200–600 kb, 'minichromosomes'.

In *C. gloeosporioides*, the minichromosomes were highly variable between isolates. DNA additions or deletions were also associated with the CLP and differences were observed within and between each biotype (Masel *et al.*, 1993). Analysis showed that

some genes found on a 1.2 Mb chromosome were unique and that high-copy-number repeat sequences were not present, suggesting that the genome of the progenitor strain underwent a large-scale deletion or addition from a genetically distinct strain. Interestingly, with a background of relatively high CLP variation within each biotype, there was little variation observed using RFLP analysis (He *et al.*, 1996).

CLPs have been observed in the insect-pathogenic fungus *B. bassiana* (Viaud *et al.*, 1996). Although there was some variability between strains, this variability was not great enough to identify genets. The CLPs observed were also correlated with telomeric, RFLP and RAPD markers. Shimizu *et al.* (1992) identified the electrophoretic karyotype in five isolates of *M. anisopliae*. Chromosomal sizes were between 1.6 and 7.2 Mbp and the total genome size between 29 and 33 Mbp. The five *M. anisopliae* isolates could be readily distinguished from one another.

Although extremely useful for genomic analysis, the drawback to this karyotyping method is that the procedure is lengthy and not necessarily discriminatory among strains. Furthermore, CLP may vary during subculturing fungi in the laboratory (e.g. *Magnaporthe grisea* (Talbot *et al.*, 1993)). It seems unlikely that CLP can be used as a technique by itself to identify the genet. However, when combined with other techniques it may be used to identify and map the positions of specific genes on various chromosomes.

Molecular markers

Base substitutions, such as insertions or deletions, in the genome can be detected with RFLP. What one requires is a probe that will identify size variations in certain parts of the digested genomic DNA. Probes that have been used to detect inter- and intraspecific variation in fungi are rDNA (Pipe *et al.*, 1995), mitochondrial DNA (mtDNA) (Hegedus and Khachatourians, 1993a) and telomeric DNA (Couteaudier and Viaud, 1997).

Cloned DNA probes are portions of the genomic DNA identified through various subtractive or differentiation screening methods that hybridize to a particular strain or species of fungus. Kosir *et al.* (1991) used a cloned DNA probe to differentiate a strain of *B. bassiana* virulent to grasshoppers, *Melanoplus sanguinipes*, from a less virulent strain. Hegedus and Khachatourians (1993b) developed DNA probes that could differentiate *B. bassiana* from several other deuteromycetous entomopathogenic fungi.

An excellent example of the utility of molecular probes in a fungal epidemiological study has been the investigation of an entomophthoralean pathogen of gypsy-moth larvae, *L. dispar*. Gypsy-moth larval populations in the eastern USA were frequently infected by an entomophthoralean fungus. Hajek and co-workers utilized RFLP analysis, allozymes and cloned DNA probes to differentiate two closely related entomophthoralean fungi, *E. aulicae* and *E. maimaiga* (Soper *et al.*, 1983; Walsh *et al.*, 1990). By applying these molecular techniques to infected gypsy-moth larvae collected in the field, it was determined that *E. maimaiga* was the infectious fungus in these epizootics (Hajek *et al.*, 1996a, b, c). This in itself was interesting since *E. maimaiga* and gypsy-moth larvae are both exotic species to North America (Hajek *et al.*, 1995). This study is one of the few long-term epidemiological studies where a biocontrol fungus, *E. maimaiga*, has been identified and tracked using molecular probes (Hajek *et al.,* 1990, 1991a; Walsh *et al.*, 1990). The information gathered also allowed for the augmentative introduction of *E. maimaiga* to control gypsy moth at the leading edge of their expanding range (Hajek *et al.*, 1990). The probes have also been used to track

fungal persistence and dispersal and to evaluate physiological versus ecological host ranges of the fungus (Hajek *et al.*, 1996a).

RAPD utilizes the polymerase chain reaction (PCR) and single primers of between 15 and 20 nucleotides in length in order to detect variability in arbitrary regions of the genome. The technique is powerful due to its relative speed and the large number of loci that can be screened. Problems associated with RAPDs include the appearance and disappearance of minor bands with different runs and variability between thermocyclers from different manufacturers (Ellsworth *et al.*, 1993). However, taking this into consideration, the appearance or absence of the major bands is a quick and easy method to identify a genet. Using RAPD analysis, wide genetic variation in isolates of *M. anisopliae* was observed on a worldwide level (Fegan *et al.*, 1993; Bidochka *et al.*, 1994; Tigano-Milani *et al.*, 1995). An RAPD study of Brazilian isolates of *M. anisopliae* by Fungaro *et al.* (1996) showed that there was large variability of soil isolates (47% similarity) compared with those isolates from a hemipteran insect (*Deois flavopicta*) host (82% similarity).

RAPD markers have been used in several epidemiological studies in entomophthoralean fungi. In 1990 and 1991 seven isolates of *Z. radicans* originating from Serbia were released into a lucerne plot in New York state, USA, to control potato leafhopper, *Empoasca fabae* (Wraight *et al.*, 1986). RAPDs were used to distinguish released isolates of *Z. radicans* from North American isolates and indicated that the Serbian isolates had successfully established at the experimental release sites (Hodge *et al.*, 1995).

Zoophthora phytonomi in North America is a pathogen of the clover-leaf weevil, *Hypera punctata*. In 1973, *Z. phytonomi* was first noted to infect alfalfa weevil, *Hypera postica*, a species introduced from Eurasia (Hajek *et al.*, 1996c). RAPD patterns distinguished two major genotypic classes of *Z. phytonomi*; one genotype was principally isolated from *H. postica*. One explanation is that one of the genotypic classes was introduced from Eurasia, perhaps with the introduced alfalfa weevil, and subsequently spread.

Amplified restriction length polymorphisms (AFLP) have been used to detect genetic variation in plant-pathogenic fungi (Majer *et al.*, 1996). It is considered to be a reliable, reproducible technique that evaluates genomic variation at ten to 100 times more sites than either RAPDs or RFLP.

Targeted PCR refers to the amplification and analysis of particular regions of the genome, either by direct sequencing or RFLP analysis of the amplified region. Neuveglise and Brygoo (1994) showed that 28S rDNA differed in size between two strains of *B. brongniartii*. Curran *et al.* (1994) investigated the internal transcribed spacer (ITS) regions and 5.8S of *Metarhizium* rDNA. Primers for targeted PCR were developed from a specific cloned DNA probe in order to differentiate *B. bassiana* from other entomopathogenic fungi (Hegedus and Khachatourians, 1995). Ribosomal DNA sequences were used to elucidate the relationships within the genus *Colletotrichum* from New Zealand fruit-rotting isolates (Johnston and Jones, 1997) and biotypes A and B infecting legumes, *Stylosanthes* spp., in Australia (Braithwaite *et al.*, 1990). ITS regions of 18 species of *Colletotrichum* were used to gain an understanding of the phylogeny and systematics within the genus (Sreenivasaprasad *et al.*, 1996). These studies show the evolutionary relationships of various strains or may be used in a broad taxonomic application for identifying a fungal species. However, they have not been shown as useful in identifying the genet. One targeted PCR-based approach for identifying genets within insect- and plant-pathogenic fungi would be to fingerprint fungi based on

microsatellite variation (Bridge *et al.*, 1997a). Identifying species-specific microsatellite markers is potentially time-consuming; however, their utility in identifying a fungal genet is effective.

If certain amplified regions are subjected to further analysis, the information may be used to identify the fungal genet. RFLP analysis of the rDNA in *M. anisopliae* showed that some strains could be differentiated according to their geographic origins (Pipe *et al.*, 1995). RFLP analysis of the *Pr1* (extracellular protease-encoding) gene amplified from various isolates of *M. anisopliae* showed a correlation of RFLP profiles with geographic origin (Leal *et al.*, 1997). Because of the spatial distribution related to rDNA or *Pr1* RFLP, these marker systems could be applied to detect an *M. anisopliae* isolate released into another geographic location.

Another approach to identifying genets of *M. anisopliae* was to utilize a variety of biochemical and molecular techniques (Bridge *et al.*, 1997a). Isozyme analysis, RAPD and protease production were used to investigate the relationships between 30 isolates of *M. anisopliae*. Similarly, an approach that utilized biochemical markers and a phylogenetic approach based on ITS nucleotide differences was used to differentiate various species and isolates within the genus *Verticillium* (Bidochka *et al.*, 1999). However, identification of a fungal genet based on several techniques is not as efficient as a single discriminating technique.

In some cases, RFLP analysis is used to discriminate among fungal pathotypes. *B. brongniartii* was analysed by RFLP analysis of ITS regions amplified by PCR (Neuveglise *et al.*, 1994) and allowed separation of the strains into distinctive subgroupings. One of the subgroupings contained strains virulent to the white grub (*Hopochelus marginalis*). Variability in the ITS regions was due to point mutations, which occurred as frequently as 14.7% and 16.7% in the ITS1 and ITS2 regions, respectively. Ribosomal DNA and mtDNA variation in *C. gloeosporioides* showed an association with geographic origin or host (avocado, banana or papaya), except for the isolates from mango (Hodson *et al.*, 1993). RAPD markers showed similar results. A mango biotype of *C. gloeosporioides* was differentiated from eight other isolates from different fruit species (Hayden *et al.*, 1994). Pathogenicity bioassays showed that isolates pathogenic to mango were specifically highly virulent to mango, while the other isolates displayed a wide host range. The isolates from mango had the same rDNA and similar mtDNA RFLP patterns, regardless of geographic origin (eastern or western hemisphere).

A method for rapidly screening a large number of isolates of *B. bassiana* and *Aspergillus niger* has recently been developed, based on direct PCR from colonies treated with Novozym 234, a powder produced from the extracellular enzymes from *Trichoderma harzianum* (van Zeijl *et al.*, 1998). This could allow for rapid screening of a large number of fungal isolates for identification after a biocontrol release.

Double-stranded RNA viruses and transposable elements

Double-stranded RNA (dsRNA) viruses are found in a wide range of fungal species (Dickinson, 1986). There was some anticipation that dsRNAs could act as markers in population studies (Burdon, 1992). However, this seems unlikely since their fidelity as markers is suspect and dsRNA transfer between fungal strains or species is not fully understood in many species of biocontrol fungi. In natural populations of insect-pathogenic fungi in Canada, dsRNAs were found in 38% and 17% of the *M. anisopliae* and *B. bassiana* isolates, respectively (Melzer and Bidochka, 1998). This study com-

pared dsRNA banding patterns from insect pathogenic fungi found in Canada with fungi (*M. anisopliae, B. bassiana* and *Metarhizium flavoviride*) found from areas as diverse as Oman, Trinidad, Benin, Mali and Australia. Double-stranded RNA banding patterns could be distinctive for an isolate or could be shared among species from diverse geographic origins. Similarly, dsRNAs may not be faithfully transmitted during subculturing of the fungus (Melzer and Bidochka, 1998). In some cases, the presence of dsRNAs is associated with reduced fitness of the pathogen (e.g. *Cryphonectria parasitica*; van Alfen *et al.*, 1975). Double-stranded RNA is conceivably transferable and is not a good indication of the genetic relationships between strains.

A transposon-like element termed *Cg*T1, unique to biotype B but not biotype A of *C. gloeosporioides*, was identified and found to contain motifs homologous to gag-like proteins, reverse transcriptase and ribonuclease (RNAse) H domains of the non-long-terminal-repeat, LINE-like class of retrotransposons (He *et al.*, 1996). PCR primers designed to amplify *Cg*T1 could be used to distinguish biotype A from biotype B. Maurer *et al.* (1997) isolated a transposable element, named *hupfer*, from *B. bassiana* by trapping it in the nitrate reductase structural gene. The transposable element had an open reading frame similar to the IS630- or *mariner-Tc*1-like transposases. Although transposable elements are common in fungi, the variability and stability of these elements is not well understood. Therefore, at this time, their utility as genetic markers is unknown.

Introduced molecular markers

Transformation of fungi is now a fairly routine procedure. However, the first requirement for transformation with a marker gene is that the expressed phenotype should not be exhibited in the indigenous population. For example, the presence of bacteria with β-glucuronidase activity limits the application of β-glucuronidase (GUS) marker systems (Jefferson, 1989). In fungi, this is not a problem. Stable GUS co-transformants of *M. anisopliae* have been produced by either electroporation or using the gene gun (St Leger *et al.,* 1995). *M. anisopliae* has also been transformed with benomyl resistance (Bernier *et al.*, 1989; Goettel *et al.*, 1989; Bogo *et al.*, 1996; Valadares-Inglis and Inglis, 1997). The difficulty with many of the insect-pathogenic fungi, such as *M. anisopliae*, is that they are resistant to antibiotics, such as hygromycin (R.J. St Leger, personal communication), for which resistance genes are available. There are also environmental concerns regarding the release of organisms with antibiotic-resistant genes.

Other options for tagging include transformation with genes encoding green fluorescent protein or luminometric markers, such as the *lux*AB gene sequences, particularly if their expression is controlled by an inducible promoter (Shaw *et al.*, 1992). Some markers affect the competitive ability of the pathogen and are therefore less useful for monitoring to predict survival. However, with an inducible promoter the introduced genes would not generally be expressed in the fungus. The most important advance provided by marker systems is the ability to measure cell activity without the requirement for extraction of cells or the need to culture organisms.

A Case-study in Monitoring a Fungal Biocontrol Release: Release of an Australian Fungal Pathogen of Grasshoppers in North America

The following is a case-study of the release of an exotic fungus to control grasshoppers in the USA. I briefly outline the fungal biology, the release programme, the public and scientific concerns raised with the release, the problems encountered with identifying the fungal genet and, finally, the outcome of the study.

The fungal pathotypes

Entomophaga grylli is a zygomycetous fungal pathogen of grasshoppers that has a worldwide distribution. Much of the work on the life-history patterns, host ranges and ecology of *E. grylli* has been carried out in conjuction with the US Department of Agriculture Agricultural Research Service (USDA-ARS) and primarily by R.I. Carruthers, M.E. Ramos, D.L. Hostetter, W.A. Ramoska and R.S. Soper. At least three pathotypes have been identified within this 'species complex', which show differences in life cycle, host range and isozyme patterns (Carruthers *et al.*, 1997). Two pathotypes (pathotypes 1 and 2) have been identified as native to North America and pathotype 3 is native to Australia. Several other genetically distinctive strains (pathotypes?) have been identified from Japan and the Philippines (Walsh *et al.*, unpublished); however, little is known of their host ranges and biology.

Pathotype 1 infects members of the grasshopper subfamily Oedipodinae, the band-winged grasshoppers. The most serious grasshopper pest in this subfamily is *Camnula pellucida*, a pest on rangeland and adjacent crops. Pathotype 1 is also characterized by the production of resting spores within the grasshopper cadaver and aerial conidia. Given adequate moisture, the fungus is able to sporulate from the intersegmental membranes of the grasshopper. These conidia may then horizontally infect other grasshoppers. Resting spores lie dormant in the soil until suitable conditions for germination occur (Carruthers *et al.*, 1997).

Pathotype 2 differs from pathotype 1 in several respects. First, it preferentially infects members of the grasshopper subfamily Melanoplinae, the spur-throated grasshoppers. A species in this subfamily is *M. sanguinipes*, a periodically serious pest on cereal crops. Pathotype 2 is characterized by the inability to produce aerial conidia and it produces resting spores in the grasshopper cadaver. Pathotype 2 also produces cryptoconidia but horizontal transmission by these spores is considered to be minimal (Carruthers *et al.*, 1997).

Pathotype 3 contains the combined characteristics of pathotypes 1 and 2. It was isolated from a cyrtacanthracridine grasshopper in Australia (Milner, 1978). In the laboratory it exhibited a broader host range and infected North American oedipodine and melanopline grasshoppers (Ramoska *et al.*, 1988). Pathotype 3 sporulated more rapidly than pathotype 1, thus allowing for horizontal transmission, but it also produces resting spores, which can lie dormant.

The biocontrol strategy

Although fungal epizootics in North America by one endemic pathotype of *E. grylli* may have caused a significant impact on one group of grasshoppers (Reigert, 1968),

the epizootic may be benign to another group of grasshoppers. In 1987, after the worst outbreak of grasshoppers in 50 years, the USDA Animal and Plant Health Inspection Service (APHIS) concluded that years of chemical control were not only environmentally harmful but chemical control was simply not working. Biological control of grasshoppers was deemed an alternative. Based on the recommendation of the Intraagency Grasshopper Integrated Pest Management committee, the implementation of pathotype 3 was determined to be a feasible tactic for the management of rangeland grasshopper populations. North American grasshopper species have had no contact with the Australian fungal pathogen and, theoretically, have not evolved resistance mechanisms to that pathogen. When an exotic pathogen is introduced, one expects the targeted host insect to be vulnerable.

From 1989 to 1991, approval was granted for the release of pathotype-3-infected grasshoppers (*Melanoplus differentialis*) at two field sites in McKenzie County, North Dakota. Approximately 5000 pathotype-3-infected grasshoppers were released by USDA-APHIS during the 3-year period at the two sites (Ramos, 1993). After the release, a population model showed that pathotype 3 was having an impact on grasshopper numbers (Carruthers *et al.*, 1997). However, it was also determined that *E. grylli* pathotypes 1 and 2 were also present at the release sites.

The controversy

In 1993 a 'News and Comments' article (Goodman, 1993) appeared in the journal *Science* outlining the negative impact that pathotype 3 could have on native non-target grasshopper species and the prairie ecosystem as a whole. Theoretical problems associated with the establishment of an exotic fungal pathotype were noted in articles authored by Lockwood (1993a, b). Lockwood speculated that, if the pest grasshopper species declined, density-dependent mechanisms could result in pathotype 3 infecting non-target grasshopper species. Low-density grasshopper species, such as *Melanoplus femurrubrum*, an intermediate host of a parasite of several bird species, or *Hesperotettix viridis*, a 'beneficial' grasshopper species that feeds on the noxious weed, snakeweed, could be affected. Concerns over the movement of pathotype 3 from the inoculation area were also raised. Infected grasshoppers still not debilitated by fungal infection could carry pathotype 3 substantial distances in 1 day.

The long-term fate, dispersal and environmental impact of pathotype 3 were entirely at question, since an adequate method of differentiating the three pathotypes from one another had not been resolved. Resting spores are indistinguishable among the pathotypes. A method of positively and easily identifying pathotypes of this obligate grasshopper pathogen was required.

Identifying the fungal pathotypes

E. grylli pathotypes had previously been shown to differ in allozyme banding patterns (Soper *et al.*, 1983). This technique required that the fungal protoplasts be grown in complex media (Grace's insect medium supplement with 10% (v/v) fetal bovine serum) from germinated resting spores or viable conidia obtained from infected grasshoppers (MacLeod *et al.*, 1980). Germination frequency of resting spores and conidia in the laboratory is very low (Stoy *et al.*, 1988) and the procedure is tedious. Once an

appropriate biomass had been obtained, the pathotypes could be differentiated based on allozyme patterns. For an obligate pathogen such as *E. grylli*, the application of this technique is logistically problematic for the evaluation of several hundred or 1000 infected grasshoppers. *E. grylli* pathotypes have also been shown to differ in rDNA polymorphisms based on RFLP analysis with a *Saccharomyces cerevisiae* rDNA probe (Walsh *et al.*, 1990). But here again relatively large amounts of good-quality DNA (*c.* 5–10 μg) need to be recovered from each infected grasshopper cadaver.

The criteria that needed to be filled in order for the technique to be suitable for a large-scale screening programme were that: (i) the technique must differentiate among the three pathotypes; (ii) only small amounts of DNA must be required; (iii) the technique must be amenable to screening large numbers of grasshoppers (of the order of 1000 infected grasshoppers); and (iv) the technique must be sensitive to *E. grylli* DNA extracted from resting spores residing in the grasshopper. We investigated three techniques in order to differentiate the three pathotypes (Bidochka *et al.*, 1995, 1997).

The first technique utilized RAPD technology to differentiate among the three pathotypes. The DNA from each pathotype that was used as the template was obtained from culture-collection isolates of the three pathotypes reared as protoplasts on Grace's insect culture media. RAPD banding patterns were specific for each pathotype and little variability was observed among strains within a pathotype (Bidochka *et al.,* 1995). However, a major obstacle was the extraction of DNA from resting spores residing in the infected grasshopper. Resting spores have thick cell walls and are recalcitrant to enzymatic or chemical digestion. The resting spores could be physically disrupted using a homogenizer. However, the DNA isolated from physically disrupted resting spores showed some degradation, possible because of the physical action of the homogenizer. When RAPD-PCR was attempted using this DNA as the template, the results were ambiguous and inconsistent.

The second technique we employed was to cut out specific bands produced by the RAPD-PCR and use these as probes against DNA extracted from *E. grylli* resting spores. Results showed pathotype-specific probes; however, the probes showed only moderate to very light signal strengths.

Finally, *Hin*dIII-digested DNA fragments from genomic DNA of the three pathotypes were cloned and screened for pathotype specificity. Figure 7.1 outlines the strategy used to clone pathotype-specific probes that showed good signal strengths. Further analysis of these probes showed that they were moderately or highly repetitive in the fungal genome. The probes also contained AT-rich regions and contained repeats of several different simple sequence motifs (Walsh *et al.*, unpublished).

Application of molecular probes to field-collected grasshoppers

Live grasshoppers were collected by sweep-netting every 7–10 days at the North Dakota release sites and areas concentric to the release sites during the summers of 1992 to 1994. The grasshoppers were identified to species, instar and adult sex and then brought to indoor facilities, where they were housed. Grasshopper mortality was checked daily and the abdomen dissected and examined for the presence of resting spores, using a microscope (Ramos, 1993). The infected grasshoppers were kept frozen (–20°C) until the application of the pathotype-specific probes.

In the laboratory, the infected grasshoppers were homogenized in order to fracture the resting spores and the DNA was extracted. DNA was dot-blotted on to nitro-

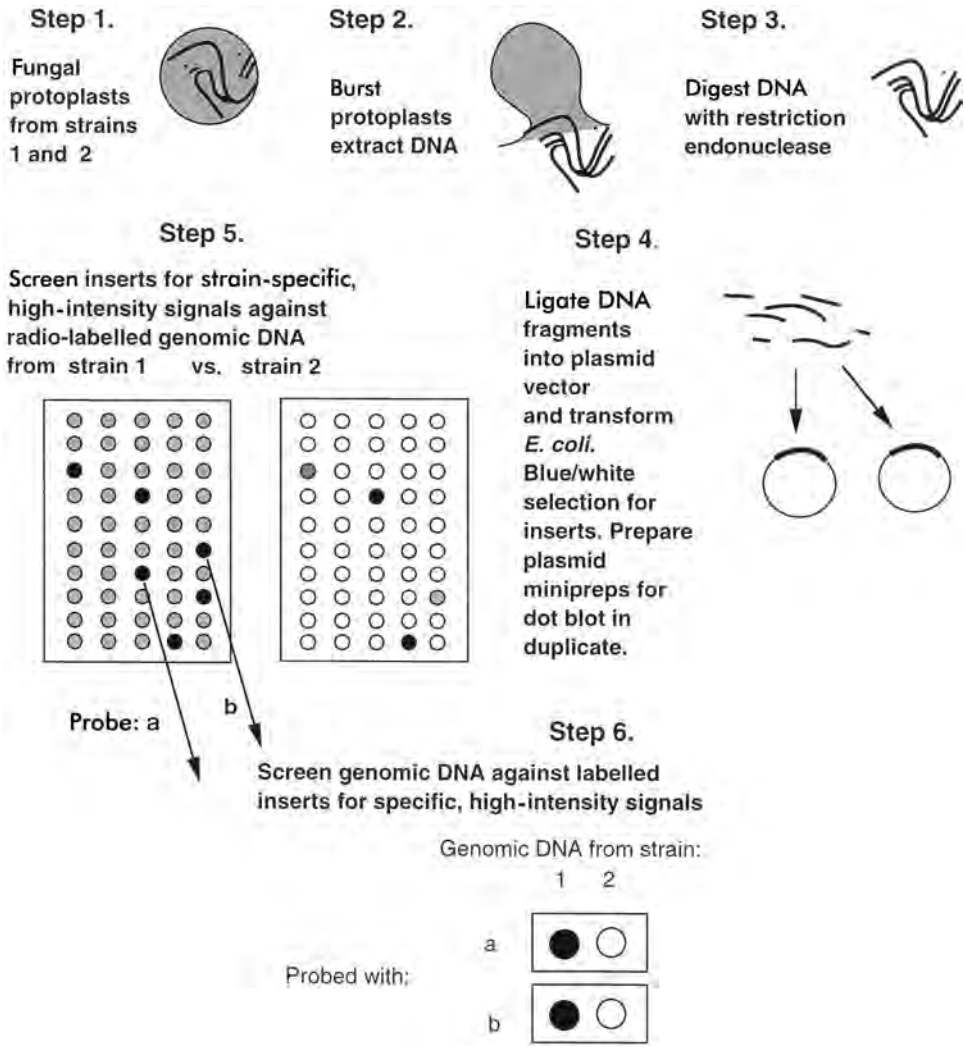

Fig. 7.1. The strategy used to develop a specific DNA probe to distinguish, in this example, strain 1 from strain 2. This strategy would be repeated for each strain for which you require a probe. The same strategy was employed to develop specific DNA probes to distinguish *Entomophaga grylli* pathotypes 1, 2 and 3. In this case, since three pathotypes were involved, three membranes would be used for hybridization against genomic DNA from a separate pathotype or genomic DNA from two pathotypes would be combined for the subtractive comparison on one membrane.

cellulose membranes in triplicate and each membrane was hybridized with a radiolabelled pathotype-specific DNA probe. Figure 7.2 shows a representative autoradiograph of 24 grasshoppers analysed for pathotype-specific infection. In total, 1216 grasshoppers were analysed (888 within 1 km of the release sites and 243 concentric to the release sites). Of the infected grasshoppers collected and analysed, 92.7% showed positive signals for one or more of the three pathotypes (Bidochka *et al.*, 1996). The percentage of *E. grylli*-infected grasshoppers at the release sites declined over 3 years

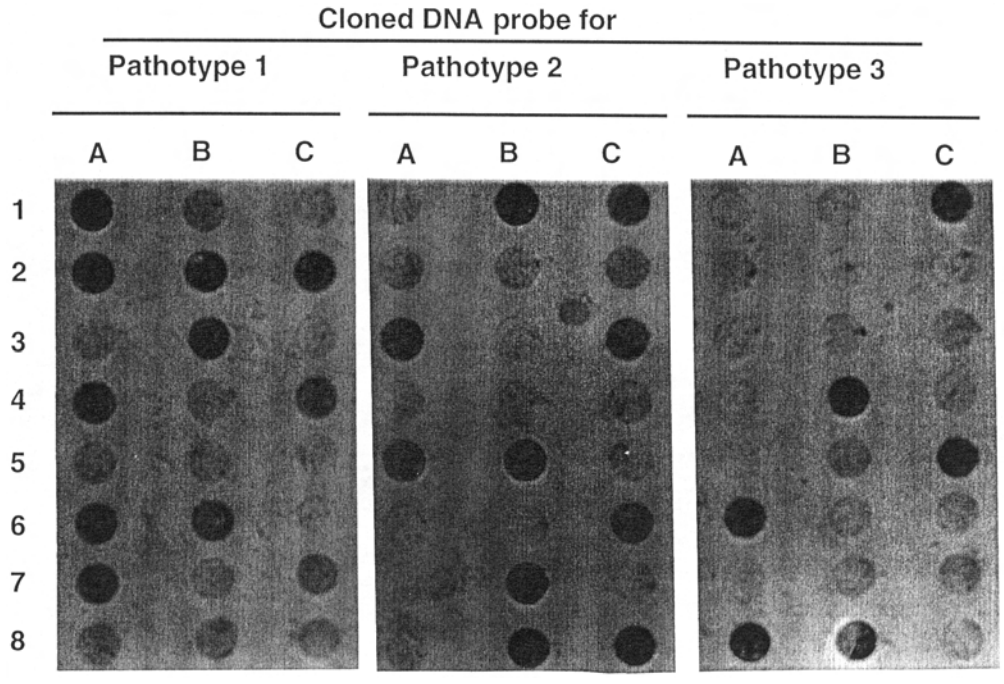

Fig. 7.2. A representative autoradiograph of 24 *Entomophaga grylli*-infected grasshoppers from which fungal DNA had been dot-blotted in triplicate and hybridized with a pathotype-specific cloned DNA probe. In this example there are no instances of grasshoppers co-infected with pathotypes 1 and 2. However, grasshopper A6 is co-infected with pathotypes 1 and 3, while grasshoppers B8 and C1 are co-infected with pathotypes 2 and 3.

(1992–1994) from 23% to 1.7% to 0%. Outside the 1 km radius from the release sites, no pathotype 3 infections were detected in *E. grylli*-infected grasshoppers.

Classical biocontrol entails the establishment of a fungal agent in the host population and relies on cycling of the pathogen. Obligate pathogens, such as *E. grylli*, are a good choice for classical biological control since they have restricted host ranges and show good pathogenicity toward their hosts. This strategy appears not to have applied in the *E. grylli* field trial. Infected grasshoppers from years after 1994 have not been evaluated and grasshopper populations have been relatively low up to 1998. The conidia are relatively ephemeral and are killed by high temperature, desiccation and solar radiation. However, the resting spores are reported to remain in the soil for several seasons and thus are present if grasshopper populations increase (Carruthers *et al.*, 1997). The capacity for dormancy in these fungi merits a continuing survey of pathotype 3. The decline of pathotype 3 in the host population and its purported lack of dispersal from the application area is probably good news in light of the controversy surrounding the release of exotic biocontrol agents.

As an indundative strategy the application of pathotype 3 appears not to be economically feasible since these fungi are not readily culturable and mass production must be done by injecting large numbers of grasshoppers for field release.

Molecular probes and the ecology of Entomophaga grylli

It was mentioned in the introduction that the release of the lacZY-engineered strain of *P. aureofaciens* provided some of the most valuable insights into the population dynamics of rhizosphere bacteria because the fate of the clone could be monitored. The ability to distinguish *E. grylli* pathotype 3 using molecular probes not only proved to be a powerful tool for monitoring a biocontrol release but has also provided some insights into the host ranges and population dynamics of *E. grylli* (Bidochka *et al.*, 1996). For example, the degree of host specificity in various grasshopper species infected by *E. grylli* pathotypes in field conditions was shown. Cross-infections of pathotype 1 in melanopline grasshoppers were more common than pathotype 2 infection in oedipodine grasshoppers. Pathotypes 1 and 2 showed between 1.1% and 15.8% cross-infection, on a grasshopper species basis, from their preferred host. Another subfamily of North American grasshoppers, in which little information on *E. grylli* infection was known, the Gomphocerinae, were found to be most susceptible to pathotype 1 infections. Multiple infections of pathotypes 1 and 2 were very rare in grasshoppers, with only one reported case out of more than 1000 *E. grylli*-infected grasshoppers screened. This suggests that there is some mechanism operating which prevents pathotype 1 and pathotype 2 infections from co-occurring. However, multiple infections of the endemic pathotypes 1 or 2 with the exotic pathotype 3 occurred in approximately 10% (9.0% at one site and 15.5% at the other site) of the *E. grylli*-infected grasshoppers. The same stringent level of exclusion appears not to be operating between the endemic pathotypes and pathotype 3. This may indicate that some mechanism may have evolved in the endemic pathotypes during host-specific infections that excludes a competing endemic pathotype. The same principle may not apply to pathotype 3, the Australian pathotype, with the North American pathotypes because they did not co-occur and compete for hosts.

The use of cloned DNA probes also showed evidence for infection preferences by the *E. grylli* pathotypes in different life stages of grasshopper species (Bidochka *et al.*, 1996). Pathotype 2 infections occurred more frequently in early-instar *M. sanguinipes* and *Melanoplus bivitattus* than in adult grasshoppers, while pathotype 1 infections occurred more frequently in adult *C. pellucida* than in the early instars. When cross-infections (e.g. pathotype 1 in melanopline grasshoppers) or pathotype 3 infections occurred, they were found in later-instar and adult grasshoppers.

Conclusions

Fungal biocontrol introductions require identification and monitoring of the introduced genet in the field in order to determine the effect of the pest control application and as part of a biological impact assessment. No one technique can provide conclusive identification of a fungal genet in the field against the endemic fungal population. The application of a certain technique will depend primarily on the fungus that is to be introduced into a site and information concerning the amount of variation that can be detected in the fungal species. If the fungal species is not endemic to the introduced site, techniques that can differentiate fungal species or pathotypes could be used. These include morphological markers, allozyme analysis or immunological markers. This will also depend on the ability to culture the fungus on artificial media. However, if a fungal species that is endemic to the area is to be released, more

discriminatory molecular analysis, such as RFLP, RAPD or cloned DNA probes, may be more appropriate. Techniques such as DNA arrays on genotyping chips have been developed (Chee *et al.*, 1996) and are being applied for discovering single-nucleotide polymorphisms (SNPs) in humans (Wang *et al.*, 1998). In the very near future, this technology could be accessible for screening fungal genets. There is also a vast field of knowledge devoted to modeling the fate of chemical contaminants in the ecosystem. This information could be utilized to model the fate of fungal biocontrol agents. The fallout from this type of analysis will be to provide information on the efficacy of fungal biocontrol agents and, in addition, to provide valuable information on pathogenic fungal epidemiology and ecology.

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References

Adams, P.B. (1990) The potential of mycoparasites for biological control of plant diseases. *Annual Review of Phytopathology* 28, 59–72.

van Alfen, N.K., Janes, R.A., Anagnostakis, S.L. and Day, P.R. (1975) Chestnut blight: biological control by transmissible hypovirulence in *Endothia parasitica. Science* 189, 890–891.

Bernier, L., Cooper, R.M., Charnley, A.K. and Clarkson, J.M. (1989) Transformation of the entomopathogenic fungus *Metarhizium anisopliae* to benomyl resistance. *FEMS Microbiology Letters* 60, 261–266.

Bidochka, M.J., McDonald, M.A., St Leger, R.J. and Roberts, D.W. (1994) Differentiation of species and strains of entomopathogenic fungi by random amplification of polymorphic DNA (RAPD). *Current Genetics* 25, 107–113.

Bidochka, M.J., Walsh, S.R.A., Ramos, M.E., St Leger, R.J., Silver J.C. and Roberts, D.W. (1995) Pathotypes in the *Entomophaga grylli* species complex of grasshoppers pathogens differentiated with rapid amplification of polymorphic DNA and cloned-DNA probes. *Applied and Environmental Microbiology* 61, 556–560.

Bidochka, M.J., Walsh, S.R.A., Ramos, M.E., St Leger, R.J., Silver J.C. and Roberts, D.W. (1996) Fate of biological control introductions: Monitoring an Australian fungal pathogen of grasshoppers in North America. *Proceedings of the National Academy of Sciences USA* 93, 918–921.

Bidochka, M.J., Walsh, S.R.A., Ramos, M.E., St Leger, R.J., Carruthers, R.I., Silver, J.C. and Roberts, D.W. (1997) Cloned DNA probes distinguish endemic and exotic *Entomophaga grylli* fungal pathotype infections in grasshopper life stages. *Molecular Ecology* 6, 303–308.

Bidochka, M.J., Kasperski, J.E. and Wild, G.A.M. (1998) Occurrence of the entomopathogenic fungi *Metarhizium anisopliae* and *Beauveria bassiana* in soils from temperate and near-northern habitats. *Canadian Journal of Botany* 76, 1198–1204.

Bidochka, M.J., St Leger, R.J., Stuart, A. and Gowanlock, K. (1999). Nuclear rDNA phylogeny in the fungal genus *Verticillium* and the relationship to insect and plant virulence, extracellular proteases and carbohydrases. *Microbiology* 145, 955–963.

Bogo, M.R., Vainstein, M.H., Aragao, F.J.L., Rech, E. and Schrank, A. (1996) High frequency gene conversion among benomyl resistant transformants in the entomopathogenic fungus *Metarhizium anisopliae. FEMS Microbiology Letters* 142, 123–127.

Braithwaite, K.S., Irwin, J.A.G. and Manners, J.M. (1990) Restriction fragment length polymorphisms in *Colletotrichum gloeosporioides* infecting *Stylosanthes* spp. in Australia. *Mycological Research* 94, 1129–1137.

Bridge, P.D., Pearce, D.A., Rivera, A. and Rutherford, M.A. (1997a) VNTR derived oligonucleotides as PCR primers for population studies in filamentous fungi. *Letters in Applied Microbiology* 24, 426–430.

Bruns, T.D., White, T.J. and Taylor, J.W. (1991) Fungal molecular systematics. *Annual Review of Ecology and Systematics* 22, 525–564.

Burdon, J.J. (1992) Host population subdivision and the genetic structure of natural plant populations. *Advances in Plant Pathology* 8, 81–94.

Burdon, J.J. (1993) The structure of pathogen populations in natural plant communites. *Annual Review of Phytopathology* 31, 305–323.

Carruthers, R.I., Ramos, M.E., Larkin, T.S., Hostetter, D.L. and Soper, R.S. (1997) The *Entomophaga grylli* (Fresenius) batko species complex: its biology, ecology, and use for biological control of pest grasshoppers. *Memoirs of the Entomological Society of Canada* 171, 329–353.

Chee, M., Yang, R., Hubbell, E., Berno, A., Huang, X.C., Stern, D., Winkler, J., Lockhart, D.J., Morris, M.S. and Fodor, S.P.A. (1996) Accessing genetic information with high-density DNA arrays. *Science* 274, 610-614.

Cook, R.J., Bruckart, W.L., Coulson, J.R., Goettel, M.S., Humber, R.A., Lumsden, R.D., Maddox, J.V., McManus, M.L., Moore, L., Meyer, S.F., Quimby, P.C., Stack, J.P. and Vaughn, J.L. (1996) Safety of microorganisms intended for pest and plant disease control: a framework for scientific evaluation. *Biological Control* 7, 333–359.

Couteaudier, Y. and Viaud, M. (1997) New insights into population structure of *Beauveria bassiana* with regard to vegetative compatibility groups and telomeric restriction length polymorphisms. *FEMS Microbiology Ecology* 22, 175–182.

Curran, J., Driver, F., Ballard, J.W.O. and Milner, R.J. (1994) Phylogeny of *Metarhizium*: analysis of ribosomal DNA sequence data. *Mycological Research* 98, 547–552.

Daniel, J.T., Templeton, G.E., Smith, R.J. and Fox, W.T. (1973) Biological control of northern jointvetch in rice with an endemic fungal disease. *Weed Science* 21, 303–307.

Dickinson, M.J. (1986) Viruses of plant pathogens. In: Kosuge, T. and Nester, E.W. (eds) *Plant–Microbe Interactions*. Macmillan Press, New York, pp. 359–382.

Dustan, A.G. (1924) *The Control of the European Apple Sucker,* Psyllia mali *Schmidb. in Nova Scotia.* Pamphlets of the Canadian Department of Agriculture 45, 13 pp.

Ellsworth, D.L., Rittenhouse, K.D. and Honeycutt, R.L. (1993) Artifactual variation in randomly amplified polymorphic DNA banding patterns. *BioTechniques* 14, 214–217.

Fargues, J., Duriez, T., Popeye, R., Robert, P.H. and Biguet, J. (1981) Immunological characterization of the entomopathogenic hyphomycetes *Beauveria* and *Metarhizium. Mycopathologia* 75, 101–108.

Fegan, M., Manner, J.M., Maclean, D.J., Irwin, J.A.G., Samuels, K.D.Z., Holdom, D.G. and Li, D.P. (1993) Random amplified polymorphic DNA markers reveal a high degree of genetic diversity in the entomopathogenic fungus *Metarhizium anisopliae. Journal of General Microbiology* 139, 2075–2081.

Ferron, P. (1978) Biological control of insect pests by entomogenous fungi. *Annual Review of Entomology* 23, 409–423.

Fungaro, M.H.P., Vieira, M.L.C., Pizzirani-Kleiner, A.A. and De Azevedo, J.L. (1996) Diversity among soil and insect isolates of *Metarhizium anisopliae* var. *anisopliae* detected by RAPD. *Letters in Applied Microbiology* 22, 389–392.

Genthner, F.J., Foss, S.S., Campbell, R.P. and Fournie, J.W. (1993) Fate and survival of microbial pest control agents in nontarget aquatic organisms. *Diseases of Aquatic Organisms* 16, 157–162.

Goettel, M.S. and Jaronski, S.T. (1997) Safety and registration of microbial agents for control of grasshoppers and locusts. *Memoirs of the Entomological Society of Canada* 171, 83–99.

Goettel, M.S., St Leger, R.J., Bhairi, S., Jung, M.K., Oakley, B.R. and Staples, R.C. (1989) Pathogenicity and growth of *Metarhizium anisopliae* stably transformed to benomyl resistance. *Current Genetics* 17, 129–132.

Goodman, B. (1993) Research community swats grasshopper control trial. *Science* 260, 887.

Guy, P.L. and Rath, A.C. (1990) Enzyme-linked immunosorbent assays (ELISA) to detect spore surface antigens of *Metarhizium anisopliae. Journal of Invertebrate Pathology* 55, 435–436.

Hajek, A.E., Humber, R.A., Elkinton, J.S., May, B., Walsh, S.R.A. and Silver, J.C. (1990) Allozyme and restriction fragment length polymorphism analyses confirm *Entomophaga maimaiga* responsible for 1989 epizootics in North American gypsy moth populations. *Proceedings of the National Academy of Sciences USA* 87, 6979–6982.

Hajek, A.E., Butt, T.M., Strelow, L.I. and Gray, S.M. (1991a) Detection of *Entomophaga maimaiga* (Zygomycetes Entomophthorales) using enzyme-linked immunosorbent assay. *Journal of Invertebrate Pathology* 58, 1–9.

Hajek, A.E., Humber, R.A., Walsh, S.R.A. and Silver, J.C. (1991b) Sympatric occurrence of two *Entomophaga aulicae* (Zygomycetes: Entomophthorales) complex species attacking forest Lepidoptera. *Journal of Invertebrate Pathology* 58, 373–380.

Hajek, A.E., Humber, R.A. and Elkinton, J.S. (1995) Mysterious origin of *Entomophaga maimaiga* in North America. *American Entomologist* 41, 31–42.

Hajek, A.E., Butler, L., Walsh, S.R.A., Silver, J.C., Hain, F.P., Hastings, F.L., Odell, T.M. and Smitley, D.R. (1996a) Host range of the gypsy moth (Lepidoptera: Lymantriidae) pathogen *Entomophaga maimaiga* (Zygomycetes: Entomophthorales) in the field versus laboratory. *Environmental Entomology* 25, 709–721.

Hajek, A.E., Elkinton, J.S. and Witcosky, J.J. (1996b) Introduction and spread of the fungal pathogen *Entomophaga maimaiga* (Zygomycetes: Entomophthorales) along the leading edge of gypsy moth (Lepidoptera: Lymantriidae) spread. *Environmental Entomology* 25, 1235–1247.

Hajek, A.E., Hodge, K.T., Liebherr, J.K., Day, W.H. and Vandenberg, J.D. (1996c) Use of RAPD analysis to trace the origin of the weevil pathogen *Zoophthora phytonomi* in North America. *Mycological Research* 100, 349–355.

Harper, J.L. (1977) *Population Biology of Plants*. New York, Academic Press.

Hayden, H.L., Pegg, K.G., Aitken, E.A.B. and Irwin, J.A.G. (1994) Genetic relationships as assessed by molecular markers and cross-infection among strains of *Colletotrichum gloeosporioides*. *Australian Journal of Botany* 42, 9–18.

He, C., Nourse, J.P., Kelemu, S., Irwin, A.G. and Manners, J.M. (1996) CgT1: a non-LTR retrotransposon with restricted distribution in the fungal phytopathogen *Colletotrichum gloeosporioides. Molecular and General Genetics* 252, 320-331.

Hegedus, D.D. and Khachatourians, G.G. (1993a) Identification of molecular variants in mitochondrial DNAs of members of the genera *Beauvaria, Verticillium, Paecilomyces, Tolypocladium*, and *Metarhizium. Applied and Environmental Microbiology* 59, 4283–4288.

Hegedus, D.D. and Khachatourians, G.G. (1993b) Construction of cloned DNA probes for the specific detection of the entomopathogenic fungus *Beauverai bassiana* in grasshoppers. *Journal of Invertebrate Pathology* 62, 233–240.

Hegedus, D.D. and Khachatourians, G.G. (1994) Isolation and characterization of conditional lethal mutants of *Beauveria bassiana. Canadian Journal of Microbiology* 40, 766–776.

Hegedus, D.D. and Khachatourians, G.G. (1995) Identification and differentiation of the entomopathogenic fungus *Beauveria bassiana* using polymerase chain reaction and single-strand conformation polymorphism analysis. *Journal of Invertebrate Pathology* 67, 289–299.

Hodge, K.T., Sawyer, A.J. and Humber, R.A. (1995) RAPD-PCR for identification of *Zoophthora radicans* isolates in biological control of the potato leafhopper. *Journal of Invertebrate Pathology* 65, 1–9.

Hodson, A., Mills, P.R. and Brown, A.E. (1993) Ribosomal and mitochondrial DNA polymorphisms in *Colletotrichum gloeosporioides* isolated from tropical fruits. *Mycological Research* 97, 329–335.

Jefferson, R.A. (1989) The GUS gene reporter system. *Nature* 342, 837–838.

Johnston, P.R. and Jones, D. (1997) Relationships among *Colletotrichum* isolates from fruit-rots assessed using rDNA sequences. *Mycologia* 89, 420–430.

Julien, M.H. (1987) *Biological Control of Weeds: a World Catalogue of Agents and their Target Weeds*, 2nd edn. CAB International, Wallingford, UK.

Keller, S. (1989) The use of blastospores of *Beauveria brongniartii* to control *Melolontha melolontha* in Switzerland. In: Jegorov, A. and Matha, V. (eds) *Proceedings of an International Conference on Biopesticides, Theory and Practices.* Czechoslovak Academy of Sciences, Ceske Budejovice, Czechoslovakia, pp. 91–98.

Kistler, H.C. and Miao, V.P.W. (1992) New modes of genetic change in filamentous fungi. *Annual Review of Phytopathology* 30, 131–152.

Kluepfel, D.A. (1993) The behavior and tracking of bacteria in the rhizosphere. *Annual Review of Phytopathology* 31, 441–472.

Kluepfel, D.A., Kilne, E.L., Skipper, H.D., Hughes, T.A., Gooden, D.T., Drahos, D.J., Barry, G.F., Hamming, B.C. and Brandt, E.J. (1991) The release and tracking of genetically engineered bacteria in the environment. *Phytopathology* 81, 348–352.

Kosir, J.M., MacPherson, J.M. and Khachatourians, G.G. (1991) Genomic analysis of a virulent and less virulent strain of the entomopathogenic fungus *Beauveria bassiana*, using restriction fragment length polymorphisms. *Canadian Journal of Microbiology* 37, 534–541.

Kuramae-Izioka, E.E., Lopes, C.R., Souza, N.L. and Machado, M.A. (1997) Morphological and molecular characterization of *Colletotrichum* spp. from citrus orchards affected by post-bloom fruit drop in Brazil. *European Journal of Plant Pathology* 103, 323–329.

Leal, S.C.M., Bertioli, D.J.M., Butt, T.M., Carder, J.H., Burrows, P.R. and Peberdy, J.F. (1997) Amplification and restriction endonuclease digestion of the *Pr*1 gene for the detection and characterization of *Metarhizium* strains. *Mycological Research* 101, 257–265.

Leslie, J.F. (1993) Fungal vegetative compatibility. *Annual Review of Phytopathology* 31, 127–150.

Liyanage, H.D., McMillan, R.T. Jr and Corby Kistler, H. (1992) Two genetically distinct populations of *Colletotrichum gloeosporioides* from Citrus. *Phytopathology* 82, 1371–1376.

Liyanage, H.D., Koeller, W., McMillan, R.T. Jr and Kistler, H.C. (1993) Variation in cutinase form two populations of *Colletotrichum gloeosporioides* from citrus. *Phytopathology* 83, 113–116.

Lockwood, J.A. (1993a) Environmental issues involved in biological control of rangeland grasshoppers (Orthoptera: Acrididae) with exotic agents. *Environmental Entomology* 22, 503–518.

Lockwood, J.A. (1993b) Benefits and costs of controlling rangeland grasshoppers (Orthoptera: Acrididae) with exotic organisms: search for a null hypothesis and regulatory compromise. *Environmental Entomology* 22, 904–914.

MacLeod, D.M., Tyrrell, D. and Welton, M.A. (1980) Isolation and growth of the grasshopper pathogen, *Entomophthora grylli. Journal of Invertebrate Pathology* 36, 85–89.

Majer, D., Mithen, R., Lewis, B.G. and Pieter, V. (1996) The use of AFLP fingerprinting for the detection of genetic variation in fungi. *Mycological Research* 100, 1107–1111.

Manners, J.M. and He, C. (1997) Recent advances in studies of anthracnose of *Stylosanthes*. IV. Molecular approaches to studies of *Colletotrichum gloeosporioides* causing anthracnose of *Stylosanthes* in Australia. *Tropical Grasslands* 31, 435–444.

Masel, A.M., Irwin, J.A.G. and Manners, J.M. (1993) DNA addition or deletion is associated with a major karyotype polymorphism in the fungal phytopathogen *Colletotrichum gloeosporioides. Molecular and General Genetics* 237, 73–80.

Masel, A.M., He, C., Poplawski, A.M., Irwin, J.A.G. and Manners, J.M. (1996) Molecular evidence for chromosome transfer between biotypes of *Colletotrichum gloeosporioides. Molecular Plant–Microbe Interactions* 9, 339–348.

Massion, C.L. and Lindow, S.E. (1986) Effects of *Sphacelotheca holci* infection on morphology and competitiveness of johnsongrass (*Sorghum halepense*). *Weed Science* 34, 883–888.

Maurer, P., Rejasse, A., Capy, P., Langin, T. and Riba, G. (1997) Isolation of the transposable

element hupfer from the entomopathogenic fungus *Beauveria bassiana* by insertion mutagenesis of the nitrate reductase structural gene. *Molecular and General Genetics* 256, 195–202.

McDermott, J.M. and McDonald, B.A. (1993) Gene flow in plant pathosystems. *Annual Review of Phytopathology* 31, 353–373.

Melzer, M.J. and Bidochka, M.J. (1998) Diversity of double-stranded RNA viruses within populations of entomopathogenic fungi and potential implications for fungal growth and virulence. *Mycologia* 90, 586–594.

Menezes, M. and Hanlin, R.T. (1996) Morphological variability of *Colletotrichum gloeosporioides* isolates from avocado trees from Northeastern Brazil. *Revista de Microbiologia* 27, 228–236.

Mills, D. and McCluskey, K. (1990) Electrophoretic karyotypes of fungi: the new cytology. *Molecular Plant–Microbe Interactions* 3, 351–357.

Milner, R.J. (1978) On the occurrence of *Entomophthora grylli*, a fungal pathogen of grasshoppers in Australia. *Journal of the Australian Entomological Society* 17, 293–296.

Milner, R.J. and Mahon, R.J. (1985) Strain variation in *Zoophthora radicans*, a pathogen on a variety of insect hosts in Australia. *Journal of the Australian Entomological Society* 24, 195–198.

Milner, R.J., Soper, R.S. and Lutton, G.G. (1982) Field release of an Israeli strain of the fungus *Zoophthora radicans* (Brefeld) Batko for biological control of *Therioaphis trifolli* (Monell) f. *maculata. Journal of the Australian Entomological Society* 21, 113–118.

Moscardi, F. (1989) Production and use of entomopathogens in Brazil. In: Roberts, D.W. and Granados, R.R. (eds) *Proceedings of the Conference on Biotechnology, Biological Pesticides and Novel Plant-Pest Resistance for Insect Pest Management.* Boyce Thompson Institute, Ithaca, New York, pp. 52–60.

Mueller, M.M., Kantola, R. and Kitunen, V. (1994) Combining sterol and fatty acid profiles for the characterization of fungi. *Mycological Research* 98, 593–603.

Neuveglise, C. and Brygoo, Y. (1994) Identification of group-I introns in the 28s rDNA of the entomopathogenic fungus *Beauveria brongniartii. Current Genetics* 27, 38–45.

Neuveglise, C., Brygoo, Y., Vercambre, B. and Riba, G. (1994) Comparative analysis of molecular and biological characteristics of strains of *Beauveria brongniartii* isolated from insects. *Mycological Research* 98, 322–328.

Pimentel, D., Acquay, H., Biltonen, M., Rice, P., Silva, M., Nelson, J., Lipner, V., Giordano, S., Horowitz, A. and D'Amore, M. (1992) Environmental and economic costs of pesticide use. *Bioscience* 42, 750-760.

Pipe, N.D., Chandler, D., Bainbridge, B.W. and Heale, J.B. (1995) Restriction fragment length polymorphisms in the ribosomal RNA gene complex of isolates of the entomopathogenic fungus *Metarhizium anisopliae. Mycological Research* 99, 485–491.

Poplawski, A.M., He, C., Irwin, J.A.G. and Manners, J.M. (1997) Transfer of an autonomously replicating vector between vegetatively incompatible biotypes of *Colletotrichum gloeosporioides. Current Genetics* 32, 66–72.

Prosser, J.I. (1994) Molecular marker systems for detection of genetically engineered microorganisms in the environment. *Microbiology* 140, 5–17.

Ramos, M.E. (1993) The isolation, implementation and evaluation of *Entomophaga praxibuli* as a potential biological control agent of North American grasshoppers. MPS thesis, Department of Plant Pathology, Cornell University, Ithaca, New York, 47 pp.

Ramoska, W.A., Hajek, A.E., Ramos, M.E. amd Soper, R.S. (1988) Infection of grasshoppers by members of the *Entomophaga grylli* species complex. *Journal of Invertebrate Pathology* 52, 309–313.

Rayner, A.D.M. (1991) The phytopathological significance of mycelial individualism. *Annual Review of Phytopathology* 29, 305–323.

Reigert, P.W. (1968) A history of grasshopper abundance surveys and forecasts of outbreaks in Saskatchewan. *Memoirs of the Entomological Society of Canada* 52, 1–99.

Reineke, A. and Zebitz, C.P.W. (1996) Protein and isozyme patterns among isolates of *Beauveria brongniartii* with different virulence to European cockchafer larvae (*Melolontha melolontha* L.). *Journal of Applied Entomology* 120, 307–315.

Roberts D.W. and Hajek, A.E. (1992) Entomopathogenic fungi as bioinsecticides. In: Leatham, G.F. (ed.) *Frontiers in Industrial Mycology*. Chapman & Hall, New York, pp. 144–159.

Rorer, J.B. (1913) The use of the green muscardine in the control of some sugar cane pests. *Phytopathology* 3, 88–92.

Rosewich, U.L. and McDonald, B.A. (1994) DNA fingerprinting in fungi. *Methods in Molecular and Cellular Biology* 5, 41–48.

Schotts, A., Dewey, F.M. and Oliver, R.P. (1994) *Modern Assays for Plant Pathogenic fungi: Identification, Detection and Quantification.* CAB International, Wallingford, UK, 267 pp.

Shaw, J.J., Dane, F., Geiger, D. and Kloepper, J.W. (1992) Use of bioluminescence for detection of genetically engineered microorganisms into the environment. *Applied and Environmental Microbiology* 58, 267–273.

Shimizu, S. and Aizawa, K. (1988) Serological classification of *Beauveria bassiana. Journal of Invertebrate Pathology* 52, 348–353.

Shimizu, S., Arai, Y. and Matsumoto, T. (1992) Electrophoretic karyotype of *Metarhizium anisopliae. Journal of Invertebrate Pathology* 60, 185–187.

Silvie, P., Dedryver, C.A. and Tanguy, S. (1990) Application expérimentale de mycélium d'*Erynia neoaphidis* (Zygomycetes: Entomophthorales) dans des populations de pucerons sur laitues en serre maraichère: Etude du suivi de l'inoculum par caractérisation enzymatique. *Entomophaga* 35, 375–384.

Soper, R.S., Bernie, M. and Martinell, B. (1983) *Entomophaga grylli* enzyme polymorphism as a technique for pathotype identification. *Environmental Entomology* 12, 720–723.

Sreenivasaprasad, S., Mills, P.R., Meehan, B.M. and Brown, A.E. (1996) Phylogeny and systematic of 18 *Colletotrichum* species based on ribosomal DNA spacer sequences. *Genome* 39, 499–512.

St Leger, R.J., Allee, L.L., May, B., Frank, D.C., Staples, R.C. and Roberts, D.W. (1992a) World-wide distribution of genetic variation among isolates of *Beauveria* spp. *Mycological Research* 96, 1007–1015.

St Leger, R.J., May, B., Allee, L.L., Frank, D.C., Staples, R.C. and Roberts, D.W. (1992b) Genetic differences in allozymes and in formation of infection structures among isolates of the entomopathogenic fungus *Metarhizium anisopliae. Journal of Invertebrate Pathology* 60, 89–101.

St Leger, R.J., Bidochka, M.J. and Roberts, D.W. (1995) Co-transformation of *Metarhizium anisopliae* by electroporation or the gene gun to produce stable GUS transformants. *FEMS Microbiology Letters* 131, 289–294.

Stoy, W.M., Valovage, W.D., Frye, R.D. and Carlson, R.B. (1988) Germination of resting spores of the grasshopper (Orthoptera: Acrididae) pathogen, *Entomophaga grylli* (Zygomycetes: Entomophthorales) pathotype 2, in selected environments. *Environmental Entomology* 17, 238–245.

Sutton, B.C. (1992) The genus *Glomerella* and its anamorph *Colletotrichum.* In: Bailey, J.A. and Jeger, M.J. (eds) Colletotrichum – *Biology, Pathology and Control*. CAB International, Wallingford, UK, pp. 1–26.

Talbot, N.J., Salch, Y.P., Ma, M. and Hamer, J.E. (1993). Karyotype variation within lineages of the rice blast fungus *Magnaporthe grisea. Applied and Environmental Microbiology* 59, 585–593.

Templeton, G.E. (1975) EPA issues first experimental use permit for biological control fungus. *Rice Journal* 78, 30.

Tigano-Milani, M.S., Gomes, A.C.M.M. and Sobral, B.W.S. (1995) Genetic variability among Brazilian isolates of the entomopathogenic fungus *Metarhizium anisopliae. Journal of Invertebrate Pathology* 65, 206–210.

Tinline, R.D. and Noviello, C. (1971) Heterokaryosis in the entomogenous fungus, *Metarrhizium anisopliae. Mycologia* 63, 701–712.

Valadares-Inglis, M.C. and Inglis, P.W. (1997) Transformation of the entomopathogenic fungus, *Metarhizium flavoviride* strain CG423 to benomyl resistance. *FEMS Microbiology Letters* 155, 199–202.

Viaud, M., Couteaudier, Y., Levis, C. and Riba, G. (1996) Genomic organization in *Beauveria bassiana*: electrophoretic karyotype, gene mapping, and telomeric fingerprint. *Fungal Genetics and Biology* 20, 175–183.

Walsh, S.R.A., Tyrell, D., Humber, R.A. and Silver, J.C. (1990) DNA restriction fragment length polymorphisms in the rDNA repeat unit of *Entomophaga. Experimental Mycology* 14, 381–392.

Wang, D.G., Fan, J.-B., Siao, C.-J., Berno, A., Young, P., Sapolsky, R., Ghandour, G., Perkins, N., Winchester, E., Spencer, J., Kruglyak, L., Stein, L., Hsie, L., Topaloglou, T., Hubbell, E., Robinson, E., Mittman, M., Morris, M.S., Shen, N., Kilburn, D., Rioux, J., Nusbaum, C., Rozen, S., Hudson, T.J., Lipshutz, R., Chee, M. and Lander, E.S. (1998) Large-scale identification, mapping, and genotyping of single-nucleotide polymorphisms in the human genome. *Science* 280, 1077–1082.

Wraight, S.P., Galiani-Wraight, S., Carruthers, R.I. and Roberts, D.W. (1986) Field transmission of *Erynia radicans* to *Empoasca* leafhoppers in alfalfa following application of dry, mycelial preparation. In: Samson, R.A., Vlak, J.M. and Peters, D. (eds) *Fundamental and Applied Aspects of Insect Pathology: Foundation of the Fourth International Colloquium of Invertebrate Pathology.* Wageningen, the Netherlands, p. 233.

Xu, Q. (1988) Some problems about study and application of *Beauveria bassiana* against agricultural and forest pest in China. In: *Chinese Society of Mycology, Study and Application of Entomogenous Fungi in China*, Vol. 1. Academic Periodical Press, Beijing, pp. 1–9.

van Zeijl, C.M.J., van de Kamp, E.H.M., Punt, P.J., Selten, G.C.M., Hauer, B., van Gorcom, R.F.M. and van den Hondel, C.A.M.J.J. (1998) An improved colony-PCR method for filamentous fungi for amplification of PCR-fragments of several kilobases. *Journal of Biotechnology* 59, 221–224.

8 Prospects for Strain Improvement of Fungal Pathogens of Insects and Weeds

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Introduction

Fungal pathogens have been recorded for virtually all groups of multicellular organisms. Plant pathologists and insect pathologists have long been interested in the fungal pathogens of their respective host groups. All classes of *Eumycotina* contain at least a few plant pathogens. Likewise, at least 90 genera and more than 700 species of fungi have been identified as closely associated with invertebrates, principally insects. Virtually every major fungal taxonomic group, except the higher basidiomycetes and dematiaceous hyphomycetes, has members pathogenic to insects (Roberts and Humber, 1981). Initially, plant pathologists were interested in protecting crop plants from fungal diseases, but more recently they have selected and/or engineered fungi for weed and plant pathogen (primarily fungi) control. A similar evolution was followed by insect pathologists. Originally, they were interested in protecting beneficial insects (primarily honey-bee and silkworm), but currently there is considerable interest in using fungal pathogens to control insect pests. Recent widely publicized problems with synthetic chemical insecticides and herbicides have stimulated this increased interest in the development of fungi as biological control agents as supplements or alternatives to these chemicals.

Biological control experiments with fungi have often produced inconsistent results, and the slow speed of kill compared with chemical insecticides has deterred commercial development (see Chapter 1). Consequently, any consideration of the suitability of a fungus for commercial purposes inevitably leads to the possibility of improving its performance, i.e. by incorporating more toxic modes of action and increasing kill rates. However, registration requests to date have been for naturally occurring fungi obtained by standard selection procedures and improved as pathogenic agents against insects and weeds by developing the techniques required for optimizing the production and stability of the inoculum (TeBeest, 1991; Roberts and Hajek, 1992; Gressel, 2000). Improvements of pathogens have been attempted through parasexual crossing and protoplast fusion (Heale *et al.*, 1989) or by conventional mutagenesis (Miller *et*

 Fungi as Biocontrol Agents (eds T.M. Butt, C. Jackson and N. Magan)

al., 1989), but genetic engineering by directed addition of one or more genes coding for pathogenicity determinants provides the most targeted and flexible approach to altering the physiology of pathogenic fungi and producing combinations of traits that are not readily identifiable in nature without the co-transfer of possibly undesirable linked characteristics.

Until recently, recombinant DNA techniques had no application in a strain improvement programme involving pathogenic fungi because there was no information available as to the nature of the genes that control either pathogenesis or specificity. This has changed dramatically and molecular biology methods have elucidated pathogenic processes in several important biocontrol agents, with the cloning of genes that are expressed when these fungi are induced by physical and chemical stimuli to alter their saprobic growth habit, develop a specialized infection structure (the appressorium) and attack the host. Some of these genes encode enzymes and toxins with demonstrated targets in the hosts. Other genes have been identified as virulence determinants because of their role in signal transduction during the production of infection structures. As a result, we have entered an era when techniques for the isolation, identification and subsequent manipulation of expression of individual genes implicated in the disease process will allow the production of transgenic fungi with improved pathogenic qualities and hopefully generate a wider interest in fungi as sources of pesticidal genes. The availability of these diverse pathogenicity genes may supplement currently used genes in producing recombinant viral, bacterial, fungal and, eventually, plant products to add to the collection of 'softer' environmentally friendly tools for integrated pest management. Up till now, pathogenic fungi have played little part in providing 'useful' pesticidal genes for transfer, which is surprising given the vast array of biologically active metabolites they produce.

Characteristics of Fungal Candidates for Biotechnological Manipulation

Several different approaches have been used for introducing pathogenic fungi into insect and weed populations. Classical biological control entails the establishment of a fungal species in an area with host populations (usually where the pathogen does not occur) and relies on the pathogen permanently cycling in pest populations. This strategy is frequently applied to the many insects and the great majority of weeds of economic importance that are not native but have been introduced from other continents or geographical areas where they are subject to fungus-induced disease. Obligate pathogens, including members of the Uredinales, Peronosporales and Ustilaginales (plant pathogens) and Zygomycetes (insect pathogens), in general make excellent classical biological control agents, as they are often restricted in their host range and are capable of aggressive pathogenicity (Roberts and Hajek, 1992; Hajek and St Leger, 1994; Quimby and Birdsall, 1995). Biological control of a weed with a fungus began in the early 1970s with the introduction of a rust fungus to control rush skeleton weed (Adams and Line, 1984). The greatest success has been with the use of rusts (*Puccinia jaceae*) for the control of diffuse knapweed (*Centuria diffusa*) and skeleton-weed (*Chondrilla juncea*) control with the use of *Puccinia chondrillina* (Quimby *et al.*, 1991; Kennedy, 1996). Biological control of insects with fungi dates back at least 100 years. Notable recent successes include *Entomophaga maimaiga* to control gypsy-moth (*Lymantria dispar*) populations (Hajek *et al.*, 1990) and *Erynia radicans* to control the

spotted alfalfa aphid invasion of Australia in the mid-1970s (Milner *et al.*, 1982; Carruthers and Soper, 1987). These mostly obligate pathogens are not suitable for use as mycoherbicides or mycoinsecticides in an inundative strategy because they are not readily culturable and/or mass production must be done on the living host, which is not economically feasible.

Pathogens suitable for use in inundative inoculations are generally readily culturable in natural or artificial substrates and are able to produce infective units readily in culture. These properties, combined with the ease with which many facultative pathogens can be genetically altered, make them more amenable to analysis and manipulation at the molecular level than more fastidious pathogens. A further related consideration is that commercial products, including Collego (*Colletotrichum gloeosporiodes* f. sp. *aeschynomene* vs. northern joint-vetch) (developed by Upjohn Corp. with rights currently held by Ecogen), *C. gloeosporioides* f. sp. *malvae* vs. round-leaved mallow (developed by PhilomBios) and DeVine (*Phytophthora palmivora* vs. strangle-vine) (Abbott laboratories), are hemibiotrophic pathogens. They initially colonize plants in a biotrophic manner (a characteristic of many obligate pathogens) and hence do not kill cells or invoke the immune response. However, having invaded large amounts of tissue, they then kill plant tissues and enter a destructive necrotrophic phase, killing the plant. The combination of biotrophy and necrotrophy makes these pathogens highly destructive (Greaves *et al.*, 1989).

Insect pathogens currently employed as inundative control agents are all *Deuteromycetes* (class Hyphomycetes). Insect-pathogenic fungi are of special relevance for biological control as: (i) they are the major natural means of control for many insect pest populations; and (ii) they provide the only practical means of microbial control of insects that feed by sucking plant or animal juices and for the many coleopteran pests that have no known viral or bacterial diseases. The largest programme entailing fungi for insect control is that of the People's Republic of China, where at least 1,000,000 ha of pine forest are treated every 3 years with conidia of *Beauveria bassiana* to control pine moth (Xu, 1988). Other large programmes occur in the former USSR and in the Philippines. In Brazil, *Metarhizium anisopliae* is produced by small companies or grower cooperatives and used to treat approximately 100,000 ha of sugar cane annually for spittlebug control (see Roberts and Hajek, 1992, for review). Entomopathogenic fungi may also assume importance in US agriculture and household entomology. *M. anisopliae* was registered by the US Environmental Protection Agency (EPA) in 1993 for cockroach control and in 1995 for termite control, and registration packages for the use of *B. bassiana*, another imperfect fungus, were recently approved for grasshoppers and whiteflies. *Verticillium lecanii* is registered in Europe, and there is interest in it in the USA, particularly for aphid and whitefly control.

More than 100 pathogens have been identified as having the potential for biological control of weeds and many institutions and universities are now involved in research (see Quimby and Birdsall, 1995, for review).

Biotechnology – the Potential to Provide a Vast Array of New Products

Almost all pathogens currently being used or tested as commercial products have been selected from among wild-type field strains following screening against the pest insect or weed. Acceptance of these products has been limited and they represent less than

2% of the total insecticide and herbicide market. There is a perception among farmers that, compared with conventional chemical products, biologicals are not as fast-acting, lose their effectiveness more rapidly, have a narrower host spectrum and require more knowledge to use effectively. The advanced engineered approach begins by attempting to remedy these deficiencies and could lead to designing the ideal biocontrol organism using genetic engineering. It relies on molecular biology's power of specificity to identify genes conferring pathogenicity to diverse hosts and the development of a gene bank of cloned pathogen genes, each of which controls a different virulence trait. Genetic engineering would employ these and other genes to produce a genetic fusion of many desirable characteristics into the microbe stocks. Cloning of genes from many fungi, as well as their addition and expression in fungi, have become rather simple and straightforward (reviewed by St Leger and Joshi, 1997). Genes encoding biochemical entities already implicated in pathogenicity have been cloned by a range of approaches, which include the use of heterologous DNA probes (Desjardin *et al.*, 1992; Joshi *et al.*, 1995), oligonucleotide probes or primers based on conserved regions of genes (Kusserow and Schafer, 1994) or heterologous expression (Froeliger and Leong, 1991) or the screening of expression libraries with antibodies (Osbourn *et al.*, 1994). These techniques require that pathogenicity determinants be predicted from a prior knowledge of gene function. An alternative cloning strategy is to isolate pathogenicity genes that are specifically expressed during invasion processes, as amongst these may be genes that have a key role in allowing the pathogen to establish itself in the plant or insect host. Differential hybridization (St Leger *et al.*, 1992a, c; Talbot *et al.*, 1993; Pieterse *et al.*, 1994) and differential display (Joshi *et al.*, 1998) techniques allow isolation of infection-regulated genes without making any assumptions about their products. A similar approach – technically less demanding but considerably more expensive – is Expressed Sequence Tag (EST) analysis. Our ongoing EST project to identify the full range of genes expressed during the infection process by the 'generalist' (wide-host-range entomopathogen) *M. anisopliae* strain ME1 and the 'specialist' (narrow-host-range entomopathogen) *Metarhizium flavoviride* strain 324 has allowed us to identify thousands of genes expressed during pathogenicity (www.http:tegr.umd.edu). This collection provides a resource of genes both for the genetic improvement of entomopathogenic fungi and for other biotechnological applications (e.g. insect-resistant plants). Complementary approaches for the identification of pathogenicity genes involve the generation of pathogenicity mutants by random mutagenesis (e.g. REMI), with subsequent characterization of the induced mutations (Bolker *et al.*, 1995). The great promise of these 'Black box' techniques is that they will identify currently unsupected stratagems of pathogen attack.

Strain improvement can be achieved in a variety of ways, from random selection of (ultraviolet (UV)/chemical-induced) mutants to site-directed homologous gene replacement techniques. The technique chosen depends upon the availability of suitable selectable markers (e.g. antibiotic resistance), transformation systems and the desired phenotypic change. In recent years a number of robust methodologies for fungal transformation have been developed, including Ca^{2+}/polyethylene glycol (PEG)-mediated protoplast transformation, electroporation and particle bombardment (reviewed by St Leger and Joshi, 1997). Combinations of desired genes can then be created by the mating of suitable strains (not available for deuteromycete fungi). In the absence of a sexual stage, the parasexual cycle (anastomosis) may be used (Messias and Azevedo, 1980; Bello and Paccola-Meirelles,1998), or a 'forced' union can be

achieved by means of protoplast fusion techniques (Viaud *et al.*, 1998). Unfortunately, there is still no effective way to stably transform biotrophic fungi.

Characteristics that might Benefit from Genetic Manipulation

There are many common threads running through previous studies on plant and insect pathogens and many of the insights, research methods and aims developed for one system also apply to the other. Both entomopathogens, e.g. *Metarhizium* spp., and plant pathogens, e.g. *Colletotrichum* spp., infect their hosts via conidia, which attach, swell and form a germ tube upon contact with a suitable host (St Leger *et al.*, 1989; Dickman *et al.*, 1995). The germling then develops an appressorium, a terminal swelling in the germ tube, from which a narrow infection peg eventually penetrates the external cuticular surface. Many of the apparent differences between these two pathogens arise from the fact that *M. anisopliae* penetrates a (mostly) proteinaceous insect cuticle, while *Colletotrichum trifolii* penetrates a (mostly) carbohydrate plant cuticle. For both plant and insect pathogens, however, fungal perception of and response to its host are likely to be critical in dictating the sequence of events that culminate in a successful infection and will therefore be important targets for molecular manipulation. It is likely that several broad classes of pathogenicity genes are involved in these processes. Some genes encode receptors that detect either directly or indirectly the presence of the host (e.g. a guanosine triphosphate (GTP)-regulated adenylate cyclase, tyrosine protein kinases, serine and threonine protein kinases, and phosphoprotein phosphatases (reviewed in St Leger, 1993; Dickman *et al.*, 1995)). These act to change second-messenger levels or are themselves activated by second messengers to trigger differentiation. Activation of such receptors and signal transduction pathways may result in the induction of generic pathogenicity genes. These could include another class of pathogenicity genes that inactivate host defences, such as the detoxifying enzymes produced by *Gaemannomyces graminis* and *Gloeocercospora sorghi* (which both detoxify preformed inhibitors of fungal growth) (Osbourn *et al.*, 1994; Van Etten *et al.*, 1994) and *Nectria haematococca* (which detoxifies the pea phytoalexin pisatin) (Van Etten *et al.*, 1994). Other pathogenicity genes may encode toxins that are required for disease symptoms, e.g. non-specific toxins, such as trichothecenes, produced by a number of *Fusarium* spp. (Oliver and Osbourn, 1995), and cytochalasins and destruxins, produced by *M. anisopliae* or host-selective toxins, produced by members of the genus *Cochliobolus* (reviewed by Oliver and Osbourn, 1995). A fourth category of pathogenicity genes encodes enzymes that allow the fungus to overcome host barriers. To determine whether such pathogenicity genes exist and what the characteristics of each class are, it is necessary to characterize multiple genes conferring pathogenicity to diverse hosts, preferably from several pathogen species. This will allow the development of a gene bank of cloned pathogen genes, each of which controls a different virulence trait. The availability of these genes raises the possibility of creating novel combinations of insect specificity by expressing them in other fungi, as well perhaps as bacteria or viruses, if that would produce an improved pathogen.

The broad classes of pathogenicity genes detailed above suggest that directed changes to alter virulence could result from the manipulation of nearly every aspect of fungal developmental biology (summarized in Table 8.1).

We have now isolated genes (as mentioned above) from the entomopathogenic fungus *Metarhizium* spp. involved in all of these developmental processes (S. Screen

Table 8.1. Gene products that could be manipulated to enhance virulence

Receptors that detect the presence of the host
Enzymes that facilitate penetration of the host
Gene products that inactivate host defences
Toxins that are required for disease symptoms

and R. St Leger, unpublished), providing a resource of useful genes for strain improvement. In addition, we have analysed the biochemical and molecular mechanisms underlying the complex regulatory mechanisms controlling appressorium formation, enzyme production and penetration in *M. anisopliae* (Screen *et al.*, 1997, 1998; St Leger *et al.*, 1998). Understanding these molecular mechanisms is a prerequisite for making full use of this resource.

An immediate issue of prime importance is how to select those genes which offer the greatest immediate potential in improving the efficacy and reliability of fungi for pest control. The following is a wish list of characteristics that might benefit from genetic improvement.

Improving virulence – speed of kill

A major deterrent to the development of fungi as pesticides has been that it can take 5–15 days post-infection to kill the targeted pest. Based on this slow speed of action, fungi, as well as many other microbial control agents, were considered to have poor commercial efficacy. An obvious solution to this problem is genetic engineering, the idea being to add a new gene to the fungus that would allow the fungus to kill the plant or insect host more quickly and/or to prevent insects from feeding after infection. Most attention has been focused on the speed with which pathogens are able to infect the host, as this is believed to contribute significantly to escape from environmental hazards and to aggressive pathogenicity. For phytopathogens, the plant cell wall is the major barrier to infection and is composed of an array of polysaccharides and protein (Walton, 1994). Penetration of this barrier is believed to involve a combination of mechanical pressure and enzymatic degradation of cell-wall components. Cell-wall-degrading enzymes are produced by single genes and hence genetic manipulation offers attractive possibilities for enhancing pathogenesis by improving the ability of fungi to penetrate and colonize host tissues. However, disruptions of genes encoding cutinases (Stahl and Schafer, 1992; Crowhurst *et al.*, 1997; van Kan *et al.*, 1997), xylanases (Abel-Birkhold and Walton, 1996), pectinase (Scott-Craig *et al.*, 1990; Centis *et al.*, 1997) and cellulase (Sposato *et al.*, 1995) have not dramatically reduced pathogenicity, perhaps due to the redundancy of the encoding genes, i.e. residual activities remain after gene disruption (Mendgen *et al.*, 1996). Presumably, production of mixtures of compounds that affect a number of systems increases the adaptability of the pathogen and minimizes the possibility of resistance developing to the principal toxin(s). In any event, gene disruption is unlikely to provide an efficient way of assessing function of these secreted proteins if their effects can substitute for each other. The fact that the role of these genes in pathogenicity remains uncertain does not preclude them from being used for strain improvement of weed pathogens. In fact, while the multiplicity of these molecules provides a major challenge with respect to establishing

the function of each molecule in pathogenicity, the variability of molecules with activity against host substrates increases the range of tools naturally available for developing biotechnological procedures for pest control.

We have developed a strategy of developing transformation and vector systems to introduce depolymerases and toxins into insect pathogens that normally lack them or to alter their mode of action in a way that would increase speed of kill. For example, as activation of fungal infection processes involves the expression of many inducible proteins, constitutive expression provides a direct strategy for engineering enhanced virulence. This may override effects produced by physical and chemical signals that induce a transient expression of actions of the gene. The most attractive initial candidates for this approach include genes encoding cuticle-degrading enzymes and toxins, as these have often been shown to be active synergistically *in vitro* against insects (reviewed in St Leger, 1993), and, since the active agents are encoded by single genes, they should be highly amenable to manipulation by gene transfer.

The insect pathogen *M. anisopliae* produces multiple cuticle-degrading proteases that are encoded by several gene families. We used *M. anisopliae* to develop the first genetically improved entomopathogenic fungus (St Leger *et al.*, 1996c). Additional copies of the gene encoding the regulated cuticle-degrading Pr1 protease were inserted into the genome of *M. anisopliae* under the control of an *Aspergillus pgd* promoter such that the gene was constitutively overexpressed.

In contrast to the wild type, transgenic strains continued to produce Pr1 in the haemocoel of *Manduca sexta* caterpillars following penetration of the cuticle. This caused extensive melanization in the body cavity and cessation of feeding 40 h earlier than controls infected with the wild type. Pr1 was found to act indirectly by activating a trypsin-like enzyme that is involved in a cascade terminating in prophenoloxidase activation. This was facilitated by Pr1 possessing pathogenic specializations that distinguish it from similar molecules produced by saprophytes. Thus Pr1 is resistant to proteinase inhibitors (serpins) present in insect blood and even to being in a melanizing milieu, mimicking the insect defence response (St Leger *et al.*, 1988). Insects killed by transgenic strains and extensively melanized were very poor substrates for fungal growth and sporulation. This reduces transmission of the recombinant fungi providing a degree of biological containment (St Leger *et al.*, 1996b). It is also consistent with the new emphasis of using entomopathogenic fungi as 'contact insecticides' that achieve a quick kill (Prior, 1992).

Other *M. anisopliae* molecules also show pathogenic specializations, symptomatic of the fact that fungi may have spent millions of years of evolution refining chemicals that subdue their hosts; this makes their proteins choice candidates for producing improved transgenic organisms (St Leger and Bidochka, 1996). This suggests that we can use the multifarious secreted compounds produced by the entomopathogens themselves as a resource for their genetic improvement. This is important, as the use of homologous genes, albeit under altered regulation, provides an experimental design that seems inherently unlikely to raise public concern.

Restricting or widening their specificities

One of the positive environmental attributes of many naturally occurring fungi is that their reported host ranges are limited to a small number of plants or insects that do not include beneficial species. In this way, they are seen as environmentally safer than

chemicals, which may have more widespread effects. Certainly, many obligate pathogens show a specificity that is unequivocal. For example, *Erynia variabilis* is restricted to certain small dipteran flies, in part by a requirement for oleic acid to induce germination (Kerwin, 1984). However, many of the facultative pathogens currently being considered for pest control are less fastidious and have a broad host range. Even *C. gloeosporioides* f. sp. *aeschynomene* (Collego), which was initially thought to be highly specific to its leguminous target, northern joint-vetch (*Aeschynomene virginica*), can also cause low-level infections in several crop legumes (TeBeest, 1988). Fortunately, environmental risks can be reduced by strain selection or by innovative techniques such as the development of auxotrophs (Quimby and Birdsall, 1995). For example, *Rhizoctonia solani* is a broad-spectrum pathogen with the potential to control difficult weeds. Selected strains vary in their mode of attack and in their pathogenicity and may be able to be used against target weed species with low risk to non-target species (Caeser *et al.*, 1993). However, the limited range of some insect pathogens has also been a deterrent to commercial development, because this generally limits the potential market size. Furthermore, a narrow host range limits the usefulness of a microbial pesticide if a crop is attacked by a diverse group of pests, as is often the case. The interests of commercial profitability and protection of non-target species may collide over the issue of target range. A grower wants to apply just one agent to control all insect pests and one agent to control weeds on his/her crop. At present, when developing specific pathogens, industry has to cover the costs of registration, production and marketing for a pathogen for each pest. Very few pests are important enough to justify such an effort. Clearly, a major goal in developing fungal pathogens of plants and insects is to restrict or widen their specificity. This has been achieved with bacteria. Avirulence genes have been isolated from several plant-pathogenic bacteria, and the transfer of avirulence genes from one strain of bacterium to another has been shown to restrict the host range of the recipient organism (Staskawicz *et al.*, 1984). Likewise, Sandoz Agro (Palo Alto, California) used recombinant DNA techniques to transfer delta-endotoxin genes between *Bacillus thuringiensis* (*Bt*) strains to produce varieties that kill multiple types of insects. Compared with bacteria, less is known concerning the basis of host specificity for fungal pathogens, and what is known is limited to a very few model organisms. In some strains, adhesion of spores is host-specific. For example, conidia of an *M. anisopliae* isolated from the scarab beetle *Cetonia aurata* readily attached to *C. aurata* cuticle, but failed to adhere to a related non-host scarab (Fargues, 1984), indicating that specific attachment may be the earliest event in this host–pathogen interaction (Boucias and Pendland, 1991), as in fungal–plant interactions (Nicholson, 1996). Al-Aidroos and Bergeran (1981) reported that a gene determining specific adhesion by *M. anisopliae* spores was linked to that for brown spore colour, but the molecular basis for such specific adhesion remains to be established.

The absence of genes responsible for cuticle-degrading enzymes would presumably prevent penetration of host barriers. For example, while several studies employing gene disruption have not confirmed a role for cutinase in pathogenicity to plants, insertion of a cutinase gene from *N. haematococca* into an opportunistic wound pathogen enabled it to infect an intact host (Dickman *et al.*, 1989). Many studies on host range and specificity in plant pathogens have focused on detoxification of plant substances by fungi. For example, oat-attacking isolates of *G. graminis* are insensitive to the toxic effects of the oat saponin, avenacin, which is degraded by the pathogen enzyme avenacinase. Gene disruption of avenacinase produced mutants incapable of

infecting oats but fully pathogenic to a non-saponin-containing host (wheat), providing genetic evidence that saponin detoxification determines host range (Osbourn *et al.*, 1994). Interestingly, while *N. haematococca* mutants disrupted in the pisatin demethylase (PDA) gene do not show a reduction in pathogenicity (van Etten *et al.*, 1994), introduction of the gene into PDA-deficient isolates with a low level of pathogenicity to pea conferred PDA activity and increased pathogenicity (Ciufetti and van Etten, 1996). This supports the contention that negative results following gene disruption do not exclude a role for a gene in strain improvement and that genes encoding detoxifying proteins could be used to broaden the host range of a microorganism that is otherwise not pathogenic. Toxins produced by fungi can also influence specificity. Within members of the genus *Cochliobolus* (reviewed by Oliver and Osbourn, 1995), toxin production has been implicated in determining: (i) the virulence of race T of *Cochliobolus heterostrophus* to maize lines with Texas-type cytoplasmic male sterility (*cms-T*) (T-toxin); (ii) the pathogenicity of *Cochliobolus carbonum* race 1 to certain maize varieties bearing the dominant allele of the *Hm* gene (HC-toxin) – these varieties are resistant to non-toxin-producing races; and (iii) the ability of *Cochliobolus victoriae* to cause blight on Victoria oats (victorin). Toxin production is controlled by a single gene in each of the three species and hence readily amenable to genetic manipulation.

To date, little is known concerning the biochemical or molecular basis of host specificity for weed and insect pathogens. However, it seems likely from the above that responsible genes could control: adhesion (surface features favourable to surface attachment); the ability to exploit conditions (nutrients, humidity, specific recognition factors) on the cuticle surface; resistance to inhibitory compounds; the ability to overcome structural and chemical barriers to penetration; and the ability to produce toxins that damage hosts and weaken host defenses.

Reducing inoculum

The advantages of lower inoculum levels would make the processes of adhesion and formation of infection structures attractive possibilities for development. Altering events that occur subsequent to this, e.g. the production of cuticle-degrading enzymes, may affect speed of kill but without influencing the amount of inoculum required to kill an insect host (St Leger *et al.*, 1996b). Biochemical studies have shown that appressorium formation by *M. anisopliae* involves disruption of calcium gradients, redirecting cell-wall synthesis from the growing hyphal tip to the entire surface of the cell, reducing extension growth and producing a swollen appressorium (St Leger, 1993). A similar model may also apply to cellular differentiation of the plant pathogen *C. trifolii* (Dickman *et al.*, 1995). In both fungi, various protein kinases, including calcium- and calmodulin-dependent kinases and cyclic AMP (cAMP)-dependent kinases, were shown to function during fungal development and differentiation. It is evident that unifying themes exist in the manner in which disparate plant and animal pathogens respond to environmental signals. To utilize the specific molecular machinery involved in signal transduction for biotechnology it will be necessary: (i) to understand the relationship between the formation of infection structures and the expression of virulence; (ii) to determine which signal transduction mechanisms operate in the early stages of infection as compared with the later stages of development; and (iii) to determine how utilization of a complex array of host signals (nutrients, thigmotropic stimuli, chemical recognition factors) may facilitate the deployment of pathogen responses. Exploiting

this, it may be possible to produce transgenic pathogens expressing the relevant genes necessary to reduce the time expended by the fungus in penetrating host surfaces. This could reduce the susceptibility of the fungus to hostile environmental conditions and inoculum loads, hasten host death and provide a more effective strategy for pest control by fungal pathogens. To date, this has not been done, but several candidate genes have been isolated. Exogenously added cAMP stimulated appressorium formation by the rice blast fungus, *Magnaporthe grisea*, while gene disruption reduced formation of infection structures (Mitchell and Dean, 1995) and demonstrated an additional role for cAMP signalling in plant penetration (Xu and Mengden, 1997). Another signal transduction pathway required for appressorium formation involves a MAP kinase called Pmk1 (Xu and Hamer, 1996). A similar MAP-kinase signalling pathway may operate in the unrelated plant pathogen *Ustilago maydis* (for review see Kahmann *et al.*, 1995), the human pathogen *Candida albicans* (Kohler and Fink, 1996) and the insect pathogen *M. anisopliae* (S. Screen and R. St Leger, unpublished data), suggesting that a common signal transduction pathway may have evolved to regulate pathogenic growth in a variety of fungi (Hamer and Holden, 1997). However, elements of this pathway are also present in non-pathogens, so it will be important to learn what components are specific to pathogens and what signals activate these pathways. This would facilitate exploitation of this pathway for biotechnology.

Altering persistence

The development of pathogens for classical biological control depends on their being able to recycle through host populations. However, most pathogens currently being considered for genetic enhancement would be applied in an inundative manner and there are both environmental and commercial reasons why it would not be beneficial for an introduced or transgenic pathogen to persist into the next season. Thus, commercial interests in developing pest control agents rely on the probability of achieving profitability through repeat sales. The ecological relationships between fungi and their hosts are not well understood, so it is difficult to access the possibility that foreign or engineered fungi will displace native populations or possess some other unanticipated properties that would warrant mitigation. Elimination of a fungus from nature would be highly problematic and fungal spores can survive for years in soil. Therefore, it would seem prudent to engineer fungi in such a way that they would be at a selective disadvantage in nature. In this context, spore-killing factors, double-stranded RNA and viruses and specific metabolite control of the introduced genes suggest possible approaches (Koltin *et al.*, 1987). Auxotrophs of *Sclerotinia sclerotiorum* have been developed which require pyrimidine to grow. These auxotrophs can be spot-applied to a broad spectrum of weeds, with pyrimidine added to allow activity. When the pyrimidine is depleted, the fungus dies out (Miller *et al.*, 1989).

Obtaining tolerance to environmental constraints

Genetically based resistance to desiccation and temperature extremes would be a distinct advantage, both during infection and during product preparation and storage. However, these properties appear to be governed by polygenic mechanisms too complex to be readily amenable to genetic manipulation. Immediate advances are likely to

come from strain selection, and the discovery of an isolate of *V. lecanii* from aphids that can grow at unusually low humidities (Drummond *et al.*, 1987) suggests that this trait would be worth seeking in other fungi (Prior, 1992).

Resistance to fungicides

If a fungus is to be used as part of an integrated pest management programme, it will be advantageous for the fungus to be resistant to certain fungicides (Greaves *et al.*, 1989). This can be accomplished by strain selection, mutation or gene transfer.

Tracking Transgenic Fungi in the Field

Unlike most classical biocontrol procedures, it is unlikely that researchers would seek to permanently establish an engineered agent in the environment. The 'first'-generation product would probably be localized and temporary because of reduced potential for secondary infection. There is an inherent uncertainty because of the paucity of our knowledge concerning the fate of fungal genotypes at the population and ecosystem level. In fact, there is no information available on survival of individual genotypes (clones) of entomopathogenic fungi in nature, nor is there experimentally derived information on gene transfer from populations of genetically engineered pathogenic fungi to wild-type or other fungal species.

The desire to release transgenic organisms into the environment is providing a powerful motivation for studies on microbial ecology. Regulatory bodies, businesses developing products, as well as scientists themselves, are seeking systems that balance relative benefits with relative risks. These risks include potential effects on human health and environment, concerns that engineered organisms might cause ecological perturbations by replacing related organisms and the potential for the foreign material to be transferred to other organisms (Wood, 1994).

To obtain information on the survival of specific genotypes of entomopathogenic fungi in nature and to quantify with precision the epidemiological effects of genetic modifications, it will be necessary to develop methods to monitor the pathogen's survival and migration within the background of the complex microbial communities into which they will be introduced. Allozymes provided the first unambiguous markers available in sufficient numbers to enable reliable genetic studies of entomopathogenic fungi (St Leger *et al.*, 1992b). Random amplification of polymorphic DNA (RAPD) provided additional markers (Bidochka *et al.*, 1994). Strain-specific DNA probes were produced to track a wild-type Australian strain of *Entomophaga grylli* introduced into the USA for grasshopper control (Bidochka *et al.*, 1996).

Nucleic acid probing is highly specific and allows inocula to be tracked if the detected DNA sequence is stable, but gives no indication of the viability or activity of a specific population and is a poor tool for predicting the environmental impact of an inoculum. The application of molecular markers (i.e. introduced genes conferring distinctive phenotype properties) has greater potential for increasing our understanding of environmental microbiology. GUS expression has been found to be a practical means of identifying and localizing the active biomass of marked strains under different environmental conditions (St Leger *et al.*, 1995). In combination with techniques such as DNA probes and clamped homogeneous electrical field (CHEF) pulsed-field gel

electrophoresis analysis, marker genes will allow the analysis of potential gene transfer to indigenous fungal strains, i.e. by determining whether fungi retain the marker elements in their original form. To this end, it would probably be a wise precaution to construct transformants containing two or three different marker genes in the genome, as it is unlikely that multiple markers would all be lost at once if gene transfer occurred.

Problems in the Development and Commercialization of Genetically Engineered Fungi

The unacceptable broad chemical toxicity of many pesticides, regulatory considerations, economics and pest resistance have led to a marked decline in the number of chemical options available to growers for insect and weed control (see Krimsky and Wrubel, 1996, for review). Microbial pesticide producers regard this as an opportunity to promote the advantageous qualities of their products to farmers. Furthermore, while it costs $40 million and takes 4 years to develop and register a new chemical insecticide in the USA, it cost Mycogen only $1 million to bring each of its recombinant *Bt* products to market. However, even with the incentives to reduce reliance on chemical insecticides, microbial pesticides have had very little market success. At present, two commercial mycoherbicides are used on a relatively large scale in the USA, while worldwide only six species of insect pathogen are employed for pest control (TeBeest, 1991; Roberts and Hajek, 1992; Charnley, 1997; Gressel, 2000). To date, the promising techniques of genetic engineering have not produced any new commercial fungal products. It is necessary to consider why their application is so limited. A review of the literature, in particular articles by Wood (1994), Krimsky and Wrubel (1996), NABC6 and the US Congress, Office of Technology assessment OTA-ENV-636, reveals the following key points:

1. Fuelled by lavish venture capital and enthusiasm for biotechnology, a flush of biocontrol companies went public in the 1980s to exploit the potential biologicals offered as environmentally benign alternatives to chemicals.
2. The widely predicted demand for biologicals never materialized and many companies downsized. Biocontrol now makes up less than 2% of the global pesticide market.
3. Even this fraction is threatened as new pesticide chemistries come on-line.
4. On the plus side, current federal biotechnology policy is designed to stimulate innovation and to enable the US biotechnology industry to achieve hegomony in global markets. The thrust is a minimalist, cost-effective, priority-driven approach, requiring a burden of proof that regulation is warranted. The burden of proof is then on those who advance a risk scenario and, as agency resources are scarce, responsible officials carefully choose the risks of highest concern.
5. Evidence suggests that early regulatory inaction or confusion kept firms from investing in transgenic microorganisms. Companies do not make a similar case today, as biotechnology in the USA is not burdened with over-regulation. There is, however, significant divergence between American and European regulations.
6. Technical problems, especially lack of efficacy, are probably more important than any government constraints in explaining the relatively slow progress of the industry. Environmental, health and safety considerations do not sell pest control products to most farmers. The bottom line for most farmers is how well the new products work, how easy they are to use and how much they cost.

7. Companies developing biological pesticides see themselves as part of the larger pest control industry and not as an alternative industry seeking to replace conventional insecticides.
8. Many innovations in agricultural biotechnology are science-driven rather than need-driven. Industry has developed powerful tools to manipulate organisms and is seeking ways to develop products using these techniques that will generate economic value. Hence the thrust of the biotechnology industry is not to solve agricultural problems as much as it is to create profitability.
9. Major companies have targeted larger mainstream farmers rather than small organic operations (traditional mainstay of biocontrol products) – even though large farms and their customers (grocery chains, food processors) require the high cosmetic standards achievable with chemical products.
10. The chemical model emphasizes major crops and cheap, stable products that are easy to scale up and use. Most biocontrol agents fit this model poorly. Their success will depend on the ability to improve the efficacy and consistence of products and to provide consistent support to farmers and extension personnel on the techniques needed to maximize effectiveness and avoid pest resistance problems.
11. If genetic engineering succeeds in creating microbial pesticides that are more equivalent to conventional pesticides (more toxic modes of action, increased kill rates and extended environmental persistence), scientists will have engineered out the very characteristics of target specificity and short field persistence that make current microbial pesticides relatively benign.

A further consideration is that microbial pesticides will have to compete with genetically engineered plants and the number of field tests of transgenic plants dwarfs that of genetically engineered microbes. Two factors may help explain this. First, our understanding of the biology and ecology of microorganisms is limited, especially when compared with higher plants and animals. Therefore more uncertainty is associated with predicting the probability of untoward effects associated with the environmental release of microbes. Secondly, unlike field tests of transgenic plants, even small-scale field releases of genetically engineered microbes are difficult to contain (Wood, 1994; Krimsky and Wrubel, 1996).

The US agricultural market for herbicides in 1994 was more than $3.9 billion (thousand million) annually, compared with $1.1 billion for insecticides and $0.9 billion for fungicides (Krimsky and Wrubel, 1996). The greatest opportunity in herbicide usage presented by the advent of gene transfer technology has been to create new crop strains that are resistant to herbicides so that broad-spectrum weed killers, such as Round-Up, will selectively control weeds in crops (see Cole, 1994, for review). Transformation of crops to herbicide resistance is among the most controversial applications of biotechnology to agriculture. A prominent theme of the agricultural biotechnology industry is that genetically engineered crops will reduce the use of pesticides and are thus environmentally beneficial and should aid in the development of sustainable agriculture. Crops engineered with the *Bt* protein to kill insects have been cited by industry as an example. Likewise, entomopathogenic fungi represent an unconsidered, and therefore untapped, reservoir of pesticidal genes for the production of insect-resistant plants – an important consideration, given that the lack of 'useful' pesticidal genes for transfer has been a major constraint in the implementation of biotechnology in crop protection (Gatehouse *et al.*, 1992). In contrast, the ability of herbicide-resistant crops to reduce herbicide use is very doubtful. In fact,

agrochemical companies see herbicide-resistant crops as a way of increasing the value of certain herbicides by expanding their range of uses to fields containing growing crops (Krimsky and Wrubel, 1996). If herbicide-resistant crops provide an extension of high-input chemically intensive agriculture in the USA then the genetic enhancement of mycoherbicides provides an opportunity for biotechnology to reduce herbicide use.

Pathogenic Fungi as a Possible Resource of Genes in Producing Recombinant Viral and Plant Products

Producing an engineered biocontrol insect-pathogenic fungus may not always be the most effective means of delivering a fungal anti-insect gene (depending on the insect pest and host plant in question). A very profitable direction for research could be to use genes encoding the battery of hydrolytic enzymes, toxins and other anti-insect components produced by various entomopathogenic fungi to improve the performance of pathogens that already possess a set of suicide mechanisms (thus alleviating public concerns and reducing the possibility of environmental damage), or to directly introduce anti-insect genes into the genome of plants. In this case, pioneer products have already paved the way for registration. One such strategy for containing recombinant strains is the co-occlusion process for baculovirus populations (reviewed by Wood, 1994). The potential of using baculoviruses for insect pest control has long been recognized as they have minimal environmental impact and high target specificity. At present, baculoviruses compete inadequately with classical insecticides, partly because of their slow speed of action. An important goal of genetically engineering these viruses has been to improve their ability to kill target insects rapidly. An example of this type of approach has been the construction of improved baculovirus insecticides containing toxin genes from spiders or mites (Stewart *et al.*, 1991). Several groups are also investigating the potential of insect hormones or hormone regulators, but with little success so far at improving pathogen performance (Possee, 1993).

Based on the potential utility of foreign gene inserts constructed to date, particularly toxins, the search for additional pesticidal genes is clearly a commercial priority. The insertion and expression of additional genes is performed very simply. The primary limitation in this area has been the availability of pesticidal genes (Stowell, 1994; Wood, 1994). Arthropods have provided a major resource for toxins, but analysis of these is usually hampered by the extremely small quantities of material available. Fungi are much more amenable to molecular analysis, and the screening of fungal genes encoding fast-acting proteins could greatly increase the scope of such studies. To confirm that the baculovirus expression system can produce a biologically active toxin from *M. anisopliae* complementary DNA (cDNA), we have introduced the Pr1 gene into the *Autographa californica* nuclear polyhedrosis virus (ACMNPV) to produce Pr1-BEV. Time to death following infection by Pr1-BEV was reduced by about 50% compared with controls, indicating that the recombinant Pr1 retained its toxic capacity (Huang *et al.*, 1999).

Protection of crops from their insect pests is already a major goal of plant genetic engineering. Fungal genes with anti-insect activity could be used in plant transformation procedures so that the insect will be forced to encounter the gene product when feeding or interacting with the specific host plant. Future success in developing transgenic plants will benefit from the availability of as wide as possible a range of suitable genes. In spite of the vast range of compounds available from fungi, up to now fun-

gal genes have played little part in the 'invisible revolution' resulting from the implementation of biotechnology in crop protection. To date, the vast majority of the work on incorporation of microbial genes, as well as on all other aspects of microbially mediated biocontrol, centres around *Bt* toxins. As a result of relying on a single agent for biocontrol, there is mounting concern that the evolution of resistance to *Bt* toxin will greatly reduce its utility (Krimsky and Wrubel, 1996). Thus far, there are few alternative insecticidal genes and the lack of useful genes for transfer has become a major constraint on this technology. This again is a strong reason for widening the scope of searches for genes, and entomopathogenic and other fungi could provide a major untapped reservoir of insect resistance genes to supplement *Bt* toxin. Fortunately, pioneering products engineered with *Bt* crystal protein genes have already paved the way for registration and for strain acceptance by breeders, farmers and consumers (Krimsky and Wrubel, 1996). Recombinant microbial products and transgenic plants could potentially have complementary roles to play in plant protection. Combinational integration of the large repertoire of anti-insect genes from fungi into ongoing plant breeding programmes or into alternative vectors should make an important contribution to effective, durable crop protection.

References

Abel-Birkhold, P. and Walton, J.D. (1996) Cloning, disruption and expression of two endo-β 1, 4-xylanase genes, *XYL2* and *XLY3* from *Cochliobolus carbonum. Applied Environmental Microbiology* 62, 4129–4135.

Adams, E.B, and Line, R.F. (1984) Biology of *Puccinia chondrillina* in Washington. *Phytopathology* 74, 742–746.

Al-Aidroos, K. and Bergeran, D. (1981) Use of the parasexual cycle to relate spore adhesiveness to virulence in the entomopathogenic fungus, *Metarhizium anisopliae. Genetics* 91 (suppl. 1), 52.

Bello V.A. and Paccola-Meirelles, L.D. (1998) Localization of auxotrophic and benomyl resistance markers through the parasexual cycle in the *Beauveria bassiana* (Bals.) Vuill entomopathogen. *Journal of Invertebrate Pathology* 72 (2), 119–125.

Bidochka, M.J., St Leger, R.J. and Roberts, D.W. (1994) Differentiation of species and strains of entomopathogenic fungi by random amplification of polymorphic DNA (RAPD). *Current Genetics* 25, 107–113.

Bidochka, M.J., Walsh, S.R.A., Ramos, M.E., St Leger, R.J., Silver, J.C. and Roberts, D.W. (1996) Fate of biological control introductions: monitoring an Australian fungal pathogen of grasshoppers in North America. *Proceedings of the National Academy of Sciences USA* 93, 918–921.

Bolker, M., Bohnert, H.U., Braun, K.H. and Kahmann, R. (1995) Tagging pathogenicity genes in *Ustilago maydis* by restriction enzyme-mediated integration (REMI). *Molecular Genetics* 248, 547–552.

Boucias, D.G. and Pendland, J.C. (1991) Attachment of mycopathogens to cuticle: the initial event of mycosis in arthropod hosts. In: Cole, G.T. and Hoch, H.C. (eds) *The Fungal Spore and Disease Initiation in Plants and Animals*. Plenum Press, New York, pp. 101–128.

Caeser, A.J., Rees, N.E., Spencer, N.R. and Quimby, P.C. (1993) Characterization of *Rhizoctonia* spp. causing disease of leafy spurge in the Northern Plains. *Plant Diseases* 77, 681–687

Carruthers, R.I. and Soper, R.W. (1987) Fungal diseases. In: Fuxa, J.R. and Tanada, Y. (eds) *Epizootiology of Fungal Diseases*. John Wiley & Sons, New York, pp. 357–416.

Centis, S., Guillas, I., Sejalon, N., Esquerre-Tugaye, M.T. and Dumas, B. (1997) Endopolygalacturonase genes from *Colletotrichum lindemuthianum*: cloning of *CLPG2* and

comparison of its expression to that of *CLPG1* during saprophytic and parasitic growth of the fungus. *Molecular Plant-Microbiol Interactions* 10, 769–775.

Charnley, A.K. (1997) Entomopathogenic fungi and their role in pest control. In: Wicklow, D.T. and Soderstrom, B.E. (eds) *The Mycota* IV *Environmental and Microbial Relationships*. Springer, Berlin, pp. 185–201.

Ciuffetti, L.M and van Etten, H.D. (1996) Virulence of a pisatin demethylase-deficient *Nectria haematococca* MPVI isolate is increased by transformation with a pisatin demethylase gene. *Molecular Plant-Microbe Interactions* 9, 787–792.

Cole, D.J. (1994) Molecular mechanisms to confer herbicide resistance. In: Marshall, G. and Walters, D. (eds) *Molecular Biology in Crop Protection.* Chapman & Hall, London, pp. 146–176.

Crowhurst, R.N., Binnie, S.J., Bowen, J.K., Hawthorne, B.T., Plummer, K.M., Rees-George, J., Rikkerink, E.H.A. and Templeton, M.D. (1997) Effect of disruption of a cutinase gene (cutA) on virulence and tissue specificity of *Fusarium solani* f. sp. *Curcurbitae* race 2 toward *Cucurbita maxima* and *C. moschata. Molecular Plant-Microbial Interactions* 10, 355–368.

Desjardins, A.E., Gardner, H.W. and Weltring, K.M. (1992) Detoxification of sesquiterpene phytoalexins by *Gibberella pulicaris* (*Fusarium sambucinum*) and its importance for virulence on potato tubers. *Journal of Industrial Microbiology*, 9, 201–211.

Dickman, M.B., Podila, G.K. and Kolattukudy, P.E. (1989) Insertion of cutinase gene into a wound pathogen enables it to infect intact host. *Nature* 342, 446–448.

Dickman, M.B., Buhr, T.L., Warwar, V., Truesdell, G. and Huang, C.X. (1995) Molecular signals during the early stages of alfalfa anthracnose. *Canadian Journal of Botany* 73 (suppl. 1), S1169-S1177.

Drummond, J., Heale, J.B. and Gillespie, A.T. (1987) Germination and effect of reduced humidity on expression of pathogenicity in *Verticillium lecanii* against the glasshouse whitefly *Trialeurodes vaporariorum. Annals of Applied Biology* 111, 193–201.

Fargues, J. (1984) Adhesion of the fungal spore to the insect cuticle in relation to pathogenicity. In: Roberts, D.W. and Aist, J.R. (eds) *Infection Processes of Fungi – Conference Report.* Rockefeller Foundation, New York, pp. 99–110.

Froeliger, E.H. and Leong, S.A. (1991) The α mating-type alleles of *Ustilago maydis* are ideomorphs. *Gene* 100, 113–122.

Gatehouse, A.M.R., Boulter, D. and Hilder, V.A. (1992) Potential of plant-derived genes in the genetic manipulation of crops for insect resistance. In: Gatehouse, A.M.R., Hilder, V.A. and Boulter, D. (eds) *Plant Genetic Manipulation for Crop Protection.* CAB International, Wallingford, UK, pp. 155–182.

Greaves, M.P., Bailey, J.A. and Hargreaves, J.A. (1989) Mycoherbicides: opportunities for genetic manipulation. *Pesticide Science* 26, 93–101.

Gressel, J. (2000) Molecular biology of weed control. *Transgenic Research* 9, 355–382.

Hajek, A. and St Leger, R.J. (1994) Interactions of entomopathogenic fungi and insect hosts leading to epizootic development. *Annual Review of Entomology* 39, 293–323.

Hajek, A.E., Humber, R.A., Elkinton, J.S., May, B., Walsh, S.R.A. and Silver, J.C. (1990) Allozyme and RFLP analysis confirm *Entomophaga maimaiga* responsible for 1989 epizootics in North American gypsy moth populations. *Proceedings of the National Academy of Sciences USA* 87, 6979–6982.

Hamer, J.E. and Holden, D.W. (1997) Linking approaches in the study of fungal pathogenesis: a commentary. *Fungal Genetics and Biology* 21, 11–16.

Heale, J.B., Isaac, J.E. and Chandler, D. (1989) Prospects for strain improvement in entomopathogenic fungi. *Pesticide Science* 26, 79–92.

Huang, X.P., St Leger, R.J., Davis, T.R., Hughes, P.R. and Wood, H.A. (2000) Construction and characterization of a recombinant AcMNPV with a fungal gene encoding an insecticidal protease. *Journal of Invertebrate Pathology* (in press).

Joshi, L., St Leger, R.J. and Bidochka, M.J. (1995) Cloning of a cuticle-degrading protease from

the entomopathogenic fungus, *Beauveria bassiana. FEMS Microbiology Letters* 125, 211–218.

Joshi, L., St Leger, R.J. and Roberts, D.W. (1998) Isolation of a cDNA encoding a novel subtilisin-like protease (Pr1B) from the entomopathogenic fungus *Metarhizium anisopliae* using differential display-RT-PCR. *Gene* 197, 1–8.

Kahmann, R., Romeis, T., Bolker, M. and Kamper, J. (1995) Control of mating and development of *Ustilago maydis. Current Opinions in Genetic Development* 5, 559–564.

van Kan, J.A.L., van't Klooster, J.W., Wagemakers, C.A.M., Dees, D.C.T. and van der Vlught-Bergmans, C.J.B. (1997) Cutinase A of *Botrytus cinerea* is expressed, but not essential, during penetration of *Gerbera* and tomato. *Molecular Plant–Microbial Interactions* 10, 30–38.

Kennedy, A.C. (1996) Molecular biology of bacteria and fungi for biological control of weeds. In: Gunasekaren, M. and Weber, D.J. (eds) *Molecular Biology of the Biological Control of Pests and Diseases of Plants.* CRC Press, Boca Raton, Florida, pp. 155–172.

Kerwin, J.L. (1984) Fatty acid regulation of the germination of *Erynia variabilis* conidia on adults and puparia of the lesser housefly *Fannia canicularis. Canadian Journal Microbiology* 30, 158–161.

Kohler, J.R. and Fink, G.R. (1996) *Candida albicans* strains heterozygous and homozygous for mutations in mitogen-activated protein kinase signaling components have defects in hyphal development. *Proceedings of the National Academy of Sciences USA* 93, 13223–13228.

Koltin, Y., Finkler, A. and Ben-Zvi, B. (1987) In: Pegg, G.F. and Ayres, P.G. (eds) *Fungal Infections of Plants*. Cambridge University Press, Cambridge, pp. 334–348.

Krimsky, S. and Wrubel, R. (1996) *Agricultural Biotechnology and the Environment, Science, Policy and Social Issues.* University of Illinois Press, Urbana and Chicago.

Kusserow, H. and Schafer, W. (1994) The role of polygalacturonase in the abstraction between *Penicillium alsonii* and *Arabidiopsis thaliana*. In: *7th International Symposium on Molecular Plant–Microbe Interactions, Edinburgh, 26 June–2 July,* abstract no. 442.

Mendgen, K., Hahn, M. and Deising, H. (1996) Morphogenesis and mechanisms of penetration by plant pathogenic fungi. *Annual Review of Phytopathology* 34, 367–386.

Messias, C.L and Azevedo, J.L. (1980) Parasexuality in the deuteromycete *Metarhizium anisopliae. Transactions of the British Mycological Society* 75 (3),473–477.

Miller, V., Ford, E.J., Zidack, N.J. and Sands, D.C. (1989) A pyrimidine auxotroph of *Sclerotinia sclerotiorum* for use in biological weed control. *Journal of General Microbiology* 135, 2085–2089.

Milner, R.J., Soper, R.S., and Lutton, G.G. (1982) Field release of an Israeli strain of the fungus *Zoophthora radicans* (Brefeld) Batko for biological control of *Therioaphis trifolii* (Monell) f. *maculata*. *Journal of the Australian Entomological Society* 21, 113–118.

Mitchell, T.K. and Dean, R.A. (1995) The cAMP-dependent protein kinase catalytic subunit is required for appressorium formation and pathogenesis by the rice blast fungus *Magnaporthe grisea. Plant Cell* 7, 1869–1878.

Nicholson, R.L. (1996) Adhesion of fungal propagules. In: Nicole, M. and Gianinazzi-Pearson, V. (eds) *Histology, Ultrastructure and Molecular Cytology of Plant–Microorganism Interaction*. Kluwer Academic Publishers, Amsterdam, the Netherlands, pp. 117–134.

Oliver, R. and Osbourn, A. (1995) Molecular dissection of fungal phytopathogenicity. *Microbiology* 141, 1–9.

Osbourn, A., Bowyer, P., Bryan, G., Lunness, P., Clarke, B. and Daniels, M. (1994) Detoxification of plant saponins by fungi. In: Daniels, M.J., Downie, J.A. and Osbourn, A.E. (eds) *Advances in Molecular Genetics of Plant–Microbe Interactions,* Vol. 3. Kluwer, Dordrecht, pp. 215–222.

Pieterse, C.M.J., van West, P., Verbakel, H.M., Brasse, P.W.H.M., van den Berg-Velthus, G.C.M and Govers, F. (1994) Structure and genomic organisation of the *ipiB* and *ipiO* gene clusters of *Phytophthora infestans. Gene* 138, 67–77.

Possee, R.D. (1993) Viral approaches for insect control. In: Kim, L. (ed.) *Advanced Engineered Pesticides.* Marcel Dekker, New York, pp. 99–112.

Prior, C. (1992) Discovery and characterization of fungal pathogens for locust and grasshopper control. In: Lomer, C.J. and Prior, C. (eds) *Biological Control of Locusts and Grasshoppers.* CAB International, Wallingford, UK, pp. 159–180.

Quimby, P.C. Jr and Birdsall, J.L. (1995) Fungal agents for biological control of weeds: classical and augmentative approaches. In: Reueni, R. (ed.) *Novel Approaches to Pest Management.* Boca Raton, Florida, pp. 293–308.

Roberts, D.W. and Hajek, A.E. (1992) Entomopathogenic fungi as bioinsecticides. In: Leatham, G.F. (ed.) *Frontiers in Industrial Mycology.* Chapman & Hall, London, pp. 144–159.

Roberts, D.W. and Humber, R.A. (1981) Entomogenous fungi. In: Cole, G.T. and Kendrick, B. (eds) *Biology of Conidial Fungi*, Vol. 2. Academic Press, New York, pp. 201–236.

Scott-Craig, J.S., Panaccione, D.G., Cervone, F. and Walton, J.D. (1990) Endopolygalacturonase is not required for pathogenicity of *Cochliobolus carbonum* on maize. *Plant Cell* 2, 1191–1200.

Screen, S., Bailey, A., Charnley, K., Cooper, R. and Clarkson, J. (1997) Carbon regulation of the cuticle-degrading enzyme PR1 from *Metarhizium anisopliae* may involve a trans-acting DNA-binding protein CRR1, a functional equivalent of the *Aspergillus nidulans* CREA protein. *Current Genetics* 31 (6), 511–518.

Screen, S., Bailey, A., Charnley, K., Cooper, R. and Clarkson, J. (1998) Isolation of a nitrogen response regulator gene (nrr1) from *Metarhizium anisopliae*. *Gene* 221 (1), 17–24.

Sposato, P., Ahn, J.H. and Walton, J.D. (1995) Characterization and disruption of a gene in the maize pathogen *Cochliobolus carbonum* encoding a cellulase lacking a cellulose binding domain and hinge region. *Molecular Plant–Microbe Interactions* 8, 602–609.

Stahl, D.F. and Schafer, W. (1992) Cutinase is not required for fungal pathogenicity on pea. *Plant Cell* 4, 621–629.

Staskawicz, B.J., Dahlbeck, D. and Keen, N.T. (1984). Cloned avirulence gene of *Pseudomonas syringae* pv. glycinea determines race-specific incompatibility on *Glycine max* (L.) merr. *Proceedings of the National Academy of Sciences USA* 81, 6024–6028.

Stewart, L.M.D., Hirst, M., Ferber, M.L., Merryweather, A.T., Cayley, P.J. and Possee, R.D. (1991) Construction of an improved baculovirus insecticide containing an insect-specific toxin gene. *Nature (London)* 352, 85.

St Leger, R.J. (1993) Biology and mechanisms of invasion of deuteromycete fungal pathogens. In: Beckage, N.C., Thompson, S.N. and Federici, B.A. (eds) *Parasites and Pathogens of Insects*, Vol. 2. Academic Press, San Diego, pp. 211–229.

St Leger, R.J. and Bidochka, M.J. (1996) Insect–fungal interactions. In: Soderhall, K., Vasta, G. and Iwanaga, S. (eds) *Invertebrate Immunology.* SOS Publications, pp. 443–479.

St Leger, R.J. and Joshi, L. (1997) The application of molecular techniques to entomopathogenic fungi. In: Lacey, L. (ed.) *Manual of Techniques in Insect Pathology*. Academic Press, San Diego, pp. 367–394.

St Leger, R.J., Butt, T., Goettel, M.S., Staples, R.C. and Roberts, D.W. (1988) Production *in vitro* of appressoria by the entomopathogenic fungus *Metarhizium anisopliae. Experimental Mycology* 13, 274–288.

St Leger, R.J., Frank, D.C., Roberts, D.W. and Staples, R.C. (1992a) Molecular cloning and regulatory analysis of the cuticle-degrading protease structural gene from the entomopathogenic fungus *Metarhizium anisopliae. European Journal of Bichemistry* 204, 991–1001.

St Leger, R.J., May, B., Allee, L.L., Frank, D.C. and Roberts, D.W. (1992b) Genetic differences in allozymes and in formation of infection structures among isolates of the entomopathogenic fungus *Metarhizium anisopliae. Journal of Invertebrate Patholology* 60, 89–101.

St Leger, R.J., Staples, R.C. and Roberts, D.W. (1992c) Cloning and regulatory analysis of ssgA: a gene encoding a hydrophobin-like protein from the entomopathogenic fungus, *Metarhizium anisopliae. Gene* 120, 119–124.

St Leger, R.J., Bidochka, M.J. and Roberts, D.W. (1995) Co-transformation of *Metarhizium anisopliae* by electroporation or the gene gun to produce stable GUS transformants. *FEMS Microbiology Letters* 131, 289–294.

St Leger, R.J., Joshi, L., Bidochka, M.J. and Roberts, D.W. (1996a) Characterization of chitinases from *Metarhizium anisopliae, M. falvoviride* and *Beauveria bassiana*, and ultrastructural localization of chitinase production during invasion of insect cuticle. *Applied Enviornmental Microbiology* 62, 907–912.

St Leger, R.J., Joshi, L., Bidochka, M.J. and Roberts, D.W. (1996b) Construction of an improved mycoinsecticide over-expressing a toxic protease. *Proceedings of the National Academy of Sciences USA* 93, 6349–6354.

St Leger, R.J., Joshi, L., Bidochka, M.J., Rizzo, N.W. and Roberts, D.W. (1996c) Biochemical localization and ultrastructural localization of two extracellular trypsins produced by *Metarhizium anisopliae* in infected cuticles. *Applied Environmental Microbiology* 62, 1257–1264.

St Leger, R.J., Joshi, L. and Roberts, D. (1998) Ambient pH is a major determinant in the expression of cuticle-degrading enzymes and hydrophobin by *Metarhizium anisopliae. Applied Environmental Microbiology* 64 (2), 709–713.

Stowell, L.J. (1994) Factors influencing acceptance and development of biopesticides. In: Kim, L. (ed.) *Advanced Engineered Pesticides.* Marcel Dekker, New York, pp. 249–260.

TeBeest, D.O. (1988) Additions to the host range of *Colletotrichum gloeosporioides* f. sp. *aeschynomene. Plant Disease* 72, 16–18.

TeBeest, D.O. (1991) Microbial control of weeds. Routledge, Chapman & Hall, New York.

TeBeest, D.O. (2000) Bioherbicides: opportunities, challenges and an epidemiological explanation for the effectiveness of Collego™. *Phytoparasitica* 28, 178.

Talbot, N.J., Ebbole, D.J. and Hamer, J.E. (1993) Identification and characterization of *MPG1*, a gene involved in pathogenicity from the rice blast fungus *Magnaporthe grisea. Plant Cell* 5, 1575–1590.

Van Etten, H.D., Soby, S., Wasmann, C. and McCluskey, K. (1994) Pathogenicity genes in fungi. In: Daniels, M.J., Downie, J.A. and Osbourn, A.E. (eds) *Advances in Molecular Genetics of Plant-Microbe Interactions*, Vol. 3. Kluwer, Dordrecht, pp. 163–170.

Viaud, M., Couteaudier, Y. and Riba, G. (1998) Molecular analysis of hypervirulent somatic hybrids of the entomopathogenic fungi *Beauveria bassiana* and *Beauveria sulfurescens. Applied Environmental Microbiology* 64 (1), 88–93.

Walton, J. (1994) Deconstructing the cell wall. *Plant Physiology* 104, 1113–1118.

Wood, H.A. (1994) Development and testing of genetically improved baculovirus insecticides. In: Shuler, M.L., Wood, H.A., Granados, R.R. and Hammer, D.A. (eds) *Baculovirus Expression Systems and Biopesticides*. Wiley-Liss, New York, pp. 91–102.

Xu, H. and Mendgen, K. (1997) Targeted cell wall degradation at the penetration site of cowpea rust basidiosporelings. *Molecular Microbial-Plant Interactions* 10, 87–94.

Xu, J.R. and Hamer, J.E. (1996) MAP-kinase and cAMP signaling regulate infection structure formation and pathogenic growth in the rice blast fungus *Magnaporthe grisea. Genes Development* 10, 2696–2706.

Xu, Q. (1988) Some problems about study and application of *Beauveria bassiana* against agricultural and forest pests in China. In: *Chinese Society of Mycology, Study and Application of Entomogenous Fungi in China*, Vol. 1. Academic Periodical Press, Beijing, pp. 1–9.

9

Physiological Approaches to Improving the Ecological Fitness of Fungal Bicontrol Agents

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Introduction

There have been numerous developments of microbial inoculants for the effective control of plant diseases, pests and weeds (Baker, 1987; Burge, 1988; Cook, 1993). In these areas of research, the efficacy of the biocontrol agent (BCA) has been intricately linked with the potential for the production of inocula by either liquid or solid substrate fermentation systems. While such studies have been numerous, most have concentrated on optimizing the quantity of propagules or mycelial fragments produced, with practically no consideration given to quality of inocula. Few, if any, studies have considered the very real practical problem of effective establishment of prospective BCAs in the natural environment, be it for control of pests, diseases or weeds. This can be a crucial bottleneck, limiting the consistency of control under field conditions and the widespread commercialization of BCAs. Fluctuating abiotic factors, particularly water availability, temperature, length of dew periods, microclimate, canopy type and rainfall events, all have an impact on the prospective BCA. Tolerance to such abiotic fluctuations are a prerequisite for the successful development of ecologically competent BCAs for use in the field. Unfortunately, the area of improving the ecological fitness of inocula has received very little attention, although some elegant studies on desiccation tolerance of BCAs have been published (Jin *et al.*, 1991; Jackson *et al.*, 1997). Effective environmental-stress tolerance of inocula may improve establishment, which could contribute significantly to improving the efficacy of BCAs.

There are thus four key questions that need to be addressed: (i) can one manipulate the physiology of non-xerophilic/tolerant fungi to accumulate useful endogenous reserves into inocula for improved environmental-stress tolerance? (ii) would this result in improved germination/growth under environmental stress? (iii) can this improve the establishment of inocula and conserve biocontrol potential in the field? and (iv) does ecophysiological manipulation have a role in improving the production and quality of inocula? This chapter will address these issues and present examples to demonstrate

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that studies on the quality of inocula may be a valuable component in and approach to improving the formulation and delivery of ecologically competent inocula for field use.

Abiotic Stress Tolerance of Biocontrol Agents – Background

It is well known that xerotolerant and xerophilic fungi are able to tolerate a very wide range of water availability, with *Penicillium, Aspergillus* and *Eurotium* species able to grow at 0.85 water activity (a_w) (= 85% equilibrium relative humidity (ERH)) and 0.70 a_w (= 70% ERH) (Magan, 1997). This is much wider than most BCAs used for fungal plant disease and pest control, which germinate and grow very slowly below about 0.95–0.93 a_w.

Under water-stress conditions xerophilic/xerotolerant fungi are able to synthesize compatible solutes, particularly the low-molecular-weight sugar alcohols glycerol and

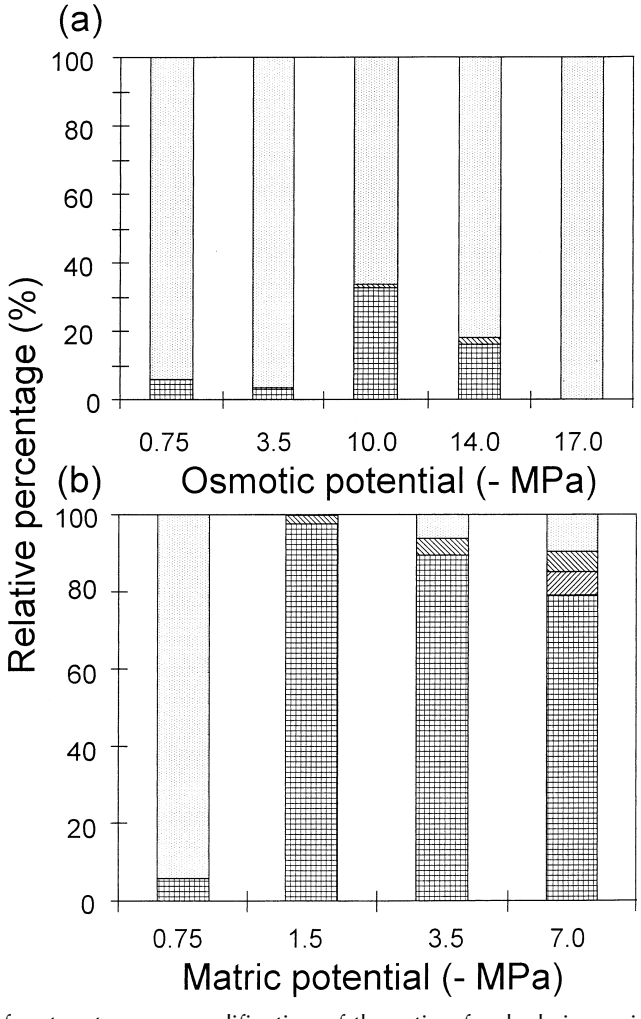

Fig. 9.1. Effect of water stress on modification of the ratio of polyols in conidia of the xerotolerant spoilage fungus *Aspergillus ochraceus.* (Adapted from Ramos *et al.,* 1999.)

erythritol (polyols), which enable their enzyme systems to continue functioning under extreme conditions of environmental stress/shock. For example, *Aspergillus ochraceus*, when grown under osmotic water stress, accumulates significantly elevated amounts of glycerol in its asexual conidia when compared with those produced under conditions with freely available water (Fig. 9.1; Ramos *et al.*, 1999). This certainly suggests that physiological manipulation of growth conditions can significantly modify the endogenous compounds synthesized and channelled into the propagules of fungi.

Interestingly, widening the environmental range for effective growth and biocontrol was also demonstrated to be possible by using low-a_w ultraviolet (UV)-mutant strains of *Metarhizium anisopliae* and *Paecilomyces farinosus* (Matawele *et al.*, 1994). This study demonstrated that the low-a_w mutant strains germinated (minima of 0.957 a_w) better and were more virulent at controlling green leafhoppers than the original wild-type strains (germination minima of 0.975 a_w). Unfortunately, while this work implied that the improved water-stress tolerance of the mutants may have been responsible, no studies were carried out to quantify the endogenous content of the propagules, although it was implied that the sugar alcohol content may have been modified.

Studies on the physiological manipulation of C:N ratios for the production of blastospores of entomogenous fungi also showed that increasing the glycogen content modified and improved the virulence against some pest species (Lane *et al.*, 1991a, b), although other studies suggest that glycogen is accumulated in the absence of stress, with polyol accumulation being more important under C and N stress (van Laere, 1989). While the stability of the low-a_w mutants was not examined, this work certainly pointed to the potential that existed for improving the quality of inocula and perhaps tolerance of a wider range of water availability and improved biocontrol.

It is also surprising that studies in relation to the control of field pests with entomogenous fungi have seldom examined the endogenous reserves present in conidia sporulating on killed insects. It is well known that conidia directly isolated from the body of the dead insect are often more virulent against pests than those obtained from repeated laboratory culture on rich artificial media. Recently, T.M. Butt and N. Magan

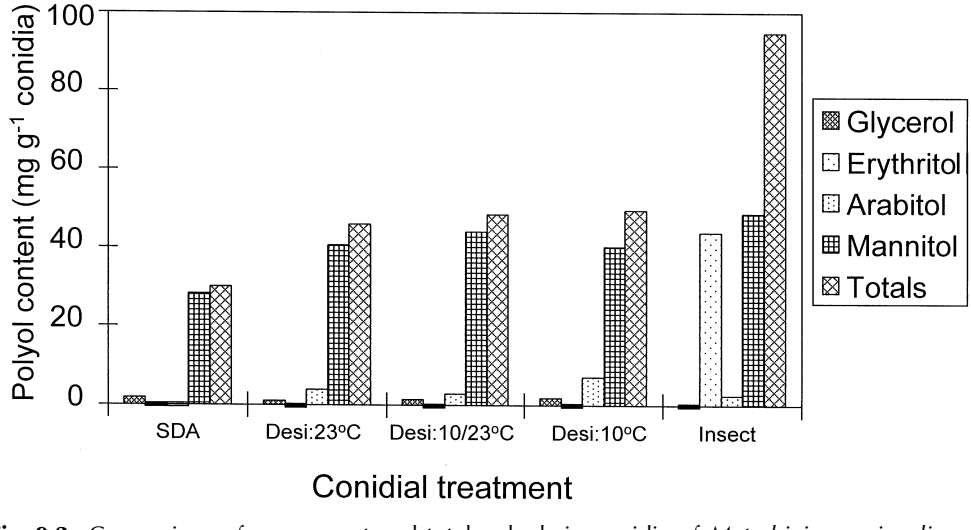

Fig. 9.2. Comparison of component and total polyols in conidia of *Metarhizium anisopliae* isolated directly from an insect, those produced on SDA and those stored under different temperature/desiccation regimes. (T.M. Butt and N. Magan, unpublished data.)

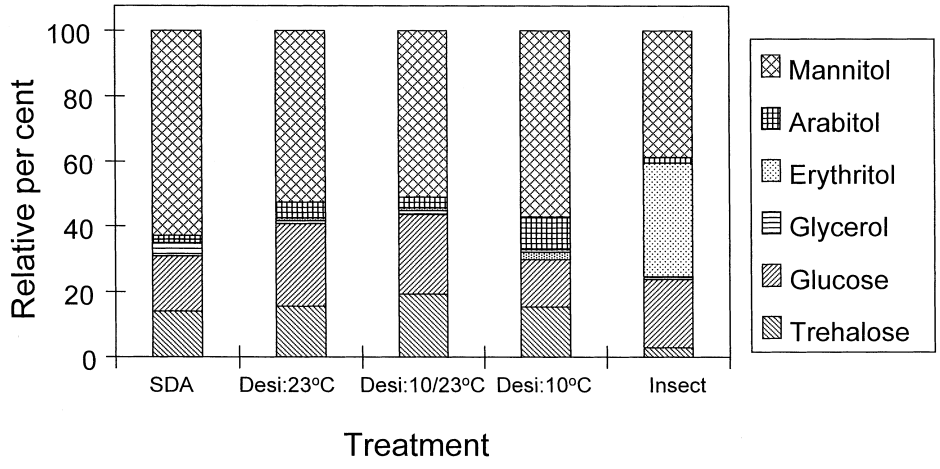

Fig. 9.3. Comparison of the ratio of sugars/polyols present in conidia of *Metarhizium anisopliae* isolated directly from insects, those produced on SDA medium and those stored under different temperature/desiccation regimes. (T.A. Butt and N. Magan, unpublished data.)

(unpublished data) examined this for the first time. They compared the quantities and profiles of sugars and sugar alcohols found in such conidia of *M. anisopliae* with those obtained from artificial media and stored under different desiccation temperatures. The quantities and ratio of endogenous sugars and sugar alcohols differed significantly in the treatments (Figs 9.2 and 9.3). The high amounts of erythritol, mannitol and glucose, with low amounts of trehalose, in conidia from killed insects were very different from those of conidia grown on richer artificial media, where mannitol, glucose and trehalose were the major reserves. This clearly demonstrates that the endogenous profiles of conidia grown on rich artificial media are significantly different from those in conidia obtained directly from insects and is indicative that the nutritional status of artificial media commonly used may not be best for the production of these BCAs. Direct comparisons now need to be made, with bioassays to correlate the relationship between such characterized inocula and the ability to kill insects. Hallsworth and Magan (1994b), in experiments with *Galleria* larvae, did find significant improvements in lethal effects over a wider humidity range. This type of information is very useful, as it suggested that ecophysiological systems could be developed to grow the BCAs under conditions that may be conducive to the synthesis and accumulation of useful compounds in the inocula/conidia in the laboratory, which can then be screened for both environmental-stress tolerance and biocontrol capability. This led to extensive research in the Applied Mycology Group, Cranfield Biotechnology Centre, in collaboration with a number of research groups, on a range of fungal BCAs, in order to understand the impact that physiological manipulations have on the synthesis and accumulation of sugars and sugar alcohols in a number of BCAs on their ecological competence; some of the results obtained in these studies, using the entomogenous fungi *Epicoccum nigrum* (for control of *Monilinia laxa* on peach twigs), *Gliocladium roseum* (for control of *Botrytis cinerea* on a variety of crops), *Candida sake* (for control of *Penicillium expansum* on apples) and *Ulocladium atrum* (for control of *B. cinerea* on a range of crops) as examples, will be described.

Ecophysiological Manipulation of Endogenous Reserves in Inocula

Water stress and temporal accumulation of sugars and polyols in inocula

Modifications of the carbon concentration of the media used can also have a significant influence on the water stress imposed on the fungus. This close interaction has seldom been recognized and in most cases has been ignored (Hallsworth and Magan, 1994a, 1995). Studies of entomogenous fungi using trehalose, glycerol, glucose or starch as a major carbon source have shown that both C concentration and time have a significant effect on the accumulation of both sugars and polyols in conidia. The time at which optimum quantities of low-molecular-weight polyols accumulated varied from 7 to 21 days. Trehalose content increased during the first 5 days and then decreased again. However, these studies were all carried out at one steady-state temperature, 25°C.

Other studies with *G. roseum*, used for the control of a range of soil-borne and foliar diseases, including *B. cinerea*, showed that the total sugars and glycerol accumulated in conidia varies with temperature and level of water stress (Fig. 9.4). Furthermore, by using either glycerol, glucose or trehalose as the major carbon source, the relative proportions of sugars and both high- and low-molecular-weight sugar alcohols varied significantly (Fig. 9.5). Studies with the BCAs *E. nigrum, C. sake* and *U. atrum* have all recently demonstrated that the endogenous contents of sugars and sugar alcohols can all be significantly modified using this approach (Pascual *et al.*, 1996; Frey and Magan, 1998; Teixido *et al.*, 1998a).

Improvements in growth and viability of modified inocula

The next key question is whether it is indeed possible for the modifications demonstrated above to be translated into improved germination capacity over a wider range

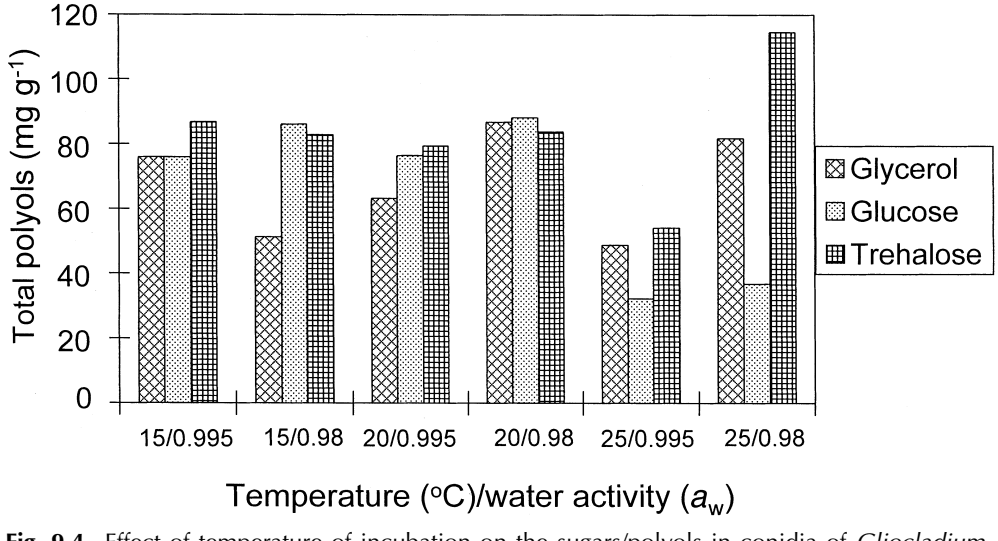

Fig. 9.4. Effect of temperature of incubation on the sugars/polyols in conidia of *Gliocladium roseum* grown on different media. (N. Magan, unpublished data.)

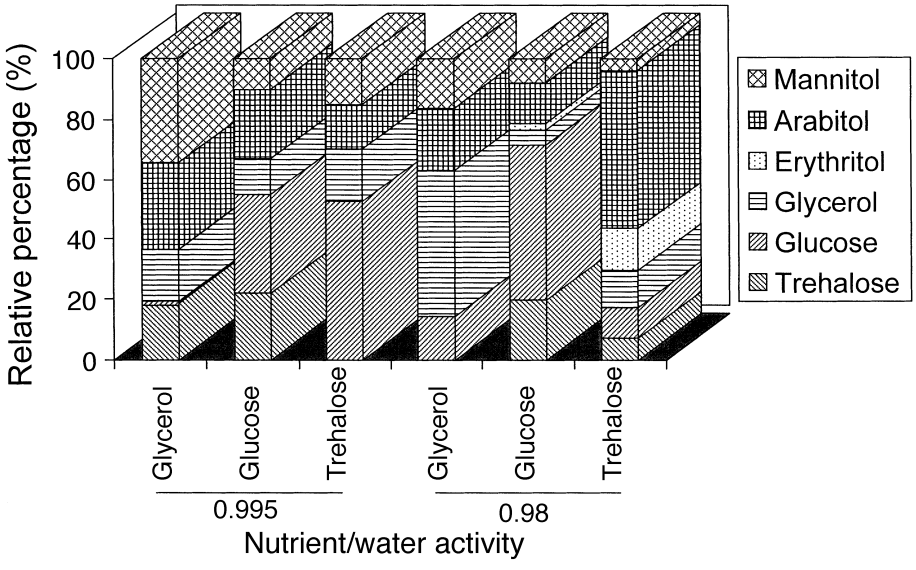

Fig. 9.5. Comparison of relative amounts of sugars/polyols in conidia of *Gliocladium roseum* at 25°C obtained from cultures grown on different media substrates. (N. Magan, unpublished data.)

of environmental stress conditions, and perhaps also more rapidly than unmodified inocula grown on rich unmodified media.

Improved viability, germination and germ-tube extension under water stress

The capacity for withstanding wider water-availability ranges was tested by using weak water-based agar media, modified with polyethylene glycol 200/400 and 600, or mixtures thereof, to avoid using solutes that might be taken up directly by propagules or yeast cells. The inocula were prepared in a diluent of the same a_w as the test agar plates over the range 0.995 (freely available water) to 0.90 or 0.88 a_w (= 90–88% ERH), representing much drier conditions than normally tolerated by these BCAs. In some cases, richer potato-dextrose- or yeast/glucose-based media were also used.

Studies with entomogenous fungi demonstrated that significant improvements in germination could be obtained with characterized modified than with unmodified conidia of *Beauveria bassiana, M. anisopliae* and *P. farinosus* (Table 9.1). It is clear that under marginal conditions for germination the modified inocula were able to germinate, while unmodified inocula could not. Studies with the cells of the yeast *C. sake* used for control of *Penicillium* rot of apples, modified by culture in weak-nutrient yeast broth media modified with either glucose or glycerol to 0.96 a_w, also demonstrated that a greater number of modified yeast cells were viable over a range of water availabilities (0.95–0.93 a_w) than unmodified yeast cells, which were significantly more sensitive (Teixido *et al.*, 1998b).

In contrast, using conidia of dematiaceous BCAs, such as *E. nigrum* and *U. atrum*, where significant physiological modifications of endogenous reserves were possible, no improvement in germination was achieved under water-stress treatment conditions. This suggests that larger, heavily pigmented spores from harsher phyllosphere environments may already, to a large extent, have evolved toleration of environmental stress.

Table 9.1. Mean percentage germination of conidia of *Beauveria bassiana, Metarhizium anisopliae* and *Paecilomyces farinosus* from different treatments over a range of water availability, at 25°C. The germination media contained 13.3 g l^{-1} glucose, 5.0 g l^{-1} mycological peptone and polethylene glycol (PEG) 600 or PEG 600 + PEG 200. (Adapted from Hallsworth and Magan, 1995.)

	Water activity			
Treatment	0.989 (14 h)	0.951 (44 h)	0.935 (61 h)	0.923 (240 h)
B. bassiana				
Control	76.3	5.7	0[a]	0*
SDA + KCl	96.3	59.7	38.0	0*
Glycerol	92.0	87.3	51.0	0*
Trehalose	90.3	16.7	0[a]	0*
LSD ($P < 0.05$)	15.1	13.4	8.2	
M. anisopliae				
Control	0	0	0	0
SDA + KCl	81.3	77.0	49.7	0*
Glycerol	92.7	61.3	51.3	69.0
Trehalose	2.3	0	0	0*
LSD ($P < 0.05$)	7.5	10.7	7.6	
P. farinosus				
Control	47.3	0	0[a]	0[a]
SDA + KCl	43.0	42.7	10.0	0[a]
Glycerol	80.0	86.7	48.7	17.7
Trehalose	4.0	0	0[a]	0[a]
LSD ($P < 0.05$)	22.9	8.0	6.3	

[a]No conidia from these treatments had germinated by 240 h.
SDA, Sabouraud dextrose agar; LSD, least significant difference.

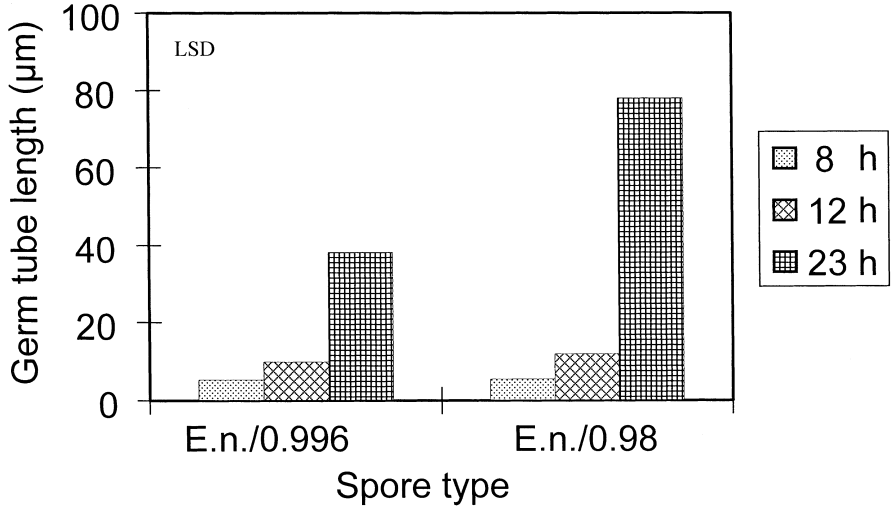

Fig. 9.6. Comparison of the temporal germ-tube extension of characterized spores of *Epicoccum nigrum* produced on unstressed (0.996 water activity) and stressed (0.98 water activity) media and germinated on water agar at 0.935 water activity. (From Pascual *et al.,* 1998.)

After germination, the next important phase is germ-tube extension and establishment, or appressorium formation for some entomogenous species. Detailed studies were made under the different water-stress regimes described previously, which demonstrated that germ-tube extension of modified conidia of *E. nigrum* and *U. atrum* could be significantly improved under water stress. Polyols such as glycerol can be a source of energy and utilized to promote subsequent growth and development. Indeed, in the case of *E. nigrum*, in some weak media, this improvement was maintained during *in vitro* mycelial colony development (Pascual, 1998). Figure 9.6 shows an example of the improved germ-tube extension observed with characterized conidia of *E. nigrum* obtained from colonies grown on low-a_w-stress media (Pascual, 1998).

Trehalose is an important sugar as it prevents damage by replacing water in dehydrated phospholipid membranes in yeasts and filamentous fungi. This inhibits transition of the liquid crystalline phase to the gel phase and in so doing preserves the cell membranes (Crowe *et al.*, 1984). This is critical in desiccation tolerance and maintaining the integrity of cell membranes during wetting and drying cycles. Such tolerance is also an important parameter, especially when BCAs are being formulated as a wettable powder. Studies by Jin *et al.* (1991) with *Trichoderma harzianum* demonstrated that, by manipulating the growth conditions with polyethylene glycol (PEG), the trehalose content of the conidia could be significantly increased and this enabled the inocula to survive desiccation better than control unmodified conidia. Although the PEG molecular weight and concentrations used were not detailed, this study pointed to the importance of considering this approach where desiccation tolerance is of critical importance. Trehalose may be more important in some groups of fungi than in others. For example, the non-xerotolerant BCA yeast *C. sake* accumulated trehalose rapidly (Teixido *et al.*, 1998a, b) and was implicated in the improved viability of cells at lowered a_w. Other elegant studies, by Jackson and Bothast (1990) and Schisler *et al.* (1991) showed that the C:N ratio influenced the relative accumulation of protein and lipid reserves in *Colletotrichum truncatum* spores, affecting biocontrol efficacy and the rate of germination. However, the C:N ratio did not affect *C. truncatum* desiccation tolerance. Jackson and Schisler (1992) and Jackson *et al.* (1997) have also pointed to variation in C:N ratios and limitation as a means of improving the desiccation tolerance of mycoherbicides (*C. truncatum*) and entomogenous species (*P. farinosus*). It needs to be recognized that the effect of ecophysiological manipulation on the endogenous accumulation of reserves varies with fungal species and groups, and that the type of modification must be appropriate for the needs. For example, if increased glycerol is required, solute stress modifications may be important, while C:N limitation and ratios may affect and increase trehalose accumulations in inocula.

Recent studies by Wilson and Lindow (1994a, b) with bacterial BCAs have also suggested that the competitiveness of individual strains could be estimated with the *in vitro* niche overlap index (NOI), derived from *in vitro* carbon utilization profiles (NOI is the proportion of the C compounds utilized by one species that is also utilized by the competing species). They hypothesized that the effectiveness of a strain as a preemptive biocontrol agent of epiphytic phytopathogenic bacteria was proportional to the ecological similarity between the BCA and the target pathogen in the phyllosphere of the host plant. They suggested that NOIs > 0.9 indicated that two competing species competed for and occupied the same niche, while those with < 0.9 occupied separate niches. NOIs have been used in studies to understand the interactions between mycotoxigenic *Fusarium* causing ear rot of maize grain and other mycoflora. Interestingly, it was found that the NOIs varied depending on both water availability and temper-

ature (Marin *et al.*, 1998). However, practically no studies have examined whether manipulation of endogenous reserves might affect the NOIs of potential BCAs in relation to environmental factors. The first such study was conducted recently by Pascual (1998), who compared the NOIs of the peach pathogen *M. laxa* with those of the BCA *E. nigrum* grown on unstressed (0.996 a_w) and stressed media (0.98 a_w). The NOIs changed with water stress, suggesting that the relative niche occupation by the antagonist and the pathogen may vary and that the potential for niche exclusion will be influenced by the quality of the inoculum (the water-stress-tolerant and the unmodified strain). This suggests that endogenous modifications of the polyols and sugars can also modify competitiveness based on the NOI system suggested by Wilson and Lindow (1994b).

Improvements in Biocontrol in the Field

The critical component of this strategy is whether biocontrol can be either conserved or improved under a range of environmental factors. To this end, a series of studies were carried out to test the low-a_w-stress inocula described previously. With *B. bassiana* and *M. anisopliae*, tests were carried out using *Galleria* larvae in bioassay systems over a range of humidity regimes and temperatures not commonly used in the laboratory. Table 9.2 shows that at low relative humidity levels, regardless of temperature, the death of the larvae was improved by the modified inocula when compared with the efficacy of unmodified conidia produced on rich-nutrient media. The modified spore inocula were produced on low-water-stress media modified with glycerol, and com-

Table 9.2. Mean percentage mycosis of *Galleria mellonella* larvae over a range of equilibrium relative humidities (ERH), at 25°C. Larvae were inoculated with different characterized treatments. Cultures were grown on either SDA (control, unmodified treatment) or SDA modified with glycerol to 0.96 water activity with glycerol (glycerol treatment) at 25°C for 14 days. Least significant differences between treatments were: 9 days: 18.9, 24.5 and 24.1; 15 days: 22.8, 22.3 and 7.9 for *Beauveria bassiana, Metarhizium anisopliae* and *Paecilomyces farinosus,* respectively. (Adapted from Hallsworth and Magan, 1994b.)

Treatment	Time (days)	100% ERH	86.5% ERH	78.2% ERH
B. bassiana				
Control	9	56.7	11.6	3.9
Glycerol	9	80.0	34.3	0
Control	15	100	11.6	3.9
Glycerol	15	96.9	58.6	0
M. anisopliae				
Control	9	66.7	23.4	0
Glycerol	9	96.5	89.5	70.0
Control	15	100	35.0	4.2
Glycerol	15	96.5	96.9	100
P. farinosus				
Control	9	76.7	48.7	0
Glycerol	9	90	89.5	83.4
Control	15	100	100	0
Glycerol	15	100	100	100

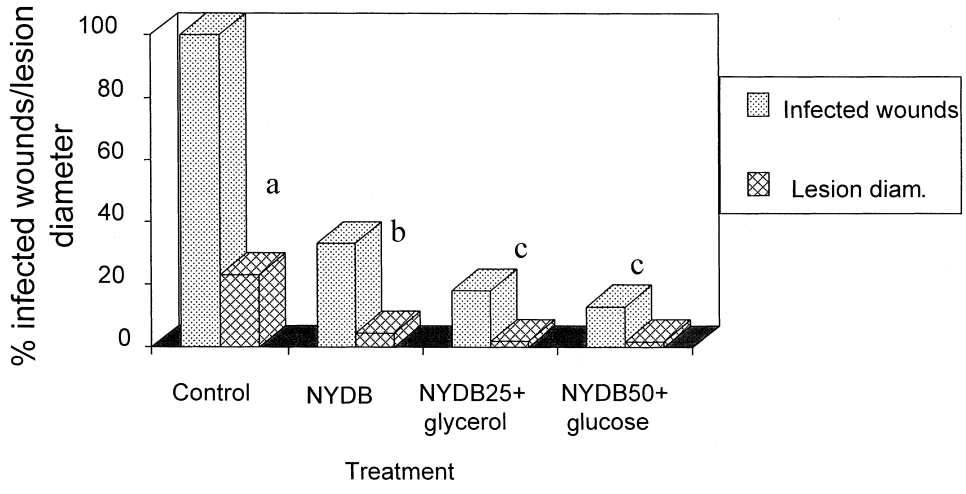

Fig. 9.7. The effect of growth medium on biocontrol capacity of *Candida sake* against postharvest *Penicillium* rot of apples. Key to treatments: NYDB, nutrient yeast dextrose broth; NYDB225+glycerol, 75% diluted medium modified with glycerol to 0.96 water activity; NYDB50+glucose, 50% diluted medium modified with glucose to 0.96 water activity. Treatments with different letters are statistically significant (P = 0.05). (From Teixido *et al.*, 1998b.)

pared with spores produced on an unmodified Sabourand dextrose agar (SDA) medium. This work has now been followed up by experiments with *B. bassiana* on leaves of whole plants, where some improvements in efficacy against aphids have been recorded (Anderson, 2000). Further whole-plant studies are needed to examine the potential for improvement in the control of insect pests, particularly under different fluctuating humidities in the leaf microclimate.

More recent studies with field spraying of different inocula of the yeast *C. sake* on apples in orchards prior to harvest and storage have demonstrated two important things. First, low-a_w-tolerant yeast cells grow better on the apple surface and such establishment can give equally good, if not better, postharvest control of *Penicillium* rot (Fig. 9.7) (Teixido *et al.*, 1998a, b, c). This work also suggested that the low-a_w-tolerant inocula could be applied at a lower concentration to obtain the same efficacy. Thus, although in some cases production of modified inocula may result in lower inoculum yields, this can be compensated for by the use of lower application rates. This could be important for the development of economic production, formulation and application systems for BCAs.

Application of *E. nigrum* inocula to peach twigs in the field has also demonstrated that better control of brown rot (*M. laxa*) could be achieved than that obtained with the unmodified inocula of *E. nigrum* or the fungicide captan. This BCA was also examined for postharvest control of *B. cinerea* on cherries under different storage humidity regimes. In this case good control was achieved at different relative humidities, but there were no statistically significant differences between inocula (Pascual *et al.*, 1999). Studies are still in progress with these inocula in field trials for assessing efficacy in the field.

Studies with *U. atrum* for control of *B. cinerea* suggests that manipulation of endogenous reserves can modify the establishment on leaf surfaces and affects the lev-

els of biocontrol achieved (Frey and Magan, 1998). These studies, however, are still in progress and more detailed analyses of the data are needed for accurate interpretation of the results to be carried out.

The above sections have thus demonstrated that the four questions posed can indeed be answered positively, which suggests that potential does exist for this approach. It should be noted, however, that the examples presented are not numerous and practically no other groups have examined this approach for improving the ecological fitness of BCAs in the field.

Future Prospects for Improving the Commercialization of Ecologically Fit Inocula

The key final question that arises is whether this approach will be useful in formulation of BCAs for field use. Many field trials are at present carried out with freshly harvested inocula. This is unrealistic in the long term for commercial exploitation. Thus, both dry and liquid formulations need to be examined for long-term storage and viability. The question of conservation of biocontrol efficacy after long-term storage then also becomes important. A fundamental understanding of the endogenous changes and accumulations of polyols and sugars in inocula of BCAs can also enable information to be obtained on any modifications to internal water and solute potentials. This can be combined with exogenous additions of specific compounds or mixtures of compounds to maintain the concentrations in the inocula for conserving viability and for the long-term stability of formulations that have ecological competence. Studies in my laboratory are now focusing on these aspects, which are critical for the effective exploitation of BCAs.

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References

Anderson, M. (2000) Manipulation of the endogenous reserves of entomopathogenic fungi for improved control of glasshouse pests. PhD thesis, Institute of BioScience and Technology, Cranfield University.

Baker, K.E. (1987) Evolving concepts of biological control of plant pathogens. *Annual Review of Phytopathology* 25, 67–85.

Burge, M.N. (1988) *Fungi in Biological Control Systems.* Manchester University Press, Manchester, UK.

Cook, R.J. (1993) Making greater use of introduced microorganisms for biological control of plant pathogens. *Annual Review of Plant Pathology* 31, 53–80.

Crowe, J.H., Crowe, L.M. and Chapman, D. (1984) Preservation of membranes in anhydrobiotic organisms: the role of trehalose. *Science* 223, 701–703.

Frey, S. and Magan, N. (1998) Improving quality and quantity of *Ulocladium atrum* for enhanced biological control of *Botrytis cinerea. Proceedings of Brighton Crop Protection Symposium – Pests and Diseases,* 4D. British Crop Protection Council, Farnham, UK, pp. 305–306.

Hallsworth, J.E. and Magan, N. (1994a) Effect of carbohydrate type and concentration on polyhydroxy alcohol and trehalose content of conidia of three entomopathogenic fungi. *Microbiology – UK* 140, 2705–2713.

Hallsworth, J.E. and Magan, N. (1994b) Improved biological control by changing polyols/trehalose in conidia of entomopathogens. In: *Proceedings of the Brighton Crop Protection Conference – Pests and Diseases,* pp. 1091–1096.

Hallsworth, J.E. and Magan, N. (1995) Manipulation of intracellular glycerol and erythritol enhances germination of conidia at low water availability. *Microbiology – UK* 141, 1109–1115.

Jackson, M.A. and Bothast, R.J. (1990) Carbon concentration and C–N ratio influences submerged culture conidiation by the potential bioherbicide *Colletotrichum truncatum* NRRL 137371. *Applied and Environmental Microbiology* 56, 3435–3438.

Jackson, M.A. and Schisler, D.A. (1992) The composition and attributes of *C.truncatum* spores are altered by the nutritional environment. *Applied and Environmental Microbiology* 58, 2260–2265.

Jackson, M.A., McGuire, M.R., Lacey, L.A. and Wraight, S.P. (1997) Liquid culture production of desiccation tolerant blastospores of the bioinsecticidal fungus *Paecilomyces fumosoroseus. Mycological Research* 101, 35–41.

Jin, X., Harman, G.A. and Taylor, A.G. (1991) Conidial biomass and desiccation tolerance of *Trichoderma harzianum* produced at different water potentials. *Biological Control* 1, 237–243.

van Laere, A. (1989) Trehalose, reserve and/or stress metabolite. *FEMS Microbiology Reviews* 63, 201–210.

Lane, B.S., Trinci, A.P.J. and Gillespie, A.T. (1991a) Endogenous reserves and survival of blastospores of *Beauveria bassiana* harvested from carbon and nitrogen-limited batch cultures. *Mycological Research* 95, 821–828.

Lane, B.A., Trinci, A.P.J. and Gillespie, A.T. (1991b) Influence of cultural conditions on virulence of conidia and blastospores of *B. bassiana* to the green leaf hopper. *Mycological Research* 95, 829–833.

Magan, N. (1997) Fungi in extreme environments. In: Wicklow, D.T. and Soderstrom, B. (eds) *The Mycota IV, Environmental and Microbial Relationships.* Springer, Berlin, pp. 99–114.

Marin, S., Sanchis, V., Ramos, A.J., Vinas, I. and Magan, N. (1998) Environmental factors, *in vitro* interspecific interactions, and niche overlap between *Fusarium moniliforme, F. proliferatum* and *F. graminearum, Aspergillus* and *Penicillium* spp. isolated from maize grain. *Mycological Research* 102, 831–837.

Matawele, P., Trinci, A.P.J. and Gillespie, A.T. (1994) Mutants of entomopathogenic fungi that germinate and grow at reduced water activities and reduced relative humidities are more virulent to *Nephotettix virescens* (green leafhopper) than the parental strain. *Mycological Research* 98, 1329–1333.

Pascual, S. (1998) Studies on production and ecophysiology of fungal antagonists for improved biological control of *Fusarium oxysporum* f. sp. *lycopersici* and *Monilinia laxa*. PhD thesis, Institute of BioSciences and Technology, Cranfield University, UK.

Pascual, S., Magan, N. and Melgarejo, P. (1996) Improved biological control of peach twig blight by physiological manipulation of *Epicoccum nigrum.* In: *Brighton Crop Protection Conference – Pests and Diseases*, British Crop Protection Council, Farnham, UK, pp. 411–412.

Pascual, S., Magan, N. and Melgarejo, P. (1998) Improvement of growth and biocontrol ability of *Epicoccum nigrum* under water stress. In: *Proceedings of 7th International Congress of Plant Pathology, Edinburgh,* British Crop Protection Council, Farnham, UK, p. 6.52.

Ramos, A.J., Magan, N. and Sanchis, V. (1999) Osmotic and matric potential effects on growth,

sclerotial production and partitioning of polyols and sugars in colonies and spores of *Aspergillus ochraceus. Mycological Research* 103, 141–147.

Schisler, D.A., Jackson, M.A. and Bothast, R.J. (1991) Influence of nutrition during conidiation of *Colletotrichum truncatum* on conidial germination and efficacy in inciting disease in *Sesbania exaltata. Phytopathology* 81, 587–590.

Teixido, N., Vinas, I., Usall, J., Sanchis, V. and Magan, N. (1998a) Ecophysiological responses of the biocontrol yeast *Candida sake* to water, temperature and pH stress. *Journal of Applied Microbiology* 84, 192–200.

Teixido, N., Vinas, I., Usall, J. and Magan, N. (1998b) Improving ecological fitness and environmental stress tolerance of the biocontrol yeast *Candida sake* by manipulation of intracellular sugar alcohol and sugar content. *Mycological Research* 102, 1409–1417.

Teixido, N., Vinas, I., Usall, J. and Magan, N. (1998c) Control of blue mold of apples by preharvest application of *Candida sake* grown on media with different water activity. *Phytopathology* 88, 960–964.

Wilson, M. and Lindow, S.E. (1994a) Ecological similarity and coexistence of epiphytic ice-nucleating (Ice+) *Pseudomonas syringae* strains and a non-ice-nucleating (Ice–) biological control agent. *Applied and Environmental Microbiology* 60, 3128–3137.

Wilson, M. and Lindow, S.E. (1994b) Coexistence among epiphytic bacterial populations mediated through nutritional resource partitioning. *Applied and Environmental Microbiology* 60, 4468–4477.

10 Production, Stabilization and Formulation of Fungal Biocontrol Agents

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Introduction

The past decade has seen many important advances in the field of applied mycopathology. This progress has been extremely broad-based, but especially pronounced in the areas of commercial-scale mass production, propagule stabilization and product formulation. This chapter reviews some of the problems that have constrained the commercial development of mycopathogens, the progress achieved in solving these problems and the potential for continued progress and successful commercialization.

Mycoinsecticides

Introduction

Over the past 10 years there has been an unprecedented increase in activities related to the commercial development of entomopathogenic fungi. Table 10.1 lists more than 30 trade-named mycoinsecticide products now registered or under development worldwide, and this tabulation does not include numerous non-commercial preparations developed by government research institutions in such countries as China (Feng *et al.*, 1994). Discussions in this section will address many of the important technological advances that have stimulated the recent increase in research and development activities.

Table 10.1. Some mycopesticide products registered or under commercial development for microbial control of various insect pests.

Pathogen	Product trade name	Company or government	Active ingredient[a]	Formulation	Principal target pests
Beauveria bassania	Bea-Sin	Agrobiológicos del Noroeste (Agrobionsa), Mexico	Conidia	WP	Pepper weevil, boll weevil, whiteflies
	Boverin	USSR (former)	Aerial or sub-merged conidia and/or blasto-spores	WP	Colorado potato beetle, codling moth
	Boverol-spofa	Czechoslovakia	Conidia	WP	Colorado potato beetle
	Conidia	Hoechst Schering AgrEvo, Colombia	Conidia	WDG	Coffee-berry borer
	Mycotrol/ BotaniGard	Mycotech, USA	Conidia	WP, ES, OF	Whiteflies, aphids, dia-mondback moth, thrips, grasshoppers
	Naturalis	Troy Biosciences, USA	Conidia	ES	Whiteflies, aphids, thrips
	Ostrinil	Natural Plant Protection (NPP), France	Conidia	G	European corn-borer
	Proecol	Productos Biológicos para el Agro (Probioagro), Venezuela	Conidia	WP	Army worms
Beauveria brongniartii	Beauveria	Eric Schweizer Seeds,	Conidia	G/WC	European
	Schweizer	Switzerland	Conidia	G	cockchafer
	Betel	NPP, France			White grubs
	Biolisa Kamikiri	Nitto Denko, Japan	Conidia	WC	Cerambycid beetles
	Engerlingspilz	Andermatt Biocontrol AG	Conidia	G/WC	European cockchafer
	Melocont	Kwizda, Austria	Conidia	G/WC	European cockchafer
Lagenidium giganteum	Laginex	AgraQuest, USA	Mycelium	AS	Mosquitoes
Metarhizium anisopliae	Bio-Blast	EcoScience, USA	Conidia	WP	Termites
	BioGreen	Bio-Care Technology, Australia	Conidia	G	Red-headed cockchafer
	Bio-Path	EcoScience, USA	Conidia	WC	Cockroaches
	BIO 1020	Bayer AG, Germany	Mycelium	G	Black vine weevil
	Cobican	Probioagro, Venezuela	Conidia	WP	Sugar-cane spittlebug

Table 10.1. continued

Pathogen	Product trade name	Company or government	Active ingredient[a]	Formulation	Principal target pests
	Metabiol	PlanTerra – Produtos Biotecnológicos, Brazil	Conidia	WP	Pasture spittlebug
	Metarhizium Schweizer	Eric Schweizer Seeds, Switzerland	Conidia	G/WC	White grubs
	Metarril	PlanTerra, Brazil	Conidia	WP	Sugar-cane spittlebug
	Meta-Sin	Agrobionsa, Mexico	Conidia	WP	Pepper weevil, boll weevil, sugar-cane borer
M.anisopliae var. *acridum*	Green Muscle	Biological Control Products, South Africa	Conidia	WP, OF	Grasshoppers, locusts
Paecilomyces fumosoroseus	Bemisin	Probioagro, Venezuela	Conidia	WP	Whiteflies
	Pae-Sin	Agrobionsa, Mexico	Conidia	WP	Whiteflies
	PreFeRal/ PFR 97	Thermo Trilogy, USA	Blastospores	WDG	Whiteflies
Verticillium lecanii	Mycotal	Koppert Biological Systems, the Netherlands	Conidia	WP	Whiteflies, thrips
	Vertalec	Koppert, the Netherlands	Blastospores	WP	Aphids

[a]Conidia refers to aerial conidia unless otherwise indicated.
AS, aqueous suspension; ES, oil-based emulsifiable suspension; G, granular; OF, oil flowable; WC, whole culture; WDG, water-dispersible granular; WP, wettable powder.

Commercial-scale mass production

Production efficiency

The propagules of entomopathogenic fungi responsible for dispersal and infection under natural conditions are the aerial conidia (spores produced in air on conidiogenous cells). Most entomopathogenic hyphomycete fungi produce large quantities of small (< 10 μm), hydrophobic conidia in dense masses. Conidia of many species have strong hydrophobic walls, which confer environmental stability. These characteristics contribute significantly to production efficiency and storage stability and have made conidia the propagules of choice for most commercial formulations (Table 10.1). However, even under favourable conditions, individual conidia of the common hyphomycete entomopathogens are not highly infectious, and regression slopes are low (Wraight and Carruthers, 1999). This, in large part, accounts for the high doses (10^{13}–10^{14} spores ha^{-1}) typically required to control pests in the field (Bartlett and Jaronski, 1988).

The capacity to produce 10^{13} spores at a cost competitive with the per hectare costs of chemical insecticides has historically represented an important goal in commercial development. This level of production efficiency has now been realized for at least one fungus; automated technologies for solid-substrate production of aerial conidia of *Beauveria bassiana* have been developed in several countries (Feng *et al.*, 1994). Production technologies developed in China are claimed to support applications of $1.5–3 \times 10^{13}$ conidia ha^{-1} at the remarkably low cost of US$2–3; however, detailed economic analysis of these systems is not available. Liquid-surface culture technologies developed in Eastern Europe are also highly productive with respect to total nutrient requirements on a dry-weight basis; however, efficiency is limited by space requirements. Currently, the most efficient technology for *B. bassiana* conidia production in the West is that of Mycotech Corp. of Butte, Montana (Bradley *et al.*, 1992). Mycotech's computer-controlled system with forced aeration generates yields of approx. 10^{13} conidia kg^{-1} of a solid substrate occupying 1 l of fermenter space. This number of conidia in various formulations is currently retailed in the US for < $20.

It is probable that this technology can be adapted for production of other fungi, including *Metarhizium anisopliae*; however, the exceptional efficiency of *B. bassiana* production is at least partly due to the small size of the conidia (2–3 μm diam.). Dorta and Arcas (1998) reported a maximum *M. anisopliae* yield of 1.85×10^{12} conidia l^{-1} of solid substrate in fermentations with forced aeration. Historically, the most commonly employed method for mass production of *Metarhizium* conidia has been culture on solid substrates (usually whole grains) in plastic bags or other small containers. Mendonça (1992) reports that these low-technology systems can generate *M. anisopliae* yields of 10^{13} conidia kg^{-1} of rice; however, recent reports by other researchers indicate consistent yields of only $1–5 \times 10^{12}$ conidia kg^{-1} (Dorta and Arcas, 1998; Jenkins *et al.*, 1998).

Despite these advances in solid-substrate culture, difficulties remain with many species. *Paecilomyces fumosoroseus*, for example, requires light for optimal production of aerial conidia (Sakamoto *et al.*, 1985), and efficient production of conidia thus requires surface culture or periodic agitation of particulate substrates to increase exposure to light. *Verticillium* conidia develop in sticky globules produced at relatively low densities on a diffuse growth of hyphae. *Aschersonia* conidia are produced in convolutions or pits in a dense stroma. Conidia of entomophthoralean fungi are usually large (>10 μm), thin-walled and highly susceptible to desiccation. These various growth characteristics limit mass culture productivity and greatly complicate the harvest and formulation processes.

Many fungi that are difficult to produce efficiently on solid substrates can be readily cultured in liquid media. In this environment, depending on the species or isolate, Hyphomycetes can be induced to grow in various forms, including thin-walled, single-celled hyphal bodies (some forms referred to as 'blastospores') and submerged conidia. The latter are produced either directly from blastospores (microcycle conidiation) or by conidiogenous cells that form on submerged hyphae. Submerged conidia resemble aerial conidia but are substantially less hydrophobic. In some cases, hyphal bodies and submerged conidia of Hyphomycetes can be as virulent as or more virulent than aerial conidia (Jenkins and Thomas, 1996; Jackson *et al.*, 1997; Vandenberg *et al.*, 1998; Lacey *et al.*, 1999), suggesting considerable potential for formulation and application as microbial insecticides.

Currently, in terms of the number of propagules that can be produced in a unit volume of culture media, production of blastospores and other submerged propagules is less

efficient than production of aerial conidia. Liquid cultures become increasingly viscous, and thus difficult to aerate, as concentrations increase. For *P. fumosoroseus*, this problem is encountered when concentrations rise above approximately 2×10^{12} blastospores l^{-1} (M.A. Jackson, unpublished). Similar maximum yields of blastospores of *B. bassiana* have been reported (Fargues *et al.*, 1979). This per litre rate of *B. bassiana* blastospore production is one-fifth the rate of aerial conidia production achieved with the Mycotech solid–substrate system, and researchers have suggested that yields of 5×10^{12} blastospores l^{-1} must be achieved before the use of *B. bassiana* blastospores becomes economically feasible (Feng *et al.*, 1994). Hyphal bodies of entomophthoralean fungi are substantially larger than hyphomycete blastospores, and production of these propagules is consequently even less efficient in terms of the number of fungal cells producible in a litre of culture medium. A recently patented process for the mass production of *Entomophaga aulicae*, for example, provides yields of 3.5×10^{8} hyphal bodies l^{-1} (Nolan, 1998).

Liquid-culture production of fungal mycelia for formulation as granules represents an even greater problem in terms of production efficiency. The number of granules producible per unit of fermentation medium is obviously lower than the number of microscopic spores, and production costs can be prohibitive if large numbers of granules are needed to effect rapid pest control in a complex, three-dimensional pest habitat, such as soil (Schwarz, 1995) or a dense crop canopy (Wraight *et al.*, 1986).

Comparisons such as those discussed above are useful but must be viewed with caution. Ultimately, production efficiencies of various culture systems cannot be assessed and compared solely on the basis of numbers of propagules produced per litre of fermenter space. Other factors that must be considered are discussed below in the section on potential.

Product quality

Quality control is one of the most critical concerns in any industrial-scale production system. Repeated *in vitro* culture can lead to attenuation of fungal pathogens, and methods to preserve pathogen virulence, including manipulations of media composition, have been explored for many years (e.g. Schaerffenberg, 1964). However, the most common solution to this problem still involves storage of large quantities of fungal inoculum taken directly from the insect host or from a limited number of *in vitro* passages (e.g. Jenkins *et al.*, 1998).

A logical development from efforts to prevent loss of pathogen virulence has involved attempts to employ physiological manipulations not just to preserve but to enhance virulence. Lane *et al.* (1991b), for example, reported that blastospores of *B. bassiana* produced under nitrogen-limited conditions adhered and germinated better on leafhopper cuticle than those from carbon-limited cultures. Recent studies have also focused on manipulation of culture conditions to produce fungal conidia with enhanced reserves of polyhydroxy alcohols (polyols), materials known to accumulate in fungal cells and support metabolic activities at low water activity (a_w). Hallsworth and Magan (1994a, 1995) reported that conidia of several hyphomycetes with elevated levels of glycerol and erythritol were able to germinate and grow more rapidly at reduced water activities (a_w as low as 0.887 in the case of *M. anisopliae*) and were more virulent than unmodified conidia against *Galleria* larvae incubated at 86.5% relative humidity (RH). The relationship between endogenous reserves and the ecological fitness and virulence of fungal biological control agents (BCAs) is dealt with by Magan in Chapter 9 of this book.

Ultimately, various traits of fungal pathogens, including host range, production

capacity, stability and virulence, will be manipulated through mutagenesis or parasexual recombination and strain selection (Heale *et al.*, 1989) or genetic (molecular) engineering. Engineering efforts have already produced transformants of *M. anisopliae* with resistance to a commonly used fungicide (Bernier *et al.* 1989; Goettel *et al.*, 1990) and a 25% faster speed of kill (St Leger *et al.*, 1996).

Potential

There is considerable potential for improving the efficiency of fungus production in solid-substrate systems. Bradley *et al.* (1992) reported that pilot systems have produced yields of 3×10^{13} conidia kg^{-1} substrate (1 l of fermenter volume), a greater than twofold increase over the current operational system. Production of fungal propagules in liquid culture at equally high levels of efficiency is a more difficult challenge. Yields of 5×10^{12} blastospores l^{-1} have been claimed for small-spored strains of *Verticillium lecanii*, but yields of other fungi are substantially lower (Latge *et al.*, 1986). On the other hand, yield per unit volume is only one factor in economic analysis. Compared with solid substrates such as whole grains, liquid-culture media contain much less nutrient material on a dry-weight basis. This could translate into lower production and waste-product disposal costs. The rapid growth of fungi in liquid fermentation is another important consideration. Fargues *et al.* (1979) reported growth of *B. bassiana* to concentrations of 2×10^{12} blastospores l^{-1} within 45 h and, recently, media and methods stimulating growth of *P. fumosoroseus* to concentrations of 10^{12} blastospores l^{-1} within 40 h have been developed (Jackson, 1997). Maximum production of aerial conidia on solid substrates requires substantially longer, e.g. 10–14 days for production of *M. anisopliae* var. *acridum* (formerly identified as *Metarhizium flavoviride*) and *B. bassiana* (Samsináková *et al.*, 1981; Jenkins *et al.*, 1998). Several liquid-fermentation cycles could be completed within this time, with efficiency possibly approaching that of aerial-conidia production. In the case of mass-culture systems developed for the production of vegetative forms, such as hyphae or hyphal bodies, which must sporulate to be effective, efficiency assessments must take into account the sporulation capacity of the final product and the fact that some use strategies (e.g. those based on autodissemination or epizootic initiation) may require only small amounts of material.

Submerged conidia are smaller than blastospores and thus theoretically producible in higher concentrations. Unfortunately, in many standard, low-cost culture media, they are produced less commonly and in lower numbers than blastospores (e.g. Vidal *et al.*, 1998). Recent work indicates, however, that production of these propagules is highly dependent on fungal species and isolates, and yields can also be increased by manipulating culture conditions (Rombach, 1989). There is considerable scope for increasing the efficiency of liquid-culture production systems. Jenkins and Prior (1993) recently reported growth of *M. anisopliae* var. *acridum* submerged conidia to concentrations of 1.5×10^{12} conidia l^{-1} within 7 days in an inexpensive medium.

The work on strain improvement through physiological manipulations via modification of culture conditions and genetic manipulations via selection of mutant or novel recombinant strains and genetic engineering is still in early stages of development, and technologies have not proved feasible on an operational scale (especially with respect to commercial production capacity of altered strains). Nevertheless, these technologies clearly have enormous potential to produce improved pathogens for microbial control. Physiological manipulation and parasexual recombination might

provide a means for developing improved fungal BCAs with fewer registration and safety concerns than genetic engineering.

Stabilization of fungal propagules

Aerial conidia

Another important constraint to the development of fungi as microbial control agents has been poor long-term storage stability (defined as maintenance of propagule viability). Stability for 18–24 months at room temperature (*c.* 25°C), needed to increase market competitiveness, has remained an elusive goal; however, considerable progress has been made. Stability has most often been achieved by mixing spores with various materials, and therefore the topic of formulation to increase stability is a major focus of this discussion.

Initial studies of the storage stability of entomopathogenic fungi were conducted with small samples of unformulated conidia exposed to a broad range of temperature and moisture conditions. These studies demonstrated that RH was an important factor in the moderate-temperature storage stability of several fungi, including *M. anisopliae*, *B. bassiana* and *Paecilomyces* spp. (Clerk and Madelin, 1965; Kawakami and Mikuni, 1965; Daoust and Roberts, 1983). Conidia of one strain of *M. anisopliae* retained high levels of viability for at least 18 months under moist conditions (97% RH) at 26°C; however, no dry conidia of any fungus remained stable for more than a few months at this temperature (including those maintained at 0% RH over anhydrous calcium chloride).

The first dry formulation of conidia of an entomopathogenic fungus which retained high viability for 1 year without refrigeration was described in an unpublished report by Ward and Roberts (1981); 78% of *B. bassiana* aerial conidia formulated in attapulgite clay remained viable after 12 months of storage at 26°C, compared with only 6% of unformulated conidia. Moisture conditions were not reported and, while the clay clearly extended spore survival, its mode of action was not known. Ward (1984) hypothesized various mechanisms, including a possible role in keeping moisture available to the fungal spores. Soon thereafter, Chinese researchers described clay formulations of *B. bassiana* with comparable stability, but, in this case, drying of both conidia and formulation ingredients was controlled and quantified, and stability was ultimately correlated with moisture content. Shi (1988) reported that conidia in a clay formulation with < 10% moisture stored at room temperature remained 91% viable for 480 days and 70% viable for 780 days. Chen *et al.* (1990) reported similar stability of an attapulgite clay formulation stored for 12 months at 26°C. Feng *et al.* (1994) reported that these and other studies ultimately led to the establishment of drying to < 5% moisture as a standard protocol for *B. bassiana* production in China. These results represented an important breakthrough in our understanding of factors affecting the long-term stability of conidial formulations and, since the mid-1990s, moisture content has became one of the most actively studied parameters in the formulation of entomopathogenic fungi (Wraight and Carruthers, 1999). It now appears that the capacity of clay to enhance spore survival may relate primarily to its desiccating properties. In a recent study, Moore and Higgins (1997) found no improvement in the stability of *M. anisopliae* var. *acridum* conidia predried to 7% moisture content and formulated with and without various clays.

Composition of the storage atmosphere is another important factor in fungal stability. Storage under nitrogen or enriched CO_2 atmospheres and also under vacuum have been known since the 1960s to enhance short-term stability of aerial conidia (Clerk and Madelin, 1965) and, recently, Miller (1995) claimed that removal of oxygen from the storage atmosphere preserved viability of *M. anisopliae* conidia for 12 months at 37°C.

Considerable effort has been devoted recently to the evaluation of various oils for formulation of aerial conidia (see section on formulation for improved efficacy). The reported effects of vegetable- and petroleum-based oils on the stability of conidia stored over a broad range of temperatures are highly variable and even contradictory (Wraight and Carruthers, 1999). Inconsistencies probably derive from the great variety of oils investigated and the highly variable sensitivities of different fungal species and strains (Jaronski, 1997). Nevertheless, selected oils are highly compatible with the hydrophobic conidia of some hyphomycete species. Paraffinic oil formulations of *B. bassiana* are claimed to have a shelf-life of approximately 1 year at 25°C (S.T. Jaronski, personal communication). *M. anisopliae* var. *acridum* appears to be less amenable to moderate-temperature storage in oil, but is stable for > 1 year at 17°C (Moore and Higgins, 1997).

Hyphal bodies and mycelia

Notwithstanding this substantial progress with hyphomycete conidia, stabilization of mycelia and hyphal bodies from liquid cultures remains a difficult challenge. Blachère *et al.* (1973) reported that blastospores of *Beauveria brongniartii* formulated with sugar and stored at 23°C in sealed ampules under vacuum or nitrogen atmospheres retained viability for at least 8 months. A patent submitted in 1983 claimed that free water was a critical parameter in the stabilization of *B. bassiana* blastospores. Blastospores encapsulated in xanthan-carob gel and dried through a controlled process to an a_w of 0.07 retained 100% viability for 1 year (Jung and Mugnier, 1989). Jung and Mugnier (1989) suggested that polysaccharide encapsulation protected the blastospores from the extreme conditions associated with spray-drying, an economical commercial drying process. More recently, Stephan *et al.* (1997) reported that spray-dried blastospores of *M. anisopliae* var. *acridum* retained 68% viability after storage for 1 year at 20°C. However, none of these techniques has been successfully applied on an industrial scale. Andersch *et al.* (1990) reported that the stability of *M. anisopliae* mycelium pellets (BIO 1020®) was enhanced by storage under vacuum, but neither this treatment nor drying to <10% moisture conferred long-term, moderate-temperature stability (viability was lost after 5 months at 20°C). To our knowledge, 5–6 months is the maximum reported room-temperature stability of hyphal-body or mycelium formulations of entomopathogenic fungi; however, the results of Knudsen *et al.* (1990) suggest that longer shelf-life of mycelium formulations may be achievable with alginate encapsulation. In order to assess and compare the stability of various formulations, it is important that future researchers clearly differentiate between viability of individual hyphal bodies and that of hyphal-body aggregates (especially granules of dried blastospores).

In the cases of fungi propagated as thin-walled hyphal bodies or mycelia in liquid culture, stability becomes an important issue not only with respect to storage, but also during harvest following production. In this regard, lack of desiccation tolerance has been the most important constraint. Materials that function as drying protectants, especially sugars, have been used for many years to stabilize fungal propagules during

desiccation (Crowe *et al.*, 1987). Approaches have varied from the simple incorporation of sugars to encapsulation with starches, flours and polysaccharide gels. These technologies were recently exploited for stabilization of entomophthoralean fungi. Working with *Zoophthora radicans*, McCabe and Soper (1985) developed a desiccation-stabilization process involving extraction of the liquid-culture medium from the harvested mycelium, re-saturation with a maltose solution (10%), incubation to initiate conidiogenesis and then processing through a series of steps with controlled temperatures and drying rates. The original procedure called for storage of the dry mycelium frozen or under refrigeration. At the time of its development, this process represented a major breakthrough in the handling of these difficult fungi, and the process was readily adapted for desiccation stabilization of other entomophthoralean and hyphomycete species (Rombach *et al.*, 1986; Roberts *et al.*, 1987; Li *et al.*, 1993). However, commercial potential remains limited to those species that are mass-producible in relatively inexpensive media, and no modifications of the protocol have yet produced formulations with long-term, room-temperature storage stability.

In recent years, considerable research has focused on the development of special packaging to maintain optimum storage environments. Formulations stabilized by drying and storing under vacuum, for example, require waterproof packaging that is also impermeable to atmospheric gasses, including water vapour. The most commonly used packaging materials with this property are polyethylene–aluminium foil laminates (e.g. Jenkins *et al.*, 1998). On the other hand, fungal products needing oxygen to survive require very different packaging. Polyethylene alone can form an effective barrier against moisture loss or gain, while allowing exchange of oxygen and CO_2 (Miller, 1995).

Submerged conidia

Liquid-fermentation researchers are becoming increasingly interested in developing products based on submerged conidia. This approach has been stimulated by numerous reports of the greater stability of these propagules compared with hyphal bodies. Few studies, however, have actually quantified and compared the desiccation and storage stabilities of the different spore types. Work by Hegedus *et al.* (1992) indicates that, at low temperature (–70°C), the stability of *B. bassiana* submerged conidia is intermediate to that of blastospores and aerial conidia.

Potential

The goal of developing dry powder or dry oil formulations of fungal entomopathogens stable for 18–24 months at room temperature, as called for by commercial developers (Couch and Ignoffo, 1981; Miller, 1995), has not yet been realized, but rapid progress is being made. Recent claims of product stability are impressive and represent important advances in formulation and packaging techniques. Mycotech Corp. claims that their second-generation wettable powder of *B. bassiana* conidia (Mycotrol® 22WP) has a shelf-life of > 12 months at 25°C (S. Jaronski, personal communication), and Jenkins *et al.* (1998) report that conidia of *M. anisopliae* var. *acridum* stored in plastic-lined foil bags with small packets of silica gel are stable for > 1 year at 30°C. The recent discovery that rapid rehydration following dry storage can kill *Metarhizium* conidia and result in erroneous measures of viability should advance research in this area (Moore *et al.*, 1997). Unfortunately, the effects of such factors as drying rate, moisture content, storage atmosphere and rehydration conditions on the stability of blas-

tospore- and mycelium-based formulations of most entomopathogenic fungi remain poorly understood. Because the conidia of Entomphthorales are not amenable to desiccation, researchers have pursued the development of technologies for commercial-scale production of resting spores. Progress in this field is reviewed by Pell *et al.* in Chapter 4 of this book.

Persistent problems with the stability of blastospores and mycelia are stimulating alternative solutions for production and marketing. Technologies are currently being developed for the automated, on-site production of blastospores to supply fresh material for immediate application to insect-infested plants or soil (M.A. Jackson, unpublished observations). Initial development is targeting relatively small-scale production systems, such as those in greenhouses or orchards; however, advances in liquid-culture production could open larger markets. Another solution to the stability problem could involve 'just in time' production and direct sale of fresh mycoinsecticide products to growers (circumventing traditional warehouse-based distribution systems). Pest-control strategies employing live insects to disseminate fungal inoculum (Keller *et al.*, 1997; Butt *et al.*, 1998) require small amounts of fungus that could be supplied in this way.

For the small markets discussed above, cold-temperature storage might be an economically feasible means of stabilizing fungal hyphal bodies and mycelia (and also greatly extending the shelf-life of conidial formulations). Granular formulations of yeasts developed for the biocontrol of postharvest diseases are stable for 12–18 months when vacuum-packed and stored under cool, dry conditions (see below). Nevertheless, commercially viable formulation technologies for the long-term, moderate-temperature stabilization of operational-scale quantities of fungal mycelia and hyphal bodies are needed to make these control agents more competitive in the broader agricultural markets, and success in this area would represent a major advance in the development of fungi as microbial control agents. The studies cited in this work indicate a strong potential for achieving this goal in the near future.

Finally, it is important to note that the stability of fungal spores can be substantially improved by means unrelated to product formulation or packaging, including strain selection (Jaronski, 1997), controlled drying (Jung and Mugnier, 1989) and manipulation of culture conditions, especially nutrients (Lane *et al.*, 1991a; Hallsworth and Magan, 1994a; Jackson *et al.*, 1997).

Formulation of fungal propagules

Many important constraints to the commercial development of entomopathogenic fungi are being addressed through formulation. In this limited space, it is not possible to offer more than a brief overview of the principal problems and progress.

Formulation for improved handling and safety

An important problem associated with microbial control applications of Hyphomycetes relates to the hydrophobicity of the aerial conidia. This characteristic renders technical powders extremely dusty and difficult to suspend in water. In field assessments of *B. bassiana* unformulated conidial powders, Wraight and colleagues found that the preparation of large-volume aqueous suspensions of *B. bassiana* and *P. fumosoroseus* was greatly facilitated by the use of organosilicone wetting agents (Wraight and Bradley, 1996; Wraight and Carruthers, 1999). However, oils (both vegetable- and petroleum-derived)

are inherently compatible with lipophilic conidia and make superior spray carriers. They are essential ingredients for ultra-low-volume applications, capable of being atomized into small droplets (50–100 μm) that do not evaporate before hitting the target. Liquid oil formulations are easily measured and dispensed under operational field conditions. Those containing emulsifiers suspend quickly in water with minimal agitation. Important disadvantages of oil formulations include greater weight (and thus greater shipping costs) and the fact that oils may have phytotoxic properties and must be applied with caution. As previously mentioned, both vegetable- and petroleum-based oils are used for the formulation of mycoinsecticides. Vegetable oils (e.g. sunflower, canola, groundnut) have the advantage of being acceptable for organic production systems; however, they can turn rancid and leave gummy residues that clog spray equipment. In contrast, paraffinic oils evaporate quickly and leave less residue. They may be used to make viscous vegetable and mineral oils more fluid (Ibrahim *et al.*, 1999).

With respect to safety, oil formulants eliminate the dust hazards associated with dry spores (Goettel and Jaronski, 1997). Elimination of dust greatly reduces the risk of inhalation exposure and contact with the eyes. This is especially significant with respect to reducing the allergenic capacity of fungal propagules. For these reasons, use of oils is attractive not only for spray applications, but also in the harvesting of aerial conidia from mass culture. The risks associated with the production and use of fungal BCAs are discussed in more detail in Chapters 12 and 13.

Formulation for improved efficacy

Formulation to improve the efficacy of fungal spores can be viewed as having two broad objectives: (i) improving the persistence of infectious propagules or making them attractive (e.g. using baits) to increase the chances for host contact; and (ii) improving the infectivity of propagules after host contact.

IMPROVING PERSISTENCE. Fungal spores are extremely susceptible to solar radiation and therefore efforts related to the first objective have for many years focused on the development of economical ultraviolet (UV) protectants that do not interfere with the host infection process. Progress, however, has been slow. Many materials have been identified in laboratory studies that significantly increase survival times of irradiated spores; however, fungal propagules exhibit such extreme sensitivity to solar radiation (most are killed within 2 h by direct exposure) that even many-fold increases in survival time may not translate into improved efficacy under field conditions (e.g. Shah *et al.*, 1998). Among the most promising UV protectants identified thus far are stilbene brighteners, especially Tinopal LPW (Calcofluor white) (Shapiro, 1992). These materials can afford high levels of protection to conidia exposed to artificial UV sources in the laboratory and statistically significant protection in the field (Inglis *et al.*, 1995a). However, in field tests of *B. bassiana* formulated with Tinopal and applied to crested wheat-grass, linear regression showed that numbers of colony-forming units (CFU) declined by an average of 73% within 24 h and 88% within 48 h (compared with 87 and 96%, respectively, for water-formulated CFU). Two relatively inexpensive carriers (clay and oil) protected as well as Tinopal (Inglis *et al.*, 1995a). Poor protection by chemical sunscreens in the field may result from evaporation or absorption of the carrier, leaving an ineffective, thin deposit of the protectant.

Few studies have been conducted on the use of spreaders and stickers to improve persistence of fungal propagules, though such materials are clearly needed. Inglis *et al.*

(1995b) reported a 28–61% loss of unformulated conidia of *B. bassiana* from treated wheat and lucerne foliage exposed to simulated rain. Because most entomopathogenic fungi do not infect their hosts through the alimentary canal, an important concern with respect to use of stickers is that the spores should not adhere to the foliage so strongly as to prevent their being dislodged and inoculated on to the host cuticle. Oil carriers are excellent spreaders/stickers that apparently do not interfere with (and may actually enhance) host inoculation (Prior *et al.*, 1988; Ibrahim *et al.*, 1999; Inyang *et al.*, 2000).

IMPROVING INFECTIVITY. A number of laboratory studies (primarily with grasshoppers) have shown that oil formulations of aerial conidia are more efficacious than aqueous formulations under various temperature and moisture conditions (Prior *et al.*, 1988; Bateman *et al.*, 1993; Delgado *et al.*, 1997; Fargues *et al.*, 1997; Milner *et al.*, 1997; Ibrahim *et al.*, 1999). The most commonly postulated mode of action is that oil droplets adhere more strongly to the lipophilic insect cuticle than droplets of water. Also, upon contacting the cuticle, oils spread rapidly and presumably carry conidia to areas of the body that are protected from unfavourable ambient environmental conditions. However, many of the reported differences between oil and aqueous formulations are not large, especially in terms of mortality induced by doses comparable to field rates (10^7–10^9 conidia ml^{-1}), and improved efficacy due to oil formulation has not been clearly demonstrated in the field. Delgado *et al.* (1997) noted no significant differences in efficacy between oil and clay/water formulations of *B. bassiana* conidia applied against grasshoppers in open field plots. Jenkins and Thomas (1996) reported that aerial conidia of *M. anisopliae* var. *acridum* in oil were more effective against grasshoppers than submerged conidia in aqueous suspension, but an aqueous suspension of aerial conidia was not tested. Submerged conidia in oil emulsion or suspended in water were equally effective. Also, in field tests of aerial conidia of *B. bassiana* against whiteflies, wettable powder formulations suspended with organosilicone surfactants were as effective as emulsifiable oil formulations (S.P. Wraight and C.A. Bradley, unpublished). The reason for the different laboratory and field results is unknown. In some cases, efficient application may compensate for ostensibly inferior formulation (Wraight and Carruthers, 1999; Wraight *et al.*, 2000). Elucidation of the effects of oil on fungal efficacy will require much additional work.

Although fungal infection in at least some host–pathogen associations is not correlated with RH (e.g. Ferron, 1977), it is clearly moisture-dependent in many cases and also limited by extreme temperatures. Moulting of inoculated cuticle is another important constraint to fungal infection (Ferron, 1985). Thus, factors that stimulate the rapid germination and development of a fungal pathogen may enhance its capacity to exploit favourable conditions existing for limited periods. This obviously suggests a potential for increasing efficacy through the incorporation of growth stimulants (including common nutrient materials) into fungus formulations. Another approach involves the incorporation of ingredients designed to actually alter the microenvironment within which the fungus operates. Use of humectant materials such as glycerol, for example, has been suggested to provide the fungal spore with moisture for germination and host penetration. Some of the most significant work in these areas has involved formulation of *V. lecanii*. Adding nutrients and humectants to formulations of this pathogen improved efficacy against aphids and whiteflies in greenhouses (Burges, 1998). However, the use of fungal growth enhancers has not yet been demonstrated to substantially increase the efficacy of entomopathogenic fungi applied to field crops.

Researchers developing fungi for weed control have been particularly active in the investigation of humectants and have also developed oil-based invert emulsions (pathogen spores in aqueous droplets suspended in oil) to overcome moisture requirements (Boyette *et al.*, 1993). However, this technology has not been applied to entomopathogenic fungi. Applications of such materials in the sufficiently small and numerous droplets needed to provide effective coverage for insect control would be problematic; the volume of oil required would probably be prohibitively expensive and, in many cases, phytotoxic.

Discussion of the potential for combining materials with various properties into fungal formulations leads ultimately to the topic of granular formulations. Granules provide a basis for incorporation of relatively large amounts of materials, including UV protectants, nutrients to support the growth and sporulation of the fungus and baits to attract the target pest. Granules can also penetrate dense foliage to reach soil insects or carry fungal propagules deep into plant whorls or other pest habitats. Results from many studies have been promising (Latch and Kain, 1983; Rombach *et al.*, 1986; Wraight *et al.*, 1986; Schwarz, 1995; Labatte *et al.*, 1996; Delgado *et al.*, 1997) and a number of commercial granular formulations have been developed (Table 10.1). Nevertheless, operational-scale success with these technologies has been limited. Granular formulations are typically associated with high application rates, and long-term storage of mycelium-based products requires refrigeration. Even large doses of small granules applied to soil (especially if applied at a specific depth using a drill) may not immediately contact a large proportion of the pest population, and control may be slow to develop (Keller, 1992). Studies by Villani *et al.* (1994) have also revealed that insect larvae in the soil may actively avoid mycelium granules. Granular formulations designed to support the production of infectious propagules following application are further disadvantaged in that sporulation generally requires a wetter environment than germination and penetration (Ferron, 1977). These various constraints have a substantial impact on economic competitiveness in many markets (Wraight and Carruthers, 1999).

Potential

Great strides have been made in the development of user-friendly formulations of entomopathogenic fungi. Minimally formulated, dusty and difficult-to-suspend powder preparations have all but been replaced with a variety of sophisticated liquid and wettable-powder formulations with superior handling, safety and shelf-life characteristics. Recent advances, such as the development of non-dusty, highly miscible, wettable-powder formulations of *B. bassiana* aerial conidia by private industry, exemplify the great potential for continued improvements.

Incorporation of various materials into fungus formulations shows great potential for efficacy enhancement, but research has only begun. Few studies have directly compared the effects of different formulation ingredients on fungal efficacy under field conditions. Much field development research is being conducted by scientists seeking patent protection, and experimental results are not available. The development of technologies conferring moderate temperature stability on the hyphal bodies and mycelia of fungal pathogens must be achieved before the full potential of granular mycelium formulations can be realized.

Receiving considerable attention at this time is the use of various chemicals (including synthetic chemical insecticides, microbial metabolites and insect growth regulators)

to synergize the effects of fungal pathogens (Hassan and Charnley, 1989; Boucias *et al.*, 1996; Quintela and McCoy, 1998). Any chemical that interferes with normal insect physiology and development might be hypothesized to synergize fungal activity (even if only through prolongation of intermoult periods). However, the importance of rigorous field evaluations cannot be overemphasized, as synergism expressed in the laboratory may not be observed under field conditions (e.g. Delgado *et al.*, 1999). The use of fungi mixed or alternated with chemical insecticides is an important line of research with great potential, especially in an integrated pest management (IPM) context, but is beyond the scope of this chapter.

Mycoherbicides

Introduction

During the last 30 years, extensive research has been conducted to develop a 'bioherbicide approach' for controlling weedy plants. This approach uses the annual application of indigenous plant pathogens to control weed species (Templeton, 1982). Weed scientists and plant pathologists have identified more than 100 microorganisms which are candidates for development as commercial bioherbicidal agents (Templeton, 1982; Charudattan,1991; see also Chapters 1 and 6). These organisms have been selected because they exhibit specificity toward their host weed and are usually highly aggressive in inciting disease. Despite success in discovering potential mycoherbicides, only four microbes (all fungi) have been registered for commercial use in North America: *Colletotrichum gloeosporioides* (Collego®) for the control of northern joint-vetch (*Aeschynomene virginica*) in Arkansas rice-fields, *Phytophthora palmivora* (DeVine®) for the control of strangler vine (*Morrenia odorata*) in Florida citrus groves, *C. gloeosporioides* (BioMal®) for the control of round-leaved mallow (*Malva pusilla*) in various crops in Canada and *Puccinia canalichlata* (Dr. Biosedge®) for the control of yellow nutsedge (*Cyperus esculentus* L.) (Charudattan, 1991). While regulatory issues and market demand have hindered the development of some potential mycoherbicides, the overall lack of commercial success in using living microbial BCAs stems from difficulties in producing and stabilizing these agents and from the lack of consistently effective weed control in field situations (Zorner *et al.*, 1993; Auld and Morin, 1995). At present, only Collego and DeVine are marketed in the USA.

Collego and DeVine possess three characteristics that have enabled them to become commercial products: amenability to low-cost production methods, consistent weed control under field conditions and economic benefit to the farmer from their usage. Collego and DeVine are produced using submerged culture (deep-tank fermentation) methods. The ability of these fungi to produce high concentrations of infective propagules in liquid culture has led to lower production costs when compared with solid-substrate production techniques. Lower production costs are required if mycoherbicides are to compete with other control measures and to provide the end-user with an economic benefit. Economic benefit from the use of mycoherbicides is dependent upon mycoherbicide cost, crop value and the economic impact of the weed target. Collego and DeVine are used in higher-value crops, rice and citrus groves, respectively, and target weeds of considerable economic importance. These mycoherbicides are also quite effective in controlling their weed hosts under field conditions. In large part, this consistent efficacy is due to favourable environmental conditions (high RH). Northern

joint-vetch is controlled by Collego in flooded rice-fields while strangler vine is controlled by DeVine in irrigated citrus groves.

Selecting weed targets that cause significant economic losses in higher-value crops is imperative if additional commercial successes in mycoherbicide development are to be realized. The ongoing development of herbicide-resistant crops, such as soybean and cotton, will only increase the importance of selecting promising weed–crop systems for mycoherbicide development. The amenability of potential mycoherbicides to low-cost production methods must be evaluated early in the selection and development process. This factor will weigh heavily in determining commercial potential. If commercial development is the ultimate goal, extensive studies on mycoherbicides that lack potential for low-cost production should be discouraged.

The production, stabilization and formulation of mycoherbicides can, without question, have an impact on commercial potential (Boyette *et al.*, 1991). These processes can be optimized to influence not only the final cost of the product but also its biocontrol efficacy. Weed control with fungal pathogens is usually practised by applying infective spores to the target weed (see also Chapter 6). Once in contact with the weed, the fungal spore germinates and penetrates into the weed host. This infection process requires free moisture, which is often limited by environmental conditions. Thus, many mycoherbicides applied under arid environmental conditions provide inconsistent weed control due to limited or varying time periods when free moisture is available. To improve the potential for fungal BCAs to operate under field conditions: (i) weed targets must be selected in environments conducive to fungal infection (i.e. adequate free moisture); and (ii) production and formulation methods must be developed that increase the potential for infection by the mycoherbicidal spore.

The nutritional environment present in the production medium can be optimized to have a positive influence on the 'fitness' of the bioherbicidal spore. In this sense, spore 'fitness' is equated with rapid germination, high rates of appressorium formation and enhanced tolerance to desiccation for increased product shelf-life. These beneficial characteristics allow the mycoherbicidal spore to overcome significant environmental constraints to successful commercial use. Consistent weed control under field conditions requires that the mycoherbicide should remain viable during storage and rapidly infect its weed host when applied in a field situation. By optimizing the nutritional environment during spore production, appropriate endogenous reserves can be sequestered in forming spores to improve their bioherbicidal 'fitness' (Fig. 10.1). In a similar fashion, formulations can be used to improve spore adherence to the weed host, retain moisture near the germinating spore, improve spore stability during desiccation and supply germinating spores with exogenous nutritional reserves to improve their infection potential.

Optimization of the production and formulation processes is critical to the successful commercial development of mycoherbicides. Medium optimization schemes must be designed to improve propagule yield in conjunction with improvements in propagule 'fitness' for use as a mycoherbicide. This chapter will describe strategies that have been employed to optimize production and formulation processes. As examples, studies with the fungus *Colletotrichum truncatum*, a specific pathogen of the weed hemp sesbania, will be used to demonstrate the utility of these strategies.

Production methods

In general, three methods have been evaluated for the production of mycoherbicides: (i) the use of living host plants; (ii) solid-substrate fermentation; and (iii) liquid-cul-

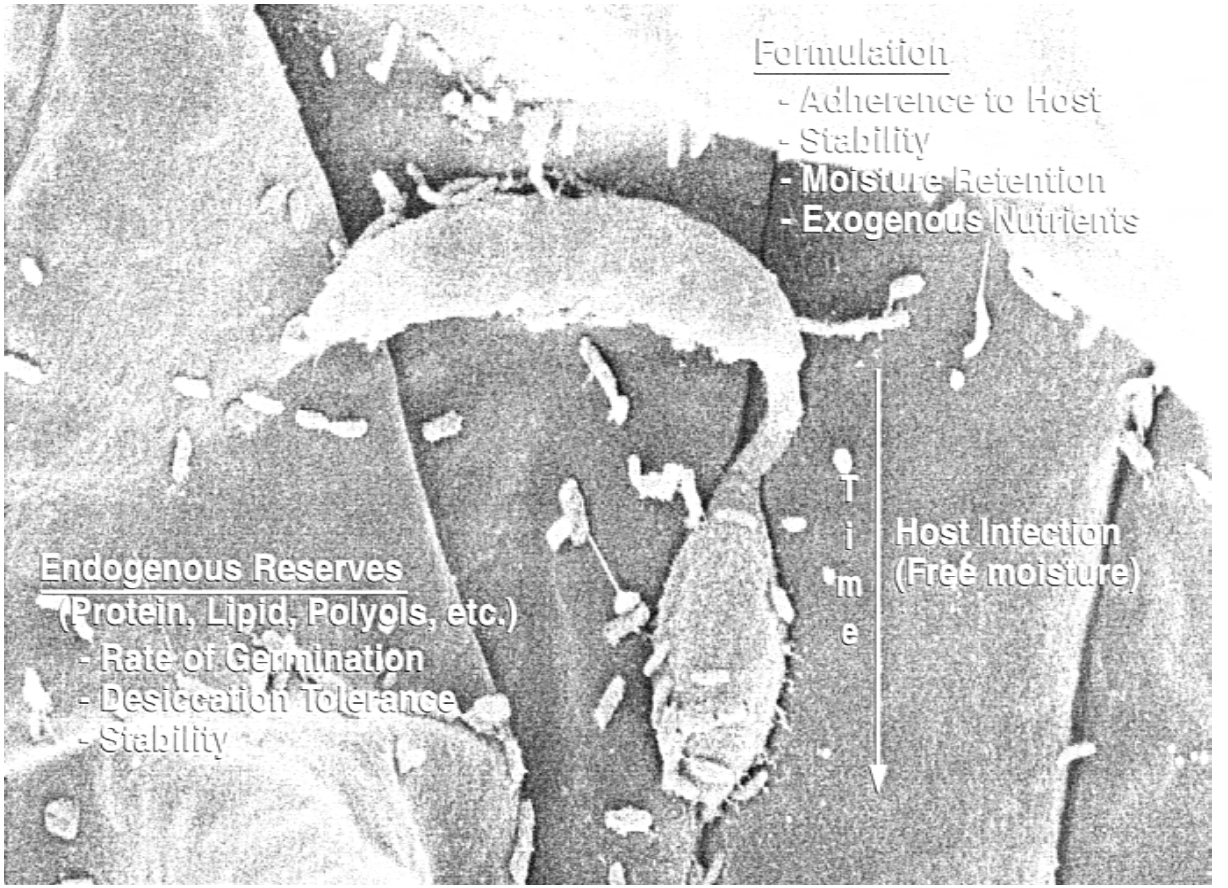

Fig. 10.1. Electron micrograph of a germinating conidium of the bioherbicide *Colletotrichum truncatum* on the leaf of the hemp sesbania (*Sesbania exaltata*). Consistent efficacy of fungal biocontrol agents under field conditions requires adequate free moisture for spore germination and penetration into the pest host. Optimizing production and formulation processes can improve biocontrol efficacy by enhancing spore fitness and environmental conditions for spore penetration into the host. (Electron micrograph provided by D.A. Schisler.)

ture fermentation. While adequate production of many potential mycoherbicides can only be achieved using living plants (rust fungi) or solid-substrate fermentations (*Alternaria* spp.), these methods are usually inefficient, and further cost-saving developments will be needed to make them commercially attractive. Analyses of the various production methods have been the subject of several in-depth reviews (Churchill, 1982; Stowell *et al.*, 1989; Stowell, 1991; Jackson, 1997).

At present, liquid-culture fermentation is the most economical method for producing microbial agents for weed biocontrol. Three of the four mycoherbicides registered for commercial use in North America are produced using liquid-culture fermentation. Both Collego and DeVine are produced in submerged culture. The use of submerged culture fermentation for the production of antibiotics, amino acids, ethanol and organic acids has provided an extensive knowledge base for optimizing processes and hardware for the liquid-culture production of mycoherbicides. Production techniques for bakers' yeast, distillers' yeast and bacterial starter cultures for the dairy industry have demonstrated that living biomass derived from liquid-culture fermentations can be produced economically and can be stabilized as dry preparations. The numerous commercial successes associated with liquid-culture fermentation have strengthened industry's acceptance of this method.

The controlled environment inherent in liquid-culture fermentation is another important advantage of this method. The homogeneity of a liquid medium simplifies production and processing methods and aids in the development of optimized nutritional conditions for production. By using submerged culture fermentations, a homogeneous nutritional environment can be maintained and monitored. In addition, environmental factors such as temperature, aeration and pH are more easily controlled compared with solid-substrate fermentations.

Optimizing production and formulation processes

Directed approaches to medium optimization are possible using liquid-culture fermentation for the production of mycoherbicides. One strategy for optimizing production is based on developing a medium that maximizes propagule yield and fitness. The first step in this optimization strategy is the development of a defined medium that supports good growth and propagule formation. The propagule of interest will depend on the BCA being evaluated and can be, for instance, fungal spores, fungal sclerotia or mycelial fragments. Once a basal medium is developed, nutritional components of the medium can be varied in a directed manner and the impact of these changes assessed in terms of propagule yield, propagule stability as a dry preparation and propagule fitness as a bioherbicidal agent. All of these factors must be considered during optimization, since all are required for an effective commercial BCA. Nutritional factors, such as carbon sources, nitrogen sources, trace metals, vitamins, carbon loading and carbon-to-nitrogen (CN) ratio can all have an influence on growth, propagule formation and biocontrol efficacy.

Once an optimized defined medium has been developed, a production medium can be formulated by replacing the nutritional components of the defined medium with low-cost, complex substrates. Use of this directed optimization strategy not only aids in the development of production media for specific mycoherbicides but also provides nutritional information that will be useful in developing production media for

other microbial BCAs. This strategy has been used to develop production techniques for numerous BCAs, including the fungus *C. truncatum.*

In a similar fashion, formulation processes must be optimized to improve propagule stability under various storage conditions, enhance biocontrol efficacy under field conditions and improve handling characteristics (Boyette *et al.*, 1996). The final product must be safe and easy to use with existing pesticide application technologies. Flowable, dispersible granules that produce low amounts of dust are desired. Formulations or adjuvants for bioherbicidal agents should be designed to improve mycoherbicide performance under field conditions by making the propagule adhere to the weed target, retaining moisture near the germinating spore or providing nutrients that stimulate rapid germination and appressoria formation. Typical mycoherbicide formulations include agricultural residues, such as flours, proteins and oils, in conjunction with inorganic compounds, such as clays and diatomaceous earth.

The development of fermentation and formulation processes must be a coordinated effort in order to reduce costs and maximize product efficacy. Fermentations that maximize propagule formation while reducing mycelial growth simplify downstream processing and formulation. It is important that formulation studies be conducted using propagules derived from typical fermentation processes. For example, liquid culture-produced spores of Collego are harvested directly from the fermentation broth by filtration with diatomaceous earth and air-dried to form the active agent (Churchill, 1982), while chlamydospores of *P. palmivora* (DeVine) are harvested as whole cultures and shipped in liquid form in refrigerated tankers to Florida citrus groves for immediate use (Kenney, 1986). In both cases, minimal processing and formulation reduced production costs, thereby increasing the likelihood that these mycoherbicidal products could be commercialized.

The case of Colletotrichum truncatum

Over the past 10 years, collaborative studies, involving microbiologists, chemical engineers, plant pathologists and formulation scientists, have been directed at developing commercial production and formulation processes for *C. truncatum* NRRL 18434, a specific fungal pathogen of the weed *Sesbania exaltata*, hemp sesbania (Boyette, 1988, 1991a, b). Initial medium optimization studies were directed at defining growth conditions for the submerged culture production of *C. truncatum* conidia. A semi-defined medium was developed, which satisfied the requirements for growth and conidiation. In this basal medium, trace metals and vitamins were included during initial experiments when various carbon and nitrogen sources were being evaluated, since these substrates may or may not contribute vitamins and metals to the medium. Numerous carbon and nitrogen sources were identified that supported submerged culture sporulation of *C. truncatum* (Jackson and Bothast, 1990).

Extensive studies with submerged cultures of *C. truncatum* led to an understanding of the nutritional regulation of propagule formation, yield and biocontrol efficacy. Two nutritional factors, carbon concentration and CN ratio, were shown to have a dramatic impact on propagule formation by submerged cultures of *C. truncatum*. Carbon concentration was shown to regulate conidiation and microsclerotia formation (Jackson and Bothast, 1990). When a carbon concentration of 4–16 g l^{-1} was used, high concentrations of conidia were produced. Carbon concentrations greater than

25 g l^{-1} inhibited conidiation and promoted the formation of highly melanized hyphal aggregates that appeared to be microsclerotia. These studies clearly demonstrated that nutrition can have a dramatic impact on fungal propagule formation.

Conidia production was measured in media with differing CN ratios at a carbon concentration of 4 or 8 g carbon l^{-1}. Media with a CN ratio of 30:1 consistently produced more conidia than media with CN ratios of 10:1 or 80:1 (Jackson and Bothast, 1990). Experiments were designed to evaluate the attributes of conidia produced under differing nutritional environments (CN ratios of 10:1, 30:1 or 80:1). Conidial attributes important to the virulence and 'fitness' of *C. truncatum* were influenced by the nutritional environment. Conidia produced in a medium with a CN ratio of 10:1 were longer and thinner than those produced in 30:1 or 80:1 media (Schisler *et al.*, 1990). Conidia from the 10:1 medium also germinated more rapidly, formed appressoria more frequently and incited more disease in hemp sesbania seedlings when compared with conidia produced in 30:1 or 80:1 media. Rapidly germinating spores should have a significant advantage in causing infection under field conditions, where limited free moisture represents a significant constraint on biocontrol efficacy. Laboratory studies by Altre *et al.* (1999) with conidia of various isolates of the entomopathogenic fungus *P. fumosoroseus* showed a positive correlation between germination speed and infectivity on larvae of the diamondback moth, *Plutella xylostella*. Studies by Egley and Boyette (1995) have shown that the use of oil adjuvants increases the rate of conidial germination and the ability of *C. truncatum* conidia to infect hemp sesbania seedlings under conditions of limited free moisture.

These CN ratio studies demonstrated that nutrition has an impact not only on spore yield but also on the biocontrol efficacy of the *C. truncatum* spore. Obviously, spore yield cannot be the sole criterion for medium optimization, since the medium that yielded the highest spore concentrations (30:1) did not produce the most effective spores in terms of infecting and killing hemp sesbania seedlings. From a practical standpoint, these results demonstrated the importance of developing standardized production protocols to evaluate potential mycoherbicides. Comparing efficacy data on spores produced in different media could be misleading, as spore efficacy may be altered by the nutritional environment.

An essential component of a medium optimization strategy involves understanding the physiological basis for changes in propagule attributes, i.e. 'why are *C. truncatum* conidia produced in media with a CN ratio of 10:1 more effective than conidia produced in 30:1 or 80:1 media?'. Compositional analyses showed that conidia produced in media with a CN ratio of 10:1 contained more protein and less lipid than the 30:1 or 80:1 conidia (Jackson and Schisler, 1992).

Substrate utilization studies showed that the 10:1 medium was nutritionally balanced – that is, *C. truncatum* cultures grown in 10:1 medium depleted both glucose and amino acids after 2 days of growth. Cultures grown in media with CN ratios of 30:1 or 80:1 depleted the amino acids, leaving excess glucose in the media, which was probably converted to lipid reserves. These data suggest an association between increased protein content and an increased rate of conidial germination. This association is supported by optimization studies which showed that media with a CN ratio between 15:1 and 20:1 produced high concentrations of *C. truncatum* conidia ($1–3 \times 10^7$ conidia ml^{-1}) that were high in protein, germinated rapidly, formed appressoria frequently and were highly efficacious in inciting disease in hemp sesbania seedlings (Jackson and Schisler, 1992).

In recent studies, media containing low concentrations of methionine, cysteine and tryptophan were shown to reduce fermentation times and increase conidial yields

(Jackson and Slininger, 1993). Again, the use of defined nutritional conditions allowed us not only to optimize propagule yield and fitness in a directed fashion but also to identify physiological changes that may regulate these differing spore attributes. Similar studies by Hallsworth and Magan (1994b, c), of entomopathogenic fungi grown on solid media, showed that conidia grown on media with differing a_w or nutritional composition sequestered different levels of polyols. Polyols and compounds like trehalose have likewise been associated with improved biocontrol efficacy and stability (Jin *et al.*, 1991; Hallsworth and Magan, 1994a).

A commercial medium for producing highly efficacious *C. truncatum* conidia was developed which yielded 5×10^7 conidia ml^{-1} in 4 days (Silman and Nelsen, 1993). Oxygen delivery requirements for the germination, growth and sporulation of *C. truncatum* in submerged culture have been optimized using bench-top fermenters (Slininger *et al.*, 1993). Unfortunately, initial attempts to stabilize conidia produced in liquid culture as dry or wet preparations eluded development (Silman *et al.*, 1993). Success in stabilizing conidia of *C. truncatum* has been achieved only recently, with carbohydrate- and flour-based formulations (Connick *et al.*, 1996; P.C. Quimby and N.K. Zidack, personal communication).

The initial difficulties in stabilizing conidial preparations of *C. truncatum* led investigators to focus on evaluating the potential of microsclerotia as mycoherbicidal propagules. Since sclerotia are generally considered to be desiccation-resistant structures that allow a fungus to survive adverse environmental conditions, microsclerotia should be amenable to drying and storage and therefore be useful as mycoherbicidal propagules. Microsclerotia of various *Colletotrichum* spp. are known to be overwintering structures, and *C. truncatum* microsclerotia are presumably overwintering structures which persist in decaying hemp sesbania biomass (Fig. 10.2; Tu, 1980; Mahmood and Sinclair, 1991; Khan and Sinclair, 1992).

Submerged culture studies confirmed that media with a high carbon loading (80 g glucose l^{-1}) supported production of 6×10^6 sclerotial particles l^{-1} within 11 days (Jackson *et al.*, 1993; Jackson and Schisler, 1995). When stored at 4°C, air-dried preparations of *C. truncatum* microsclerotia (particle size: 180–425 μm) retained > 90% viability after 4 years (M. Jackson, unpublished data). Furthermore, sclerotial propagules incorporated into soil (150 microsclerotia cm^{-3} potting soil) killed over 95% of emerging hemp sesbania seedlings in growth-chamber studies (Jackson *et al.*, 1993; Jackson and Schisler, 1995).

Flour-based formulations that enhance spore production by microsclerotia have also been shown to incite higher levels of disease in hemp sesbania seedlings (Jackson *et al.*, 1996). Flour-based 'pesta' formulations have also been shown to increase the thermal stability of *C. truncatum* microsclerotia (Connick *et al.*, 1997). For 'pesta' granules stored at 25 and 35°C, no loss in microsclerotia viability was seen after 12 and 4 months, respectively. These studies suggest that microsclerotia may be a useful form of inoculum for controlling hemp sesbania. The potential to produce high concentrations of stable, infective *C. truncatum* microsclerotia in liquid culture is the key developmental step that has allowed us to consider microsclerotia as bioherbicidal propagules. Formulation was also shown to increase biocontrol efficacy and stability.

By using a directed approach to the development of production and formulation media for the potential mycoherbicide *C. truncatum*, we have increased our understanding of how nutrition regulates propagule formation, conidial yield and conidial efficacy. These studies have also led to a method for producing *C. truncatum* microsclerotia in liquid culture. The principles that form the basis for this approach should be

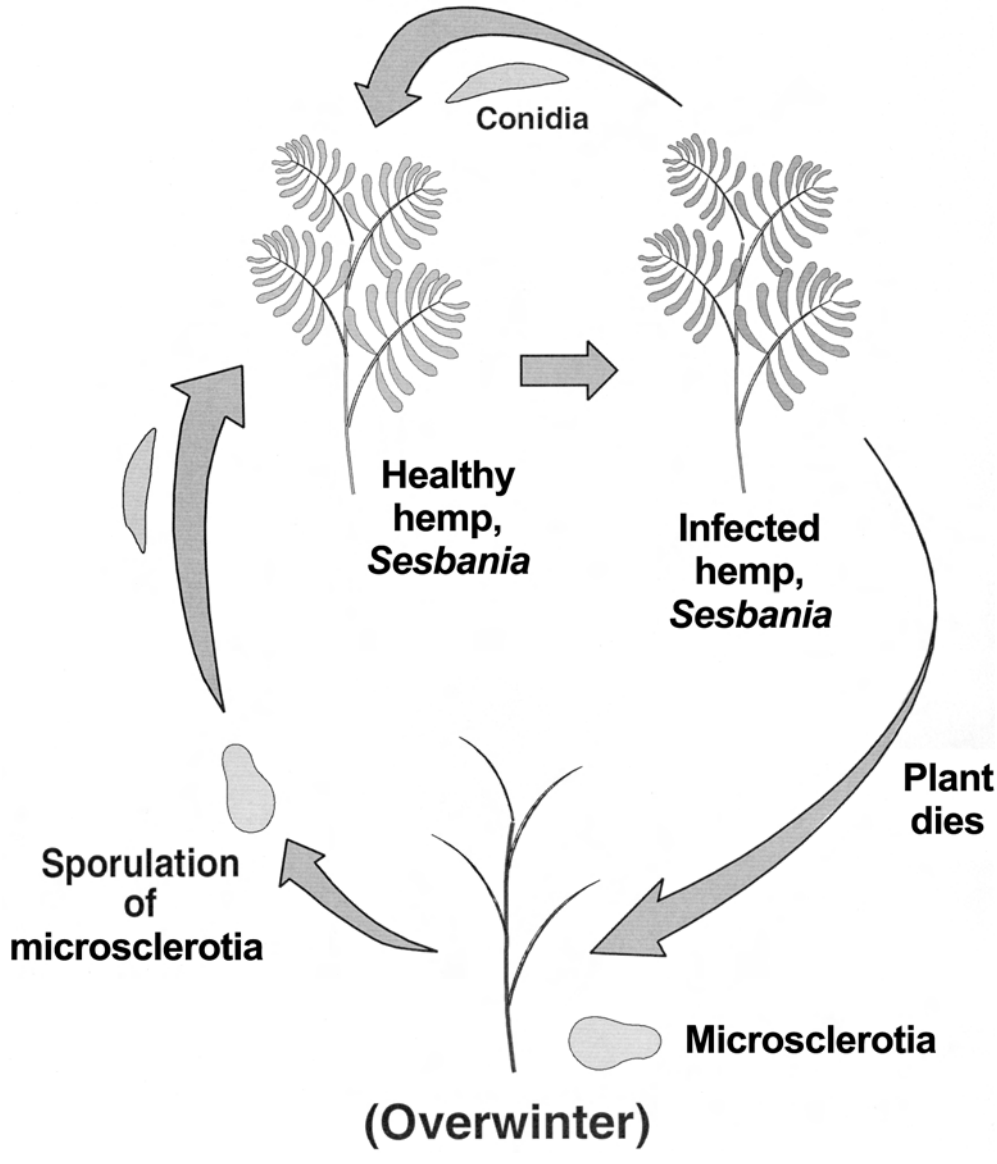

Fig. 10.2. The presumptive disease cycle for *Colletotrichum truncatum* on the weed hemp sesbania (*Sesbania exaltata*). Conidia of *C. truncatum* derived from microsclerotia or diseased plants infect healthy hemp sesbania plants. The pathogen overwinters as microsclerotia in dead plant material.

applicable to the development of any microbial BCA, not just *C. truncatum*. Studies with plant-pathogenic and entomopathogenic fungi being developed as biopesticides have corroborated these results by demonstrating that the nutritional composition of the production medium can affect propagule formation, desiccation tolerance, polyol accumulation and biocontrol efficacy (Bidochka *et al.*, 1987; Jenkins and Prior, 1993; Hallsworth and Magan, 1994a; Jackson *et al.*, 1997). Even fungi that sporulate poorly in submerged culture, such as *Alternaria* spp., may produce propagules with myco-

herbicidal potential when grown under specific nutritional conditions. Using this medium optimization strategy, the chance of finding these specific nutritional conditions is enhanced. Even more importantly, this strategy gives the researcher an opportunity to understand the impact specific nutritional conditions have on the growth, propagule formation and biocontrol efficacy of the mycoherbicide being tested.

Potential

The future of mycoherbicides will be dependent on technological advance and market opportunities. The continuing development of herbicide-resistant crops will certainly limit the market demand for these products. Potential markets for the use of fungal biopesticides will include organic farms, rangelands and low-value public lands with serious invasive weed problems. As Zorner *et al.* (1993) point out, research efforts must now be shifted from the discovery of mycoherbicides to solving the production, storage and efficacy problems that plague all mycoherbicides. Commercial interest and user acceptance of mycoherbicides as weed management tools are dependent on the development of low-cost, stable products that provide consistent weed control under field conditions. These biological constraints are not unique to mycoherbicides but, rather, general problems that have long impeded the development of microbial BCAs. Solving these problems will require research strategies that involve weed scientists, plant pathologists, microbiologists, fermentation specialists, biochemists and formulation scientists. The studies with *C. truncatum* have shown that the implementation of optimization strategies for producing and formulating mycoherbicides requires a multidisciplinary approach. Advances in mycoherbicide stabilization and field efficacy will require additional participation by formulation and application scientists. Academic, industrial and government scientists must work together so that significant advances in the commercialization of mycoherbicides continue to be realized.

Antagonistic Yeasts

Introduction

Postharvest diseases represent a major limiting factor in the long-term storage of fruits and vegetables. The susceptibility of harvested produce to postharvest disease tends to increase as the produce ripens. The major postharvest losses of fruit and vegetables are caused by fungal and bacterial pathogens. In the USA alone, postharvest decay causes an estimated 25% loss of fruit and vegetables (Wilson *et al.*, 1996). In pome fruit, the major postharvest decays are caused by such fungi as *Botrytis, Penicillium* and *Mucor* spp. In South Africa, losses due to postharvest decay of pome fruit have been estimated at 12% (J.C. Combrink, Stellenbosch, 1998, personal communication).

The application of effective fungicides just prior to or shortly after harvest generally controls postharvest decay. Fungicides, however, leave residues on fruit, which can pose safety risks to the consumer. Furthermore, disposing of used chemical pesticides poses a problem, as they may be detrimental to the environment. Consequently, fruit industries worldwide have accepted the concept of integrated fruit production (IFP). The objective of IFP is to produce high-quality fruit in harmony with the consumer and the environment. This implies minimum usage of chemicals, especially after

harvest. IFP has necessitated an entirely new approach towards control of postharvest decay. The development of fungicide-tolerant strains of postharvest pathogens has increased efforts to develop alternative approaches to the control of postharvest diseases (Spotts and Cervantes, 1986).

Biological control has been studied for the past 20 years with varying degrees of success. Many microbial antagonists of postharvest pathogens have been studied and are effective BCAs. However, few have been commercialized. The greatest obstacle in extrapolating biocontrol research from the laboratory to the packing-house has probably been the lack of commercial partners. A commercial partner is required to transform the ideas of scientists into ventures that are commercially viable and that can be used by producers without specialized equipment or techniques.

Yeasts are effective colonizers of plant surfaces under adverse environmental conditions (Droby *et al.*, 1996) and utilize available nutrients very rapidly (Droby *et al.*, 1989). Most antagonistic yeasts produce extracellular materials (mostly polysaccharides) that enhance their survival on fruit surfaces (Droby *et al.*, 1989, 1995). They also appear to be compatible with most fungicides, which adds to their usefulness as BCAs in the postharvest environment. At present, only two antagonistic yeasts have been developed commercially as BCAs and are available as commercial products. Aspire® contains the yeast *Candida oleophila*. This product is registered for commercial use in the USA and Israel. YieldPlus® contains the yeast *Cryptococcus albidus* and is registered in South Africa for commercial use.

Production

Isolation and efficacy evaluation

Antagonists isolated from fruit surfaces are good candidates for postharvest biocontrol because of their adaptation to the host and the environment (Smilanick, 1994). Various techniques and criteria have been reported for the isolation and selection of potential antagonists (Janisiewicz, 1987; Redmond *et al.*, 1987; Chalutz and Wilson, 1990; Roberts, 1990). Using fruit wash, Chalutz and Wilson (1990) isolated over 200 isolates of yeasts and bacteria indigenous to fruit surfaces with potential biocontrol activity. Potential antagonists have also been isolated from leaves, flowers, stems and fruit peel (Janisiewicz, 1987; Redmond *et al.*, 1987; Roberts, 1990; Schiewe and Mendgen, 1992). De Kock (1998) found that peeling the skin of the fruit, liquidizing it in sterile deionized water and plating serial dilutions on nutrient broth/yeast extract/dextrose agar (NYDA) allowed the isolation of most of the epiphytic microorganisms.

Unlike *in vitro* testing, *in vivo* screening of antagonistic yeasts provides essential information regarding the ability of the potential antagonists to survive on the host, possible pathogenicity towards the host, and biocontrol activity and modes of action against major pathogens (Smilanick, 1994). Screening for biocontrol activity *in vivo* entails placement of the potential antagonists and the pathogen on the host. Subsequent suppression of disease is used as a criterion for selecting antagonists for further testing. The results from *in vivo* testing are highly dependent on the physiological state of the host tissue and therefore the maturity of experimental fruit should represent the maturity stages of commercial consignments. In the case of ripe apples and pears, sugar content is much higher than in the unripe fruit, and the presence of excessive nutrients in the wound site will probably rule out nutrient competition as an effective mode of

action (because it will not be possible for the antagonist to utilize all available nutrients before the pathogen succeeds in germinating and penetrating into the host tissue).

Evaluating suitability for commercialization

Criteria with a strong view toward marketing requirements were proposed by Wilson and Wisniewski (1989) and Hofstein *et al.* (1994) to determine the suitability of a yeast strain for commercialization as a BCA. These included:

1. Genetic stability.
2. High, consistent efficacy.
3. Ability to survive under adverse environmental conditions.
4. Effectiveness against a wide range of pathogens on a variety of fruits and vegetables.
5. Amenability for growth on an inexpensive medium in fermenters.
6. Stability of the end-product during storage.
7. Non-production of secondary metabolites that may be deleterious to humans.
8. Resistance to standard fungicides.
9. Compatibility with other chemical and physical treatments applied to the specific commodity.

Consistency in effectively controlling postharvest decay in the commercial environment is a critical requirement if a yeast strain is to emerge as an economically attractive alternative to chemical control. The efficacy of antagonistic yeasts for the control of postharvest decay cannot be directly compared with that of synthetic fungicides. Most synthetic fungicides penetrate the fruit tissue to a certain extent and therefore have a residual effect. Yeast, on the other hand, remains on the fruit surface and can only protect against decay fungi in the specific location where it is applied. To demonstrate that the efficacy of an antagonist is consistent requires extensive semi-commercial and commercial trials in packing-houses. This requires large volumes of yeast, and it is therefore important at this point to know the potential of the yeast for large-scale production. Specific laboratory tests are required to determine optimum growth conditions for the yeast. These include tests to determine osmotolerance, temperature and oxygen requirements, optimum pH and optimum growth rate (in order to determine nutrient feed rates).

Characteristics required for commercial production

Yeast suitable for large-scale production should ideally have certain characteristics. They should:

1. Be unicellular, with minimal size variation.
2. Not form pseudomycelia.
3. Have a fast growth rate.
4. Be genetically stable during production.
5. Not produce slime during the exponential-growth phase.
6. Be osmotolerant.
7. Not be temperature-sensitive.
8. Be able to survive the stress conditions during filtration and drying.
9. Be compatible with commercial emulsifiers.
10. Be viable with less than 4% cell-water content.

When scaling up the production of an antagonistic yeast strain, it is important to select candidates that can be mass-produced despite their detachment from the natural habitat of growth. Mass production has to be cost-effective (Hofstein *et al.*, 1994), because the use of the product has to be promoted in the postharvest disease-control industry, which is investing only a small proportion of production costs into postharvest treatments. Growth media may therefore have to comprise industrial by-products or waste material. Antagonistic yeasts exhibit disease control in a concentration-dependent manner. Mass production will not be cost-effective if a final concentration of more than 10^{10} CFU g^{-1} is required. Although BCAs are more environmentally friendly, the sales price of the product must compete with that of currently used fungicides.

Production problems

The art of growing an antagonistic yeast successfully is, to a great extent, dependent upon the amount of technical information available on the specific yeast strain. Antagonistic yeasts appear to have many attributes, including requirements for optimum growth, that differ from those of *Saccharomyces* spp. It therefore cannot be assumed that standard principles for the large-scale propagation of *Saccharomyces* will apply to large-scale propagation of antagonistic yeasts. In general, the growth rate of yeasts is quite high, although slower than that of most rapidly growing bacteria. A longer fermentation period thus creates the opportunity for bacteria or other contaminants to overrun the culture.

Antagonistic yeasts appear to be very sensitive to low pH (below pH 5) and high temperatures (above 28°C). Low pH levels (pH 2.9–3.0) are normally used as a means of limiting bacterial contamination during fermentation. Bacterial levels increase considerably at pH levels above pH 5. Contrary to standard practices, other technologies to prevent contamination must therefore be used. It is important to be able to quantify the level of contamination at the end of production in order to certify the end-product. It is also important to have a method of identification to verify the purity of the culture during all stages of production.

Sensitivity to temperatures above 28°C puts considerable strain on the cooling system of the production facility, because most yeast factories use evaporative cooling systems. Yeast fermentation is an exothermic reaction, generating heat during the fermentation. The implication is that the fermentation temperature can never be lower than ambient, unless a different cooling system is used (e.g. using glycol or ammonia). This, once again, adds to the cost of production.

The oxygen requirements for maximum output must be determined in advance, because either over-aeration or under-aeration could have a major influence on the growth rate of the specific yeast strain. Also, contamination can often occur during early stages of propagation, since it is difficult to keep large volumes of air sterile. The polysaccharide capsule around some antagonists such as *C. albidus* and *Candida saitoana* makes the filtering and drying processes for these yeasts considerably more complicated than those required for baker's yeast. A difficult challenge thus includes the determination of optimum growth conditions that minimize capsule production.

Stabilization and formulation

A reliable and consistent product having a reproducible performance in terms of effi-

cacy requires a strict quality assurance (QA) programme. *In vitro* evaluation of efficacy does not always correlate with the desired result of disease control. A reliable *in vivo* test on wounded fruit surfaces therefore forms an important part of the QA procedure (Hofstein *et al.*, 1994). The commercial formulation should remain stable (viable and efficacious) during a storage period of at least 12 months. This can either be achieved by supplementing the yeast with protectants, carriers and a variety of compounds designated additives, or yeast cultures can be conditioned during fermentation for fluidized-bed drying. In the latter case, the yeast cream is treated with an emulsifier prior to drying, but no other additives are required. During fluidized-bed drying, the temperature of the yeast is above 50°C only when the dry-matter content of the yeast is above 88% by weight. This occurs for only 10–45 min at the end of the drying process. Fluidized-bed drying results in dried yeast granules. Granules with an average particle size of 1.0–1.7 mm diameter have a much longer shelf-life than powder formulations; the smaller surface area of the granules slows moisture absorption and oxidation. Vacuum-packing of yeast granules of this particle size will further prolong the shelf-life of the product. Storing vacuum-packed yeast under cool, dry conditions will ensure a shelf-life of at least 12–18 months. The carriers in wettable-powder or oil-based formulations of yeast must satisfy the requirements of ecological safety (Hofstein *et al.*, 1994).

Market positioning and potential

The future of postharvest biocontrol is highly dependent on the way we position the few existing commercial biocontrol products.

The first option is to enter the agrochemical market. If we choose to do this, we have to be prepared to compete head-on against synthetic fungicides and bactericides that have been used for many years. This implies a need for biocontrol products that can eradicate latent infections or at least kill pathogenic microorganisms on the fruit surface. Yeast-alone BCAs have limitations as they can only protect the surface of a host. Yeast does not have any systemic action and cannot migrate from one part of the fruit surface to another. Complete coverage of the fruit surface is therefore essential to achieve sufficient control. Thus, positioning of antagonistic yeasts in the agrochemical market may require a new generation of biopesticides that include other natural fungicides or bactericides (Wilson *et al.*, 1996).

The second option is to position our products in the 'all green' or 'all natural' category. This market is much smaller than the agrochemical market, but here we could offer our products as value-adding products. In this market our products offer protection against fruit or vegetable diseases, where no alternative is available. Biocontrol products will only have to compete against other natural products available in the same market.

The third option would be to enter the category where the yeasts are used in combination with fungicides applied at a reduced rate. In this category our products will still have to compete against chemicals in terms of efficacy. The fact that the fungicide is used in combination with a natural product does not classify it under a different category. Once a natural product has been introduced to this market, it will be difficult to withdraw and transfer it to the 'all green' category. This option should only be used as a last resort.

If the principal reason for using BCAs is consumer and environmental safety, the

'all green' category is where we should position our products. In this market we open a new world of sales opportunities. Our products will open the door for fruit exports to countries where the use of synthetic fungicides has been restricted.

Conclusion

The path to the successful commercialization and large-scale utilization of pathogenic fungi as BCAs has proved long and difficult. The great challenge that continues to confront applied mycopathology (and biological control as a whole) is that of finding grower acceptance given the extraordinary efficacy of novel pesticidal chemistries, which continue to be discovered with remarkable regularity and reliability. And yet, in the face of this challenge, research and development of biocontrol fungi has not only continued, but accelerated. These development efforts have been driven, in large part, by the great capacity of pests to resist chemical pesticides and the need to develop sustainable food production methods with minimal environmental impacts. However, it is important to recognize that the consistent progress achieved by applied mycopathologists has also provided a significant impetus. Consistent and substantial progress in the development of fungi for biological control has been realized throughout the long history of this endeavour, and progress in the past two decades has been exceptional. Recent advances in mass culture, harvest and stabilization technologies have greatly increased production efficiencies and product shelf-life, and breakthroughs in formulation technologies are leading to products not only with improved handling and application characteristics, but also with greater and more consistent efficacy. These advances represent landmark achievements that have greatly stimulated mycoinsecticide development and culminated in the creation, registration and commercialization of numerous products worldwide.

It is our belief that the ultimate potential of fungal entomopathogens will be realized not through a single breakthrough technology, but rather from a series of complementary advances. The greatest challenge – the development of mycopesticides that can consistently provide adequate pest, weed and disease control in major food production systems at costs competitive with synthetic chemical control agents – will, almost certainly, depend upon future advances in many areas of production, stabilization and formulation.

While recognizing the need for additional progress, it is not our intent to once again describe fungal pathogens as the 'control agents of the future'. The recent advances in mycopesticide development described above have already yielded products with the capacity to provide useful control of many insect pests, weeds and postharvest diseases. Greenhouse and other high-value crops, as well as organic production systems, obviously hold the greatest potential for initial commercial success with these products. On the other hand, various pests have proved sufficiently susceptible to available pathogen formulations to stimulate the commercial development of products for broader markets. Applied in well-conceived IPM systems, currently available mycopesticide products have the potential to replace much synthetic chemical pesticide use, thereby reducing chemical contamination of the environment and food supply, and to reduce selection pressures, thereby prolonging the effective lives of novel pesticidal chemistries and the highly efficient food production systems that they support.

Note

The use of product names is necessary to report factually on available data: however, the US Department of Agriculture (USDA) neither guarantees nor warrants the standard of the product, and the use of the name by the USDA implies no approval of the product to the exclusion of others that may also be suitable.

References

Altre, J.A., Vandenberg, J.D. and Cantone, F.A. (1999) Pathogenicity of *Paecilomyces fumosoroseus* isolates to the diamondback moth, *Plutella xylostella*: correlation with spore size, germination speed, and attachment to cuticle. *Journal of Invertebrate Pathology* 73, 332–338.

Andersch, W., Hartwig, J., Reinecke, P. and Stenzel, K. (1990) Production of mycelial granules of the entomopathogenic fungus *Metarhizium anisopliae* for biological control of soil pests. In: *Proceedings of the Vth International Colloquium on Invertebrate Pathology and Microbial Control, Adelaide, Australia.* Society for Invertebrate Pathology, Raleigh, North Carolina, pp. 2–5.

Auld, B.A. and Morin, L. (1995) Constraints in the development of bioherbicides. *Weed Technology* 9, 638–652.

Bartlett, M.C. and Jaronski, S.T. (1988) Mass production of entomogenous fungi for biological control of insects. In: Burge, R.N. (ed.) *Fungi in Biological Control Systems.* Manchester University Press, New York, pp. 61–85.

Bateman, R.P., Carey, M., Moore, D. and Prior, C. (1993) The enhanced infectivity of *Metarhizium flavoviride* in oil formulations to desert locusts at low humidities. *Annals of Applied Biology* 122, 145–152.

Bernier, L., Cooper, R.M., Charnley, A.K. and Clarkson, J.M. (1989) Transformation of the entomopathogenic fungus *Metarhizium anisopliae* to benomyl resistance. *FEMS Microbiology Letters* 60, 261–266.

Bidochka, M.J., Pfeifer, T.A. and Khachatourians, G.G. (1987) Development of the entomopathogenic fungus *Beauveria bassiana* in liquid cultures. *Mycopathologia* 99, 77–83.

Blachère, H., Calvez, J., Ferron, P., Corrieu, G. and Peringer, P. (1973) Etude de la formulation et de la conservation d'une préparation entomopathogène à base de blastospores de *Beauveria tenella* (Delacr. Siemaszko). *Annales de Zoologie Ecologie Animale* 5, 69–79.

Boucias, D.G., Stokes, C., Storey, G. and Pendland, J.C. (1996) The effects of imidacloprid on the termite *Reticulitermes flavipes* and its interaction with the mycopathogen *Beauveria bassiana. Pflanzenschutz-Nachrichten Bayer* 49, 103–144.

Boyette, C.D. (1988) Efficacy and host range of a recently discovered fungal pathogen for biocontrol of hemp sesbania. *Proceedings of the Southern Weed Science Society* 41, 267.

Boyette, C.D. (1991a) Control of hemp sesbania with a fungal pathogen *Colletotrichum truncatum.* US patent no. 5,034,328.

Boyette, C.D. (1991b) Host range and virulence of *Colletotrichum truncatum*, a potential mycoherbicide for hemp sesbania (*Sesbania exaltata*). *Plant Disease* 75, 62–64.

Boyette, C.D., Quimby, P.C., Connick, W.J., Daigle, D.J. and Fulgham, F.E. (1991) Progress in the production, formulation, and application of mycoherbicides. In: TeBeest, D.O. (ed.) *Microbiological Control of Weeds.* Chapman & Hall, New York, pp. 209–222.

Boyette, C.D., Quimby, P.C. Jr, Bryson, C.T., Egley, G.H. and Fulgham, F.E. (1993) Biological control of hemp sesbania (*Sesbania exaltata*) under field conditions with *Colletotrichum truncatum* formulated in an invert emulsion. *Weed Science* 41, 497–500.

Boyette, C.D., Quimby, P.C. Jr, Caesar, A.J. Jr, Birdsall, J.L., Connick, W.J., Daigle, D.J., Jackson, M.A., Egley, G.H. and Abbas, H.K. (1996) Adjuvants, formulations, and spraying systems for improvement of mycoherbicides. *Weed Technology* 10, 637–644.

Bradley, C.A., Black, W.E., Kearns, R. and Wood, P. (1992) Role of production technology in mycoinsecticide development. In: Leatham, G.F. (ed.) *Frontiers in Industrial Mycology.* Chapman & Hall, New York, pp. 160–173.

Burges, H.D. (1998) Formulation of mycoinsecticides. In: Burges, H.D. (ed.) *Formulation of Microbial Biopesticides: Beneficial Microorganisms, Nematodes and Seed Treatments.* Kluwer Academic Publishers, Dordrecht, pp. 131–185.

Butt, T.M., Carreck, N.L., Ibrahim, L. and Williams, I.H. (1998) Honey-bee-mediated infection of pollen beetle (*Meligethes aeneus* Fab.) by the insect-pathogenic fungus, *Metarhizium anisopliae. Biocontrol Science and Technology* 8, 533–538.

Chalutz, E. and Wilson, C.L. (1990) Post-harvest biocontrol of green and blue mold and sour rot of citrus fruit by *Debaryomyces hansenii. Plant Disease* 74, 134–137.

Charudattan, R. (1991) The mycoherbicide approach with plant pathogens. In: Te Beest, D.O. (ed.) *Microbiological Control of Weeds.* Chapman & Hall, New York. pp. 24–57.

Chen, C.J., Wu, J.W., Li, Z.Z., Wang, Z.X., Li, Y.W., Chang, S.H., Yin, F.M., Wang, X.P., Dai, L.Y., Tao, L., Zhang, Y.A., Tang, J., Ding, S., Ding, G.G., Gao, Z.H. and Tan, Y.C. (1990) Application of microbial pesticides in IPM. In: Chen, C.J. (ed.) *Integrated Management of Pine Caterpillars in China.* China Forestry Publishing House, Beijing, pp. 214–308.

Churchill, B.W. (1982) Mass production of microorganisms for biological control. In: Charudattan, R. and Walker, H.L. (eds) *Biological Control of Weeds with Plant Pathogens.* John Wiley & Sons, New York, pp. 139–156.

Clerk, G.C. and Madelin, M.F. (1965) The longevity of conidia of three insect-parasitizing Hyphomycetes. *Transactions of the British Mycological Society* 48, 193–209.

Connick, W.J., Daigle, D.J., Boyette, C.D., Williams, K.S., Vinyard, B.T. and Quimby, P.C. (1996) Water activity and other factors that affect the viability of *Colletotrichum truncatum* conidia in wheat flour-kaolin granules (Pesta). *Biocontrol Science and Technology* 6, 277–284.

Connick, W.J., Jackson, M.A., Williams, K.A. and Boyette, C.D. (1997) Stability of microsclerotial inoculum of *Colletotrichum truncatum* encapsulated in wheat flour-kaolin granules. *World Journal of Microbiology and Biotechnology* 13, 549–554.

Couch, T.L. and Ignoffo, C.M. (1981) Formulation of insect pathogens. In: Burges, H.D. (ed.) *Microbial Control of Pests and Plant Diseases: 1970–1980.* Academic Press, London, pp. 621–634.

Crowe, J.H., Crowe, L.M., Carpenter, J.F. and Wistrom, C.A. (1987) Stabilization of dry phospholipid bilayers and proteins by sugars. *Biochemical Journal* 242, 1–10.

Daoust, R.A. and Roberts, D.W. (1983) Studies on the prolonged storage of *Metarhizium anisopliae* conidia: effect of temperature and relative humidity on conidial viability and virulence against mosquitoes. *Journal of Invertebrate Pathology* 41, 143–150.

Delgado, F.X., Lobo-Lima, M.L., Bradley, C., Britton, J.H., Henry, J.E. and Swearingen, W. (1997) Laboratory and field evaluations of *Beauveria bassiana* (Balsamo) Vuillemin against grasshoppers and locusts in Africa. *Memoirs of the Entomological Society of Canada* 171, 239–251.

Delgado, F.X., Britton, J.H., Onsager, J.A. and Swearingen, W. (1999) Field assessment of *Beauveria bassiana* (Balsamo) Vuillemin and potential synergism with diflubenzuron for control of savanna grasshopper complex (Orthoptera) in Mali. *Journal of Invertebrate Pathology* 73, 34–39.

Dorta, B. and Arcas, J. (1998) Sporulation of *Metarhizium anisopliae* in solid-state fermentation with forced aeration. *Enzyme and Microbial Technology* 23, 501–505.

Droby, S., Chalutz, E., Wilson, C.L. and Wisniewski, M.E. (1989) Characterization of the biocontrol activity of *Debaryomyces hansenii* in the control of *Penicillium digitatum* on grapefruit. *Canadian Journal of Microbiology* 35, 794–800.

Droby, S., Chalupovicz, L., Chalutz, E., Wisniewski, M. and Wilson, C.L. (1995) Inhibitory activity of yeast cell wall materials against post-harvest fungal pathogens. *Phytopathology* 85, 1123.

Droby, S., Chalutz, E., Wisniewski, M.E. and Wilson, C.L. (1996) Host response to introduction of antagonistic yeasts used for control of postharvest decay. In: Morris, C.E., Nicot, P.C. and Nguyen-The, C. (eds) *Aerial Plant Surface Microbiology.* Plenum Press, New York, pp. 73–89.

Egley, G.H. and Boyette, C.D. (1995) Water-corn oil emulsion conidia germination and mycoherbicidal activity of *Colletotrichum truncatum. Weed Science* 43, 312–317.

Fargues, J., Robert, P.H. and Reisinger, O. (1979) Formulation des productions de masse de l'hyphomycète entomopathogène *Beauveria* en vue des applications phytosanitaires. *Annales de Zoologie Ecologie Animale* 11, 247–257.

Fargues, J., Ouedraogo, A., Goettel, M.S. and Lomer, C.J. (1997) Effects of temperature, humidity and inoculation method on susceptibility of *Schistocerca gregaria* to *Metarhizium flavoviride. Biocontrol Science and Technology* 7, 345–356.

Feng, M.G., Poprawski, T.J. and Khachatourians, G.G. (1994) Production, formulation and application of the entomopathogenic fungus *Beauveria bassiana* for insect control: current status. *Biocontrol Science and Technology* 4, 3–34.

Ferron, P. (1977) Influence of relative humidity on the development of fungal infection caused by *Beauveria bassiana* (Fungi Imperfecti, Moniliales) in imagines of *Acanthoscelides obtectus* (Col.:Bruchidae). *Entomophaga* 22, 393–396.

Ferron, P. (1985) Fungal control. In: Kerkut, G.A. and Gilbert, L.I. (eds) *Comprehensive Insect Physiology, Biochemistry and Pharmacology*, Vol. 12. Pergamon Press, Oxford, pp. 313–346.

Goettel, M.S. and Jaronski, S.T. (1997) Safety and registration of microbial agents for control of grasshoppers and locusts. *Memoirs of the Entomological Society of Canada* 171, 83–99.

Goettel, M.S., St Leger, R.J., Bhairi, S., Jung, M.K., Oakley, B.R., Roberts, D.W. and Staples, R.C. (1990) Pathogenicity and growth of *Metarhizium anisopliae* stably transformed to benomyl resistance. *Current Genetics* 17, 129–132.

Hallsworth, J.E. and Magan, N. (1994a) Improved biological control by changing polyols/trehalose in conidia of entomopathogens. In: *Brighton Crop Protection Conference – Pests and Diseases – 1994, Proceedings*, Vol. 3. British Crop Protection Council, Farnham, UK, pp. 1091–1096.

Hallsworth, J.E. and Magan, N. (1994b) Effect of carbohydrate type and concentration on polyhydroxy alcohol and trehalose content of conidia of three entomopathogenic fungi. *Microbiology* 140, 2705–2713.

Hallsworth, J.E., and Magan, N. (1994c) Effects of KCl concentration on accumulation of acyclic sugar alcohols and trehalose in conidia of three entomopathogenic fungi. *Letters in Applied Microbiology* 18, 8–11.

Hallsworth, J.E. and Magan, N. (1995) Manipulation of intracellular glycerol and erythritol enhances germination of conidia at low water availability. *Microbiology* 141, 1109–1115.

Hassan, A.E.M. and Charnley, A.K. (1989) Ultrastructural study of the penetration of *Metarhizium anisopliae* through dimilin affected cuticle of *Manduca sexta. Journal of Invertebrate Pathology* 54, 117–124.

Heale, J.B., Isaac, J.E. and Chandler, D. (1989) Prospects for strain improvement in entomopathogenic fungi. *Pesticide Science* 26, 79–92.

Hegedus, D.D., Bidochka, M.J., Miranpuri, G.S. and Khachatourians, G.G. (1992) A comparison of the virulence, stability and cell-wall-surface characteristics of three spore types produced by the entomopathogenic fungus *Beauveria bassiana. Applied Microbiology and Biotechnology* 36, 785–789.

Hofstein, R., Friedlender, B., Chalutz, E. and Droby, S. (1994) Large-scale production and pilot testing of biological control agents for post-harvest diseases. In: Wilson, C.L. and Wisniewski, M.E. (eds) *Biological Control of Post-harvest Diseases: Theory and Practice.* CRC Press, Boca Raton, Florida, pp. 89–100.

Ibrahim, L., Butt, T.M., Beckett, A. and Clark, S.J. (1999) Germination of oil-formulated conidia of the insect-pathogen, *Metarhizium anisopliae. Mycological Research* 103, 901–907.

Inglis, G.D., Goettel, M.S. and Johnson, D.L. (1995a) Influence of ultraviolet light protectants

on persistence of the entomopathogenic fungus, *Beauveria bassiana. Biological Control* 5, 581–590.

Inglis, G.D., Johnson, D.L. and Goettel, M.S. (1995b) Effects of simulated rain on the persistence of *Beauveria bassiana* conidia on leaves of alfalfa and wheat. *Biocontrol Science and Technology 5*, 365–369.

Inyang, E.N., McCartney, H.A., Oyejola, B., Ibrahim, L., Pye, B.J., Archer, S.A. and Butt, T.M. (2000) Effect of formulation, application and rain on the persistence of the entomogenous fungus *Metarhizium anisopliae* on oilseed rape. *Mycological Research* 104, 653–661.

Jackson, M.A. (1997) Optimizing nutritional conditions for the liquid culture production of effective fungal biocontrol agents. *Journal of Industrial Microbiology and Biotechnology* 19, 180–187.

Jackson, M.A. and Bothast, R.J. (1990) Carbon concentration and carbon to nitrogen ratio influence submerged culture conidiation by the potential bioherbicide *Colletotrichum truncatum* NRRL 13737. *Applied and Environmental Microbiology* 56, 3435–3438.

Jackson, M.A. and Schisler, D.A. (1992) The composition and attributes of *Colletotrichum truncatum* spores are altered by the nutritional environment. *Applied and Environmental Microbiology* 58, 2260–2265.

Jackson, M.A. and Schisler, D.A. (1995) Liquid culture production of microsclerotia of *Colletotrichum truncatum* for use as bioherbicidal propagules. *Mycological Research* 99, 879–884.

Jackson, M.A. and Slininger, P.J. (1993) Submerged culture, conidial germination and conidiation of the bioherbicide *Colletotrichum truncatum* are influenced by the amino acid composition of the medium. *Journal of Industrial Microbiology* 12, 417–422.

Jackson, M.A., Schisler, D.A. and Boyette, C.D. (1993) Microsclerotia: alternative infective propagules of the bioherbicide *Colletotrichum truncatum.* In: *Abstracts of the Annual Meeting of the American Society for Microbiology, Atlanta, Georgia.* American Society for Microbiology, Washington, DC, p. 322.

Jackson, M.A., Shasha, B.S. and Schisler, D.A. (1996) Formulation of *Colletotrichum truncatum* microsclerotia for improved biocontrol of the weed hemp sesbania (*Sesbania exaltata*). *Biological Control of Weeds* 7, 107–113.

Jackson, M.A., McGuire, M.R., Lacey, L.A. and Wraight, S.P. (1997) Liquid culture production of desiccation tolerant blastospores of the bioinsecticidal fungus *Paecilomyces fumosoroseus. Mycological Research* 101, 35–41.

Janisiewicz, W.J. (1987) Post-harvest biological control of blue mold on apples. *Phytopathology* 77, 481–485.

Jaronski, S.T. (1997) New paradigms in formulating mycoinsecticides. In: Goss, G.R., Hopkinson, M.J. and Collins, H.M., (eds) *Pesticide Formulations and Application Systems,* Vol. 17. ASTM STP 1328, American Society for Testing and Materials, Philadelphia, pp. 99–113.

Jenkins, N.E. and Prior, C. (1993) Growth and formation of true conidia by *Metarhizium flavoviride* in a simple liquid medium. *Mycological Research* 97, 1489–1494.

Jenkins, N.E. and Thomas, M.B. (1996) Effect of formulation and application method on the efficacy of aerial and submerged conidia of *Metarhizium flavoviridae* for locust and grasshopper control. *Pesticide Science* 46, 299–306.

Jenkins, N.E., Heviefo, G., Langewald, J., Cherry, A.J. and Lomer, C.J. (1998) Development of mass production technology for aerial conidia for use as mycopesticides. *Biocontrol News and Information* 19, 21N-31N.

Jin, X., Harman, G.E. and Taylor, A.G. (1991) Conidial biomass and desiccation tolerance of *Trichoderma harzianum* produced at different medium water potentials. *Biological Control* 1, 237–243.

Jung, G. and Mugnier, J. (1989) Low-water-activity inocula for biological control. US patent no. 4,886,664.

Kawakami, K. and Mikuna, T. (1965) Effects of relative humidity and temperature on the via-

bility of conidia of some muscardines. *Acta Sericologica* 56, 42–46.

Keller, S. (1992) *The Beauveria–Melolontha* project: experiences with regard to locust and grasshopper control. In: Lomer, C.J. and Prior, C. (eds) *Biological Control of Locusts and Grasshoppers.* Redwood Press, Melksham, pp. 279–286.

Keller, S., Schweizer, C., Keller, E. and Brenner, H. (1997) Control of white grubs (*Melolantha melolantha* L.) by treating adults with the fungus *Beauveria brongniartii. Biocontrol Sciences and Technology* 7, 105–116.

Kenney, D.S. (1986) DeVine – the way it was developed – an industrialist's view. *Weed Science* 34, 15–16.

Khan, M. and Sinclair, J.B. (1992) Pathogenicity of sclerotia and nonsclerotia-forming isolates of *Colletotrichum truncatum* on soybean plants and roots. *Phytopathology* 82, 314–319.

Knudsen, G.R., Johnson, J.B. and Eschen, D.J. (1990) Alginate pellet formulation of a *Beauveria bassiana* (Fungi: Hyphomycetes) isolate pathogenic to cereal aphids. *Journal of Economic Entomology* 83, 2225–2228.

de Kock, S.L. (1998) Control of post-harvest decay of fruit by means of antagonistic microorganisms. PhD thesis, University of Stellenbosch, Stellenbosch, South Africa.

Labatte, J., Meusnier, S., Migeon, A., Chaufaux, J., Couteaudier, Y., Riba, G. and Got, B. (1996) Field evaluation of and modeling impact of three control methods on the larval dynamics of *Ostrinia nubilalis* (Lepidoptera: Pyralidae). *Journal of Economic Entomology* 89, 852–862.

Lacey, L.A., Kirk, A.A., Millar, L., Mercadier, G. and Vidal, C. (1999) Ovicidal and larvicidal activity of conidia and blastospores of *Paecilomyces fumosoroseus* (Deuteromycotina: Hyphomycetes) against *Bemisia argentifolii* (Homoptera: Aleyrodidae) with a description of a bioassay system allowing prolonged survival of control insects. *Biocontrol Science and Technology* 9, 9–18.

Lane, B.S., Trinci, A.P.J. and Gillespie, A.T. (1991a) Endogenous reserves and survival of blastospores of *Beauveria bassiana* harvested from carbon- and nitrogen-limited batch cultures. *Mycological Research* 7, 821–828.

Lane, B.S., Trinci, A.P.J. and Gillespie, A.T. (1991b) Influence of cultural conditions on the virulence of conidia and blastospores of *Beauveria bassiana* to the green leafhopper, *Nephotettix virescens. Mycological Research* 7, 829–833.

Latch, G.C.M and Kain, W.M. (1983) Control of porina caterpillar (*Wiseana* spp.) in pasture by the fungus *Metarhizium anisopliae. New Zealand Journal of Experimental Agriculture* 11, 351–354.

Latge, J.-P., Hall, R.A., Cabrera Cabrera, R.I. and Kerwin, J.C. (1986) Liquid fermentation of entomopathogenic fungi. In: Samson, R.A., Vlak, J.M. and Peters, D. (eds) *Fundamental and Applied Aspects of Invertebrate Pathology.* Foundation of the Fourth International Colloquium of Invertebrate Pathology, Wageningen, the Netherlands, pp. 603–606.

Li, Z., Butt, T.M., Beckett, A. and Wilding, N. (1993) The structure of dry mycelia of the entomophthoralean fungi *Zoophthora radicans* and *Erynia neoaphidis* following different preparatory treatments. *Mycological Research* 97, 1315–1323.

Mahmood, K. and Sinclair, J.B. (1991) Effect of soil temperature on infection of soybean roots by sclerotia-forming isolates of *Colletotrichum truncatum. Plant Disease* 75, 1282–1285.

McCabe, D. and Soper, R.S. (1985) Preparation of an entomopathogenic fungal insect control agent. US patent no. 4,530,834.

Mendonça, A.F. (1992) Mass production, application and formulation of *Metarhizium anisopliae* for control of sugarcane froghopper, *Mahanarva posticata*, in Brazil. In: Lomer, C.J. and Prior, C. (eds) *Biological Control of Locusts and Grasshoppers*. CAB International, Wallingford, UK, pp. 239–244.

Miller, D.W. (1995) Commercial development of entomopathogenic fungi: formulation and delivery. In: Hall, F.R. and Barry, J.W. (eds) *Biorational Pest Control Agents: Formulation and Delivery.* American Chemical Society, Washington, DC, pp. 213–220.

Milner, R.J., Baker, G.L., Hooper, G.H.S. and Prior, C. (1997) Development of a mycoinsec-

ticide for the Australian plague locust. In: Krall, S., Peveling, R. and Ba Diallo, D. (eds) *New Strategies in Locust Control.* Birkhäuser Verlag, Basle, pp. 177–183.

Moore, D. and Higgins, P.M. (1997) Viability of stored conidia of *Metarhizium flavoviride* Gams and Rozsypal, produced under differing culture regimes and stored with clays. *Biocontrol Science and Technology* 7, 335–343.

Moore, D., Langewald, J. and Obognon, F. (1997) Effects of rehydration on the conidial viability of *Metarhizium flavoviridae* mycopesticide formulations. *Biocontrol Science and Technology* 7, 87–94.

Nolan, R.A. (1998) Growth media for entomophthoralean hyphal bodies. US patent no. 5,728,572.

Prior, C., Jollands, P. and le Patourel, G. (1988) Infectivity of oil and water formulations of *Beauveria bassiana* (Deuteromycotina: Hyphomycetes) to the cocoa weevil pest *Pantorhytes plutus* (Coleoptera: Curculionidae). *Journal of Invertebrate Pathology* 52, 66–72.

Quintela, E.D. and McCoy, C.W. (1998) Synergistic effect of imidacloprid and two entomopathogenic fungi on the behavior and survival of larvae of *Diaprepes abbreviatus* (Coleoptera: Curculionidae) in soil. *Journal of Economic Entomology* 91, 110–122.

Redmond, J.C., Marois, J.J. and MacDonald, J.D. (1987) Biological control of *Botrytis cinerea* on roses with epiphytic microorganisms. *Plant Disease* 71, 799–802.

Roberts, D.W., Dunn, H.M., Ramsay, G., Sweeney, A.W. and Dunn, N.W. (1987) A procedure for preservation of the mosquito pathogen *Culicinomyces clavisporus. Applied Microbiology and Biotechnology* 26, 186–188.

Roberts, R.G. (1990) Biological control of *Mucor* rot of pear by *Cryptococcus laurentii, C. flavus,* and *C. albidus. Phytopathology* 80, 1050.

Rombach, M.C. (1989) Production of *Beauveria bassiana* (Deuteromycotina, Hyphomycetes) sympoduloconidia in submerged culture. *Entomophaga* 34, 45–52.

Rombach, M.C., Aguda, R.M., Shepard, B.M. and Roberts, D.W. (1986) Entomopathogenic fungi (Deuteromycotina) in the control of the black bug of rice, *Scotinophara coarctata* (Hemiptera; Pentatomidae). *Journal of Invertebrate Pathology* 48, 174–179.

Sakamoto, M., Inoue, Y. and Aoki, J. (1985) Effect of light on the conidiation of *Paecilomyces fumosoroseus. Transactions of the Mycological Society of Japan* 26, 499–509.

Samsináková, A., Kálalová, S., Vlcek, V. and Kybal, J. (1981) Mass production of *Beauveria bassiana* for regulation of *Leptinotarsa decemlineata* populations. *Journal of Invertebrate Pathology* 38, 169–174.

Schaerffenberg, B. (1964) Biological and environmental conditions for the development of mycoses caused by *Beauveria* and *Metarhizium. Journal of Insect Pathology* 6, 8–20.

Schiewe, A. and Mendgen, K. (1992) Identification of antagonists for biological control of postharvest pathogens *Pezicula malicorticis* and *Nectria galligena* on apples. *Journal of Phytopathology* 134, 229–237.

Schisler, D.A., Jackson, M.A. and Bothast, R.J. (1990) Influence of nutrition during conidiation of *Colletotrichum truncatum* on conidial germination and efficacy in inciting disease on *Sesbania exaltata. Phytopathology* 81, 587–590.

Schwarz, M.R. (1995) *Metarhizium anisopliae* for soil pest control. In: Hall, F.R. and Barry, J.W. (eds) *Biorational Pest Control Agents: Formulation and Delivery.* American Chemical Society, Washington, DC, pp. 183–196.

Shah, P.A., Douro-Kpindou, O.-K., Sidibe, A., Daffe, C.O., van der Pauuw, H. and Lomer, C.J. (1998) Effects of the sunscreen oxybenzone on field efficacy and persistence of *Metarhizium flavoviridae* conidia against *Kraussella amabile* (Orthoptera: Acrididae) in Mali, West Africa. *Biocontrol Science and Technology* 8, 357–364.

Shapiro, M. (1992) Use of optical brighteners as radiation protectants for gypsy moth (Lepidoptera: Lymantriidae) nuclear polyhedrosis virus. *Journal of Economic Entomology* 85, 1682–1686.

Shi, Z.M. (1988) Technology for conidial preparation of *Beauveria bassiana*. In: Li, Y.W., Li, Z.Z., Liang, Z.Q., Wu, J.W., Wu, Z.K. and Xu, Q.F. (eds) *Study and Application of Entomogenous Fungi in China,* Vol. 1. Academic Periodical Press, Beijing, pp. 114–115.

Silman, R.W. and Nelsen, T.C. (1993) Optimization of liquid culture medium for commercial production of *Colletotrichum truncatum. FEMS Microbiological Letters* 107, 273–278.

Silman, R.W., Bothast, R.J. and Schisler, D.A. (1993) Production of *Colletotrichum truncatum* for use as a mycoherbicide: effects of culture, drying and storage on recovery and efficacy. *Biotechnology Advances* 11, 561–575.

Slininger, P.J., Silman, R.W. and Jackson, M.A. (1993) Oxygen delivery requirements of *Colletotrichum truncatum* during germination, vegetative growth, and sporulation. *Applied Microbiology and Biotechnology* 39, 744–749.

Smilanick, J.L. (1994) Strategies for the isolation and testing of biocontrol agents. In: Wilson, C.L. and Wisniewski, M.E. (eds) *Biological Control of Post-harvest Diseases: Theory and Practice.* CRC Press, Boca Raton, Florida, pp. 25–41.

Spotts, R.A. and Cervantes, L.A. (1986) Populations, pathogenicity, and benomyl resistance of *Botrytis* spp., *Penicillium* spp., and *Mucor piriformis* in packhouses. Plant Disease 70, 106–108.

Stephan, D., Welling, M. and Zimmerman, G. (1997) Locust control with *Metarhizium flavoviridae*: new approaches in the development of a biopreparation based on blastospores. In: Krall, S., Peveling, R. and Ba Diallo, D. (eds) *New Strategies in Locust Control.* Birkhauser Verlag, Basle, Switzerland, pp. 151–158.

St Leger, R.J., Lokesh, J., Bidochka, M.J. and Roberts, D.W. (1996) Construction of an improved mycoinsecticide overexpressing a toxic protease. *Proceedings of the National Academy of Sciences USA* 93, 6349–6354.

Stowell, L.J. (1991) Submerged fermentation of biological herbicides. In: Te Beest, D.O. (ed.) *Microbiological Control of Weeds.* Chapman & Hall, New York, pp. 225–261.

Stowell, L.J., Nette, K., Heath, B. and Shutter, R. (1989) Fermentation alternatives for commercial production of a mycoherbicide. In: Demain, A.L., Somkuti, G.A., Hunter-Cevera, J.C. and Rossmore, H.W. (eds) *Novel Microbial Products for Medicine and Agriculture.* Elsevier, Amsterdam, pp. 219–227.

Templeton, G.E. (1982) Status of weed control with plant pathogens. In: Charudattan, R. and Walker, H.L. (eds) *Biological Control of Weeds with Plant Pathogens.* John Wiley & Sons, New York, pp. 29–44.

Tu, J.C. (1980) The ontogeny of the sclerotia of *Colletotrichum coccodes. Canadian Journal of Botany* 58, 631–636.

Vandenberg, J.D., Jackson, M.A. and Lacey, L.A. (1998) Relative efficacy of blastospores and aerial conidia of *Paecilomyces fumosoroseus* against the Russian wheat aphid. *Journal of Invertebrate Pathology* 72, 181–183.

Vidal, C., Fargues, J., Lacey, L.A. and Jackson, M.A. (1998) Effect of various liquid culture media on morphology, growth, propagule production, and pathogenic activity to *Bemisia argentifolii* of the entomopathogenic hyphomycete, *Paecilomyces fumosoroseus. Mycopathologia* 143, 33–46.

Villani, M.G., Krueger, S.R., Schroeder, P.C., Consolie, F., Consolie, N.H., Preston-Wilsey, L.M. and Roberts, D.W. (1994) Soil application effects of *Metarhizium anisopliae* on Japanese beetle (Coleoptera: Scarabaeidae) behavior and survival in turfgrass microcosms. *Environmental Entomology* 23, 502–513.

Ward, M.G. (1984) Formulation of biological insecticides: surfactant and diluent selection. In: Scher, H.B. (ed.) *Advances in Pesticide Formulation Technology.* American Chemical Society, Washington, DC, pp. 175–184.

Ward, M.G. and Roberts, D.W. (1981) Viability of *Beauveria bassiana* conidia stored with formulation carriers and diluents, In: *Society for Invertebrate Pathology Program and Abstracts, Bozeman, Montana.* Society for Invertebrate Pathology, Raleigh, North Carolina, pp. 30–31.

Wilson, C.L. and Wisniewski, M.E. (1989) Biological control of post-harvest diseases of fruits and vegetables: an emerging technology. *Annual Review of Phytopathology* 27, 425–441.

Wilson, C.L., Wisniewski, M.E., El Ghaouth, A., Droby, S. and Chalutz, E. (1996) Commercialization of antagonistic yeasts for the biological control of post-harvest diseases of fruits and vegetables. *SIM News* 46, 237–242.

Wraight, S.P. and Bradley, C.A. (1996) Production, formulation, and application technologies for use of entomopathogenic fungi to control field crop pests. In: *V SICONBIOL, Simpósio de Controle Biológico, Anais: Conferências e Palestras, Foz do Iguaçu, Brazil.* P.J. Communicaças and Eventos Editoria, Curitiba, Brazil, pp. 170–177.

Wraight, S.P. and Carruthers, R.I. (1999) Production, delivery, and use of mycoinsecticides for control of insect pests of field crops. In: Hall, F.R. and Menn, J.J. (eds) *Methods in Biotechnology*, Vol. 5: *Biopesticides: Use and Delivery.* Humana Press, Totowa, New Jersey, pp. 233–270.

Wraight, S.P., Galaini-Wraight, S., Carruthers, R.I. and Roberts, D.W. (1986) Field transmission of *Erynia radicans* to *Empoasca* leafhoppers in alfalfa following application of a dry, mycelial preparation. In: Samson, R.A., Vlak, J.M. and Peters, D. (eds) *Fundamental and Applied Aspects of Invertebrate Pathology.* Foundation of the Fourth International Colloquium of Invertebrate Pathology, Wageningen, the Netherlands, p. 233.

Wraight, S.P., Carruthers, R.I., Jaronski, S.T., Bradley, C.A., Garza, C.J. and Galaini-Wraight, S. (2000) Evaluation of the entomopathogenic fungi *Beauveria bassiana* and *Paecilomyces fumosoroseus* for microbial control of the silverleaf whitefly, *Bemisia argentifolii. Biological Control* 17, 203–217.

Zorner, P.S., Evans, S.L. and Savage, S.D. (1993) Perspectives on providing a realistic technical foundation for the commercialization of bioherbicides. In: Duke, S.O., Menn, J.J. and Plimmer, J.R. (eds) *Pest Control with Enhanced Environmental Safety.* American Chemical Society, Washington, DC, pp. 79–86.

11 The Spray Application of Mycopesticide Formulations

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Introduction

Biopesticides have been promoted for at least the last century, but their use remains limited to < 1% of the total pesticide market. Lisansky (1997) attributes this limited growth to lack of positive promotion by authorities and the agrochemical industry. Their perceived constraints include: narrow target spectra, poor performance relative to equivalent-cost chemical control and inconsistent product quality in comparison with chemicals. The *Biopesticide Manual* (Copping, 1998) lists six fungal agents that have been commercialized as mycoinsecticides, ten agents that have fungicidal or fungistatic properties, three mycoherbicides and one nematocidal product (Table 11.1). Together these constitute a tiny proportion (some 3%) of the already small worldwide biopesticide market (Georgis, 1997). The remainder consists largely of *Bacillus thuringiensis* subspp., other *Bacillus* products and viruses.

Poor performance can be attributed to:

- product inconsistency;
- a 'chemical paradigm', which creates the perception that users must see results immediately (rather than making cost-effective improvements in yield);
- difficulty or misunderstandings in use, which can result in poor efficacy.

In most Western field crops, 'spraying' usually means use of a tractor and boom whereas, in developing countries, this often involves hand-carried equipment (knapsack sprayers). In both cases the break-up (atomization) of the spray liquid is achieved using various types of hydraulic nozzle (see Matthews, 1992). Although this technology is more than a century old, many would argue that it has yet to be surpassed for simplicity and reliability. However, as we shall show in this chapter, hydraulic application is not necessarily the most efficient technique – especially when farmers are instructed by product labels to 'spray to runoff'. Most authorities agree that spray application is highly inefficient: Graham-Bryce (1977) has pointed out that, in foliar sprays against sucking insects, only 0.02–0.03% of the insecticide was utilized by the target. The most efficient dose transfer for insecticides quoted in the literature is 6% for aerial

Table 11.1. Mycopesticide products and their application. (From Copping, 1998.)

Microbial agent	Pesticide type	Formulation/application
Ampelomyces quisqualis	**F**: powdery mildew hyperparasite	WG (with oil adjuvant): HV spray on to vines
Beauveria bassiana	**I**: Lepidoptera, Homoptera, Coleoptera	MG applied to axils/foliage WP, SC oil-based suspension: as HV sprays
Beauveria brongniartii	**I**: Scarabaeidae	MG, AL (HV sprays)
Candida oleophila	**F**: Postharvest fruit treatment	WG: spray or dip
Chondosterum purpureum	**H**: silver-leaf fungus prevents regrowth of unwanted deciduous trees	AL: sprayed or spread on to tree stumps
Colletotrichum gloeosporioides f. sp. *aeschynomene*	**H**: against *Aeschynomene virginica* (northern joint-vetch)	AL: sprayed on to weeds under high humidity
Coniothyrium minitans	**F**: prevention of *Sclerotinia*	AL: for spraying
Endothia parasitica	**F**: competes with more pathogenic *E. parasitica* strains	PA: applied to cuts or wounds of chestnut trees
Fusarium oxysporum	**F**: competes with more pathogenic *F. oxysporum* strains	SC, MG for glasshouse use
Gliocladium catenulatum	**F**: preventive esp. against *Pythium, Rhizoctonia, Botrytis, Didymella* and *Helminthosporium*	GR (WG?): granular, dip or foliar spray for seedlings and harvested produce
Gliocladium (=Trichoderma) virens	**F**: preventive esp. against *Pythium, Rhizoctonia, Fusarium, Theilaviopsis, Sclerotina* and *Sclerotium*	GR incorporated into soil
Metarhizium anisopliae	**I**: Coleoptera Isoptera Dictyoptera	GR (discontinued) DP: for termite galleries RB: ready-to-use baiting station
M. anisopliae var. *acridium (= Metarhizium flavoviride)*	**I**: Orthoptera: Acrididae	SU, OF: usually for ULV application
Myrothecium verrucaria	**N**: esp. against *Meloidogyne, Heterodera, Belonolaimus* and *Radopholus* spp.	Powder (DS?) applied as seed treatment or in soil drench
Paecilomyces fumosoroseus	**I**: Homoptera	WG for HV spraying
Phlebiopsis gigantean	**F**: prevention of *Heterobasidion annosum*	WP sprayed on to stumps
Phytophthora palmivora	**H**: against *Morrenia odorata* (strangler or milkweed vine)	AL sprayed on to weeds under high humidity
Pythium oligandrum	**F**: presentation of wide range of soil-borne pathogens	WP sprayed in glasshouses
Trichoderma harzianum	**F**: prevention of *Botrytis* and *Sclerotina*	MG soil treatment for vines and vegetables
T. harzianum and *Trichoderma viride*	**F**: preventive esp. against *Armillaria mellea, Pythium, Chondrosterium purpureum, Phytophthora, Fusarium, Rhizoctonia* and *Sclerotium*	MG, GR and others for soil application; also injected into woody crops and wound sealant
Verticillium lecanii	**I**: esp. Homoptera	WP sprayed at HV in glasshouses

F, Fungicide; **I**, Insecticide; **H**, herbicide; **N**, nematode. Formulation types conform to the Global Crop Protection Federation (GCPF, 1999) coding system (e.g. PA paste).
Solid formulations: MG, microgranules; GR, granules; DP, dusts; DS, powder for seed dressing. Solid formulations for mixing with water: WG, ~miscible granules; WP, wettable powders. Liquid formulations for mixing with water: SC, suspension concentrates. Ultra-low-volume (ULV) formulations: SU, ULV suspension; OF, oil-miscible flowable concentrate. AL is used for miscellaneous liquids applied undiluted: here mycopesticides as aqueous suspensios of spores, to be applied as soon as possible after receipt of product. HV, high-volume.

spraying of locust swarms. Higher efficiencies, with up to 30% of the tank mixture reaching the target, can sometimes be achieved with herbicides.

'Rational pesticide use' (Brent and Atkin, 1987) is an important concept in which treatment costs and the impact on non-target organisms are reduced by combining spatial and temporal precision of application with biologically specific products. Unfortunately, even with chemical pesticides, it can be difficult to define the true biological target, i.e. the site at which efficacy could be optimized (Hislop, 1987). With microbial agents, we cannot assume that simply delivering infectious propagules to the surfaces of target organisms will result in pest control. For example, complex pathogen-host attack and defence mechanisms exist against entomopathogenic fungi (Clarkson and Charnley, 1996; Blanford *et al.*, 1998) and 'chemical models' of biopesticide dose transfer may not be reflected in field results.

Table 11.1 includes several products that are formulated for specific application techniques (soil drenches, pastes for application to stumps, granules); these are applied according to manufacturers' specific instructions. However, it is the formulations for spray application that offer both the greatest challenge and the greatest opportunity for future development, and this will be the main theme addressed in this chapter.

Biological Considerations: Modes of Action

During the development of a mycoinsecticide for the biological control of locusts and grasshoppers, scientists of the international LUBILOSA[1] programme recognized that an effective delivery system requires a thorough understanding of the biological relationship with its target pest. For example, three distinct routes of fungal infection for locusts and grasshoppers have been identified:

1. Direct impaction with spray droplets. The infection or mortality resulting from 'direct contact' may vary from some 30% to > 99% 'direct hits' in the case of locusts and grasshoppers. However, this can be a very small component of the eventual dose transfer process (Bateman *et al.*, 1998).
2. Secondary pick-up of spray residues from vegetation or the soil. As with agrochemicals, secondary pick-up is often far more important than direct contact with spray droplets in most air-to-ground and ground-to-ground spray operations. Field observations suggest that spores may persist in the field for several days, depending on conditions (Langewald *et al.*, 1997).
3. Horizontal transmission (or 'secondary cycling') of the pathogen from individuals infected via the first two modes above, then death, sporulation and release of further inoculum into the environment, under suitable conditions (Thomas *et al.*, 1995).

At another level, it is extremely important to understand how the target and spray deposit interact at a biological level. For example, if the target is scale insects, the egg stage of a pest or a fungal pathogen, the mycopesticide must be brought to the pest. Application parameters are critical if the pest is sedentary and little horizontal transmission takes place; Bateman (1993) and Chapple *et al.* (2000) discuss the differences between biological and chemical active ingredients (a.i). However, if the pest is mobile, the interaction can be exploited (although here such factors as the repellency of many surfactants must be borne in mind). Although herbicide applications can be the most efficient forms of spraying (see above; Graham-Bryce, 1977), it can still be important to determine the optimal site on or near the plant for droplet deposition. The impor-

tant practical question is 'Can application be optimized, within the limits of what the grower can be asked to do?' (Chapple *et al.*, 1996).

In theory, application technology is crucial when direct contact with target insects is needed; it may be less important if secondary cycling forms the principal method of mycopathogen infection in the field (i.e. classical biological control). Thomas *et al.* (1995) have suggested that high transfer of fungal propagules to target insects by direct impaction may be deleterious for the long-term management of acridids under certain circumstances. On the other hand, motorized mist-blower ('air-blast') sprayers were considered desirable for the efficient and speedy inoculation of a classical biological control agent in Australia; the rusts *Maravalia cryptostegiae* and *Phloeospora mimosae-pigrae* were effectively applied against rubber vine and *Mimosa*, respectively (A.J. Tomley, Queensland Department of Lands, personal communication).

Several laboratory and glasshouse studies have demonstrated that smaller droplets are more efficacious for arthropod pest control than larger ones. For example, the relative efficacy of different droplet size spectra, e.g. 30–60 μm droplets, were usually optimal with oil-based insecticide spray deposits, while 60–120 μm were most efficient with aqueous droplets (Adams *et al.*, 1990). Although this important effect has yet to be demonstrated with mycopesticides, it is not unreasonable to postulate that efficacy may be enhanced with an appropriate coverage of propagules in the target zone, obtained from suitable droplet size spectra.

Some Important Concepts in Spray Application

In order to better understand the cause of the spray inefficiency described above, it is useful to reflect on the implications of the large range of droplet sizes produced by most spray nozzles. Droplets are usually described by their perceived size (i.e. diameter), whereas the dose (or number of infective particles in the case of biopesticides) is a function of their volume. This increases by a cubic function relative to diameter ($\pi.d^3/6000$ to convert μm into picolitres); thus a 50 μm droplet represents a dose of 65 pl and a 500 μm drop represents a dose of 65 nanolitres (65,450 pl). This has long been recognized as one of the most important concepts in spray application (Himel, 1969), bringing about enormous variations in the properties of droplets (Table 11.2).

Different droplet sizes have dramatically different dispersal characteristics and are subject to complex macro- and microclimatic interactions (Bache and Johnstone, 1992). Greatly simplifying these interactions in terms of droplet size and wind speed, Craymer and Boyle (1973) concluded that there are essentially three sets of conditions under which droplets move from the nozzle to the target (see Fig. 11.1):

- Sedimentation dominates: typically larger (>100 μm) droplets applied at low wind speeds; droplets above this size are appropriate for minimizing drift contamination by herbicides.
- Turbulent eddies dominate: typically small droplets (< 50 μm), which are usually considered most appropriate for targeting flying insects, unless an electrostatic charge is also present, providing the necessary force to attract droplets to foliage. (NB: the latter effects only operate at very short distances, typically under 1 cm.)
- Intermediate conditions where both sedimentation and drift effects are important. Most agricultural insecticide and fungicide spraying is optimized by using relatively

Table 11.2. Ranges of droplet and drop sizes, with their volumes and properties.

Diameter range (μm)	Volume (maximum) in picolitres or l^{-12}	Properties/function
< 10	0.52	Potentially hazardous, very fine aerosols or particles, with a progressively increasing risk of inhalation by operators (greatest at approximately 1–3 μm)
< 50	65	Aerosols (appropriate for direct contact with small insects); most of the spray droplet spectrum in a Potter tower nozzle
50–100	524	Mists/fine sprays appropriate for oil-based ULV/mist-blower spraying
75–150	1,767	Maximizes coverage with water-based insecticide and fungicide sprays
150–300	14,137	Maximizes coverage with herbicide sprays, avoiding drift especially where wind (< 2 m s^{-1}) is present
300–500	65,450	Coarse spray: maximum avoidance of drift; at > 500 μm droplets become drops and progressively less efficient at covering foliage, leading to runoff, unless total volume applied is substantially increased
985	500,000	0.5 μl drops: lowest reliable volume that can be delivered by many microapplicators (for topical dosing in bioassays)

ULV, ultra-low-volume.

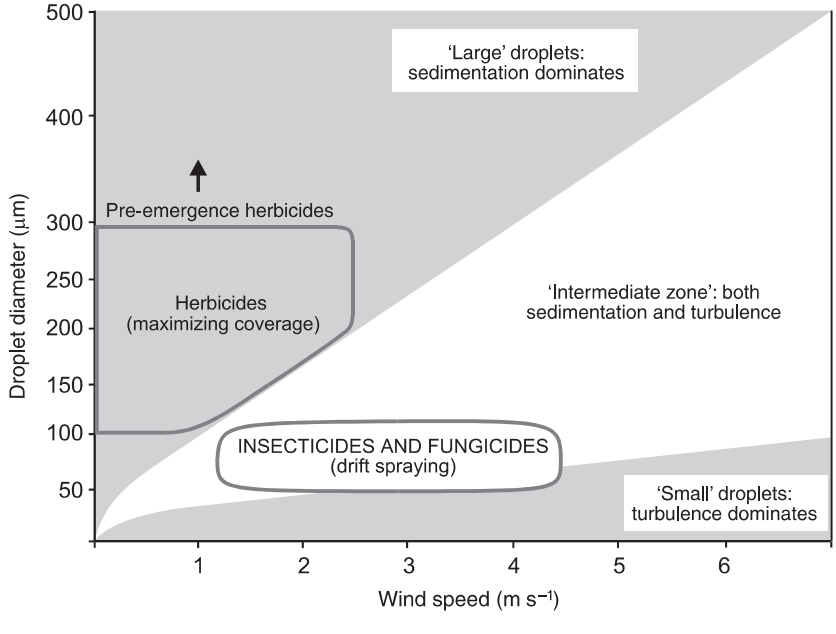

Fig. 11.1. Characteristics of 'large' and 'small' droplets. (Modified from Craymer and Boyle, 1973.)

small (say 50–150 μm) droplets in order to maximize 'coverage' (droplets per unit area), but these are also subject to drift.

Terms related to the effectiveness of application include the following:

- The efficiency of the spray application is determined by the complex phenomena

that govern droplet transport from the nozzle to the target, which are described in texts such as Bache and Johnstone (1992). As a practical measure, Courshee (1959) coined the term deposit per unit emission (DUE) to describe the amount of pesticide recovered downwind in the target zone, relative to the volume emitted from the spray nozzle (originally in locust control operations). DUE is thus an overall measure of efficiency, permitting valid comparisons of spray recovery. It is usually expressed in units such as ml m^{-1} (mean quantity recovered in a unit area) per ml m^{-1} (liquid emitted over a unit of spray).

- Coverage is defined here as the extent to which a pesticidal spray has been distributed on a target surface. The biological implication is that good coverage increases the probability that a pest will encounter a pesticide. A rule-of-thumb guide to desirable levels of coverage is shown in Fig. 11.2 (but note that this is primarily based on what is known from the application of the chemicals). Because of the relationship between the diameter and volume of a sphere, there will theoretically be a cubic increase in numbers of droplets produced in relation to their average droplet diameter.
- Retention is the amount of spray liquid retained on (mostly the leaves of) crop plants. In other words, it is the remaining proportion of pesticide that has not 'run off' (usually high-volume spraying) or been eroded by weathering. There is an interaction between formulation effects on the tenacity of a deposit and the surface of the leaf to which it adheres. Droplets often bounce on leaves that are waxy (a property that is often influenced by age) and poor retention may occur with water-based formulations, especially those with high dynamic surface tensions. On the other hand, absorption of a.i. may occur with oil-based formulations. Leaf exudates (e.g. in apples and broad beans) may also contribute to the redistribution of a pesticide. Jefree (1986) has reviewed the ultrastructure and function of trichomes and epicuticular waxes. Chapple *et al.* (2000) discuss this important subject further.

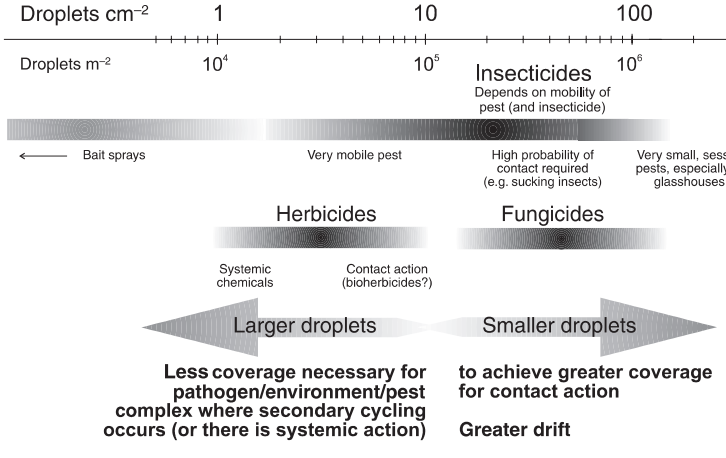

Fig. 11.2. A guide to spray droplet coverage. Horizontal bars indicate the desirable deposition in the target zone, based on chance encounter with the target. Note: (i) this diagram is for guidance only and no substitute for field evaluation; (ii) no reference has been made to the concentration, size or spreading of droplets.

- *The volume application rate (VAR)* is the amount of formulation applied per hectare. Table 11.3 gives a classification of VARs. Table 11.4 shows the theoretical coverage on plants if monodispersed droplets (i.e. all droplets have the same diameter) in these size classes were to be evenly applied at the lower limits of the ultra-low, very low, low, medium and high VAR ranges for field crops (from Matthews, 1992), assuming that all droplets were captured and retained. 'Per hectare' application often has very little relationship to the target area to be sprayed; leaf area indices of crops or weeds can range from fractions (pre-emergent weeds at the cotyledon stage) to > 5 (late-stage cereal crops). With bush and tree crops, VAR per hectare is even more inappropriate, and methods such as the unit canopy row (UCR) system have been developed where sprayer calibration is based on canopy size (Furness *et al.*, 1998).
- *Work rate* is the amount of ground (or crop) treated per hour/day and is linked to VAR. It is an important factor under certain circumstances, for example: (i) when the cost of labour is high; (ii) when a quick response is needed to a pest population that has exceeded an action threshold (especially if it is rapidly reproducing); or (iii) in migrant pest control.

Table 11.3. General classification of volume application rates (VAR in l ha^{-1}) for field and tree/bush crops. (From Matthews, 1992.)

	Field crops	Tree and bush crops
High volume (HV)	> 600	> 1000
Medium volume (MV)	200–600	500–1000
Low volume (LV)	50–200	200–500
Very low volume (VLV)	5–50	50–200
Ultra-low volume (ULV)	< 5[a]	< 50

[a] VARs of 0.25–2 l ha^{-1} are typical for aerial ULV application to forest or migratory pests.

Table 11.4. Coverage with specific droplet sizes (see Table 11.3) at different VARs if sprays consist of monodispersed drops (leaf area index taken as 1).

Monodispersed droplet size	Cross-sectional area of deposit (m^2)[a]	Droplets per m^2 (at 1 l ha^{-1})	% Cover (per ha) for lower limits of VAR classes				
			ULV (1)	VLV (5)	LV (50)	MV (200)	HV (600)
10	3.1×10^{-10}	190,985,932	6	30	NR	NR	NR
50	7.9×10^{-9}	1,527,887	1.2	6	60	NR	NR
75	1.8×10^{-8}	452,707	0.8	4	40	NR	NR
100	3.1×10^{-8}	190,986	0.6	3	30	120	NR
150	7.1×10^{-8}	56,588	0.4	2	20	80	NR
300	2.8×10^{-7}	7,074	0.2	1	10	40	120
500	7.9×10^{-7}	1,528	NR	0.6	6	24	72
985	3.0×10^{-6}	200	NR	0.3	3	12	37

[a] Single droplets (spread factor taken as 2).
NR, not realistic spraying scenario.
> 100% cover represents coalescence of droplets.

- *Controlled droplet application (CDA)* is a term probably coined by John Fryer of the Weed Research Organization in the UK (G.A. Matthews, personal communication). Bals (1969) stated that 'The efficiency of a spraying machine is inversely proportional to the range of droplets it emits, whilst the suitability for a specific problem depends on the actual size of the droplets emitted.' No atomizer is commercially available that can produce uniform (monodispersed) droplets, but rotary (spinning disc and cage) atomizers usually produce a narrower droplet size spectrum than conventional hydraulic nozzles. Therefore CDA can be considered in terms of optimizing technology to achieve a biological objective: delivering appropriately sized droplets (within practical engineering limits) for maximizing the control of a given pest target (where this is known). Unfortunately, the true biological target is often poorly defined and complex in nature, which, when combined with operational variables, makes most spraying inherently inefficient. However, there is often scope for improving existing practice (Hislop, 1987). Bals (1969) discussed the concept of producing small uniform pesticidal droplets to achieve adequate control with 'ultra-low dosage' combined with ultra-low volume (ULV) rates of application. Unfortunately, this is thought to have discouraged many chemical companies from promoting CDA techniques, since sales would be reduced (except when value could be re-added to an a.i. by proprietary delivery systems, such as the 'Electrodyn' (ED) (Coffee, 1981).
- *Droplet size* spectra generally refers to the measurement of – and statistics for describing – spray droplet spectra; they are described in the standard texts, including LeFebvre (1989) and Parkin (1992). At least two important measures of spray distributions are usually required, and the statistics used here are: (i) size: the volume median diameter (VMD), where half of the volume of spray contains droplets larger than the VMD (in μm) and the other half is in smaller droplets; and (ii) quality: the relative span, which is a dimensionless parameter calculated from the volume distribution only (see LeFebvre, 1989). A span that is substantially less than 1.0 is characteristic of a CDA spray (Bateman, 1993). With microbial control agents, particle distributions within droplet size spectra must be considered with respect to droplet volumes; this is discussed by Bateman (1993) and Chapple *et al.* (2000).
- *Spray drift*: with placement (localized) spraying of broad-spectrum or toxic chemicals, wind drift must be minimized, and considerable efforts have been made recently to quantify and control spray drift from hydraulic nozzles. On the other hand, wind drift is also an efficient mechanism for moving droplets of an appropriate size range to their targets over a wide area with ULV spraying. Himel (1974) made a distinction between exo-drift (the transfer of spray out of the target area) and endo-drift, where the a.i. in droplets falls into the target area, but does not reach the biological target. Endo-drift is volumetrically more significant and may therefore cause greater ecological contamination (e.g. where chemical pesticides pollute groundwater).

Formulations

A list of the formulation types with international standard abbreviations is given by the Global Crop Protection Federation (GCPF, 1999) and a comprehensive review on the formulation of biopesticides by Burges (1998). In brief, there are four major objectives when formulating biopesticides. Where possible the aim is to:

- stabilize agents during distribution and storage;
- aid handling and application of the product;
- protect agents from harmful environmental factors, thereby increasing persistence;
- enhance the activity of the agent at the target site.

With mycopesticides, the use of oils in formulations for spraying has shown great potential for the enhanced efficacy of insecticides (Prior *et al.*, 1988) and fungicides (Hofstein and Chapple, 1998), where the need for high humidity is also overcome. Amsellem *et al.* (1990) showed that invert emulsions (where oil constitutes the continuous phase) may eliminate the need for a minimum inoculum threshold with mycoherbicides. Oils can be pesticidal in their own right or may be phytotoxic (Wrigley, 1973), which may account for some biological activity.

The implementation of these developments for field crops is discussed by Chapple and Bateman (1997), but technical issues remain poorly understood; for example, they showed that the distribution of particles of the hyperparasitic mycofungicide *Ampelomyces quisqualis* and its emulsified oil adjuvant differs markedly before and after passage through a pump. There is a compound problem in that adjuvants will be wasted in the volume of spray droplets that are either physically too small to contain a particle or have a low probability of containing organisms. Droplet size in hydraulic nozzles can be substantially affected by the use of adjuvants (Hall *et al.*, 1993; Butler Ellis *et al.*, 1997).

Some of the interacting application parameters and how target pests might acquire the infective propagules are summarized in Fig. 11.3, emphasizing the role of formulations such as oil carriers.

Mycopesticide Application: Case-studies

Some of the technical implications of spraying mycopesticides can be illustrated with case-studies based on spray application scenarios for fungal insecticides, hyperparasitic fungicides and mycoherbicides. Measured droplet size spectra from typical sprayers are discussed with reference to product field concentrations and particle size spectra, where known. In all cases, the instrument used was a Malvern 2600 particle size analyser using model-independent analysis and the methodology described by Bateman and Alves (2000). For particle size measurements, the same instrument was fitted with a 63 mm lens and a PS1 sample cell that contained a small magnetic stirrer. Each reading consisted of a background measurement with either Shellsol T (for conidia with lipophilic cell walls) or distilled water, followed by the gradual introduction of concentrated suspensions using a pipette. A reading was taken when the obscuration of the laser was optimal in the 'illustrate live' command. The data have been exported electronically and illustrated by line graphs indicating the percentage by volume in 32 size classes. X axes are accompanied by secondary scales indicating the equivalent droplet volume in picolitres (10^{-12} l) and the probable spore loading, with 'typical' tank mixture concentrations expressed as particles (conidia, etc.) per litre.

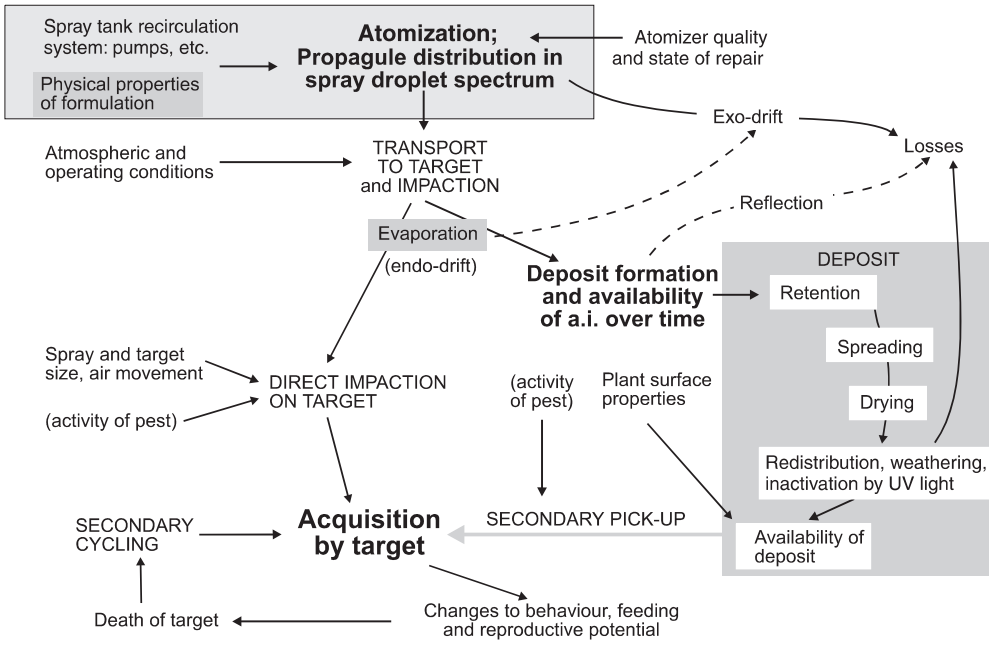

Fig. 11.3. Spray application processes and biopesticide formulations. Shaded zones represent effects that can be manipulated by formulation. This figure has been influenced by Young (1986), who describes the application of chemical pesticides. UV, ultraviolet.

Mycoinsecticides

Although fungi such as *Metarhizium anisopliae* have been known as potentially useful biological control agents for over a century, little progress was made for several decades until *Verticillium lecanii* was developed for glasshouse crops (Hall and Papierok, 1982). Even though products such as 'Vertalec'® and 'Mycotal'® became available, their use was limited to this niche market because of the need to maintain high humidity (Helyer *et al.*, 1992). During the 1990s, the LUBILOSA programme developed a mycoinsecticide ('Green Muscle'®) using oil-based formulations of an isolate of *M. anisopliae* var. *acridum*, which has been field-tested against a number of acridid pest species (Lomer *et al.*, 1999) and is now recognized as an appropriate product for locust control in environmentally sensitive areas (FAO, 1997). The use of oil overcame the need for high humidity, enhanced the efficacy of the fungus and provided a suitable carrier for ULV application (Bateman, 1997). In a series of operational trials against *Oedaleus senegalensis*, it was shown that, although the organophosphorus chemical fenitrothion achieves an impressive 'knock-down', hopper populations recover within 2 weeks of application, whereas a more profound population reduction was achieved in the plots sprayed at ULV rates with *Metarhizium* conidia (Langewald *et al.*, 1999).

Figure 11.4 illustrates the atomization of an oil-based suspension of Green Muscle SU formulation with an 'Ulva+' rotary atomizer (commonly used for small-scale ULV spraying in Africa). Spraying ULV formulations for locust control usually requires a droplet size of approximately 40–120 µm diameter (FAO, 1992) and, like many other rotary atomizers, this sprayer achieves > 80% of the spray volume as 40–120 µm

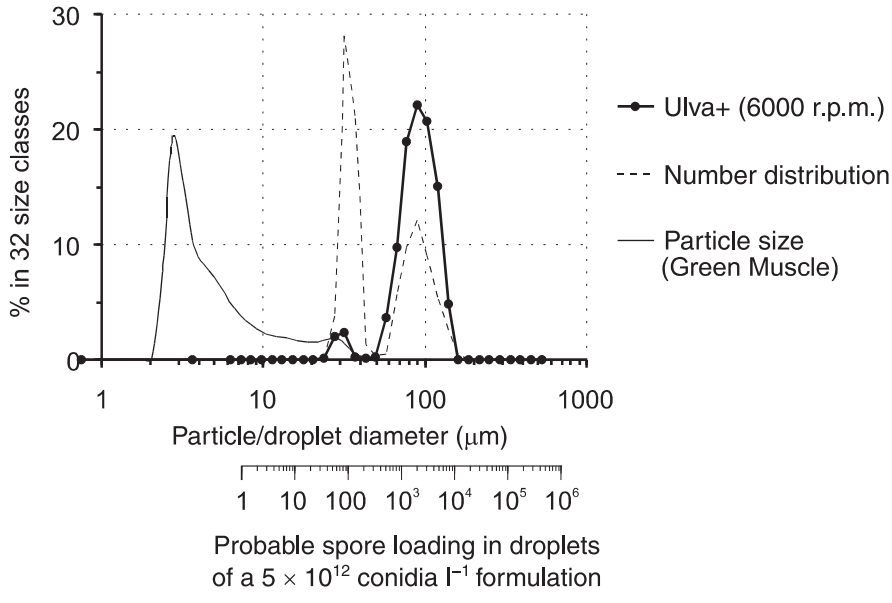

Fig. 11.4. Comparison of droplet size spectrum (volume and number distributions) of an 'Ulva+' rotary atomizer (60 ml min^{-1}) superimposed on the particle size spectrum of conidia in the 'Green Muscle' product. r.p.m., revolutions per minute.

droplets over a fairly wide range of rotational speeds (Bateman and Alves, 2000). Good-quality formulations consist of practically all single spores and most droplets contain in the region of 500–10,000 conidia with an operating concentration of 5×10^{12} conidia l^{-1} at a VAR of 1 l ha^{-1}. Only a very few droplets need to be encountered by target acridids in order to receive a dose that is lethal within 2–3 weeks.

Oil formulations of fungi such as *Paecilomyces farinosus* have been tested using other machinery, including cold foggers (Agudelo and Falcon, 1983), which are used for very small and flying insects. In Fig. 11.5 the droplet size spectra of a number of atomizers are shown juxtaposed to a particle scale of 10^{11} conidia l^{-1} (10^8 ml^{-1}). At this concentration, very few of the (aerosol-sized) droplets produced by the Microgen cold fogger would contain particles. This concentration might be more appropriate for the hydraulic nozzles illustrated, where in this scenario, a conidial application rate of 10^{13} spores might be dispersed in tank mixtures for a VAR of 100 l ha^{-1} (although with typical medium- to high-volume spraying the concentration would be up to ten times lower). The major problem here is that, at 'typical' operating pressures of around 300 kPa, much of the spray volumes of many flat-fan and commonly used hollow-cone nozzles are larger than the sizes most appropriate for covering foliage with insecticides and fungicides. For example, at 300 kPa, > 20% of the volume is in droplets of over 200 μm with the 'River Mountain' nozzle (fitted to many side-lever knapsack sprayers in China and South-east Asia). Only when high pressures (600 kPa) are applied to narrow-orifice hollow-cone nozzles (e.g. the HCX 2) are appropriate droplet spectra produced.

Figure 11.6 illustrates some of the phenomena that may occur within the tank mixture and how this might affect the contents of the droplets themselves. Preparations containing well-separated conidia are extremely important for use with ULV sprayers

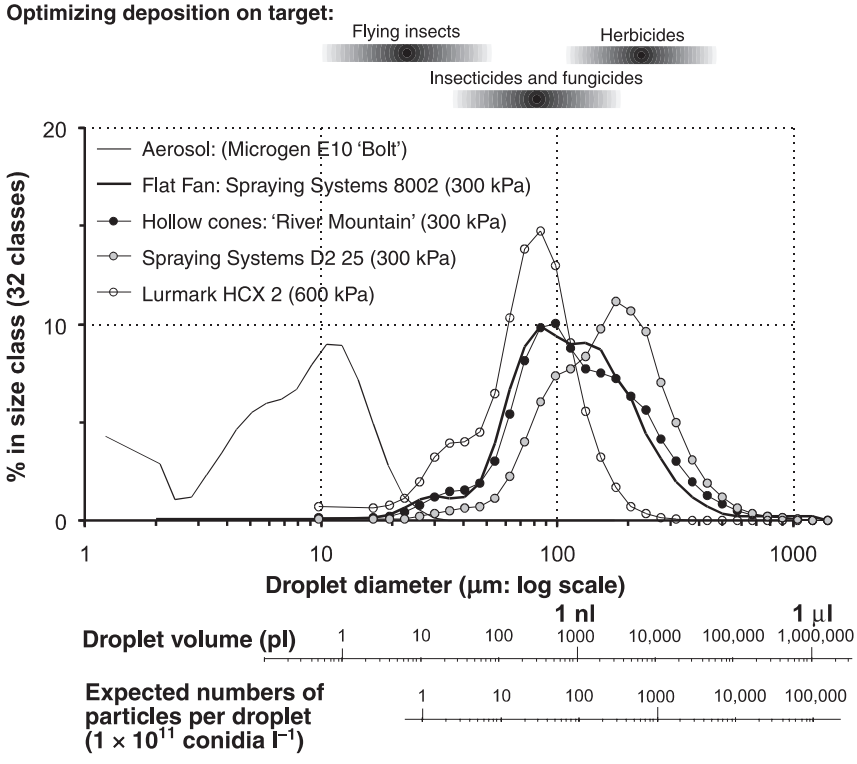

Fig. 11.5. Droplet size spectra of an aerosol generator (deodorized paraffin) and three hydraulic nozzles (water + 0.1% Agral, 300 kPa).

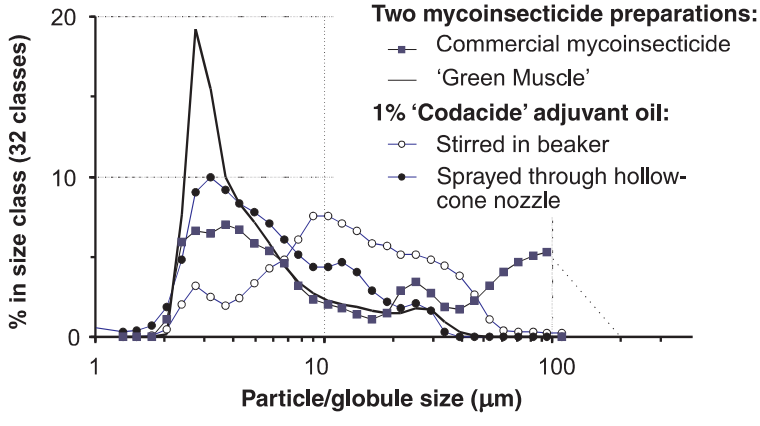

Fig. 11.6. Spectra of biopesticide formulations and emulsified oils.

and other equipment with narrow restrictions in the fluid line (Cherry *et al.*, 1999). However, problems could occur with certain commercial formulations in nozzles that produce fine sprays (quite apart from more practical filter and nozzle blockages). The action of pumping and spraying through a hollow-cone nozzle substantially broke up the globules of oil emulsions such as 'Codacide'. After passing through the sprayer, the

VMD of the globules in formulations is typically halved (and the presence or absence of conidia had little effect). This is due to the sheering force of the sprayer and pump systems, resulting in particles being more dispersed; there may therefore be an increased chance of infection with a sprayed liquid than with a formulation produced for a bioassay. Thus, although bioassays are an essential part of the development of any biological pesticide, pathogen delivery as sprayed droplets in trials is also essential.

Mycofungicides

The ultimate targets for any fungicide are the individual mycelium of the plant pathogen, but in practice this means maximum coverage on susceptible plant surfaces for protectant deposits (Mabbett, 1985). Much fungicide application research has been carried out using copper oxychloride, which being a particulate suspension may address some of the issues with microbial agents. The key practical issues are as follows:

1. Spray timing: a high work rate is especially important when protecting new foliage and young fruit, which are susceptible to infection (often by a complex of pathogens). Mabbett and Phelps (1983) found that use of low-volume and ULV applications of copper fungicides was the most practical way of achieving timely protection of seasonally induced new flushes of leaves against *Mycosphaerella citri*.
2. Spray placement: adequate coverage may be needed within the crop canopy and on the undersides of leaves (where pathogens may enter via the stomata – as in *M. citri*). Spray penetration into crops can be achieved by air assistance, provided by motorized mist-blowers and other motorized sprayers (e.g. orchard air-blast sprayers).
3. Longevity of deposit and redistribution: longevity (or persistence) is often dependent primarily on the nature of the pesticide and its formulation. The use of low-viscosity paraffinic oils (such as 'heavy alkylate' (Mabbett and Phelps, 1972)) is especially compatible with ULV application and has been shown to substantially improve the fungicide rain-fastness in comparison with water-based formulations (Mabbett and Phelps, 1983).

Fungicides perhaps require the greatest efficiency of coverage of all the pesticide types, and disease control in tree, bush and vine crops constitutes an important part of the market. Motorized knapsack mist-blowers (or air-blast sprayers) have many uses, although these sprayers were originally developed for obtaining good droplet coverage for mirid control in cocoa trees. Clayphon (1971) described the important criteria for evaluating machines, and technical requirements are now being standardized by the Food and Agriculture Organization (FAO, 1998). Because they produce relatively small droplets, mist-blowers are usually operated at lower VARs than the other types of hydraulic sprayers. They are typically used to apply water-based mixtures at 20–100 l ha^{-1} up to 250 l ha^{-1}, but low-flow-rate ULV adapters are available, achieving VARs of as little as 2 l ha^{-1} with oil-based formulations. Atomization occurs either conventionally with an air-shear nozzle or with a rotary atomizer supplied separately or (more economically) from the mist-blower manufacturers. The more expensive option is to retrofit nozzles such as the Micron 'Micronex', which can be adapted to fit on to the air outlet of most mist-blowers.

A comparison of droplet size spectra at one flow rate (200 ml min^{-1}) is shown in Fig. 11.7, with four atomizers and two formulations. The secondary X axis shows the probable spore loading with a 2.5×10^{10} conidia l^{-1} tank mixture concentration. This

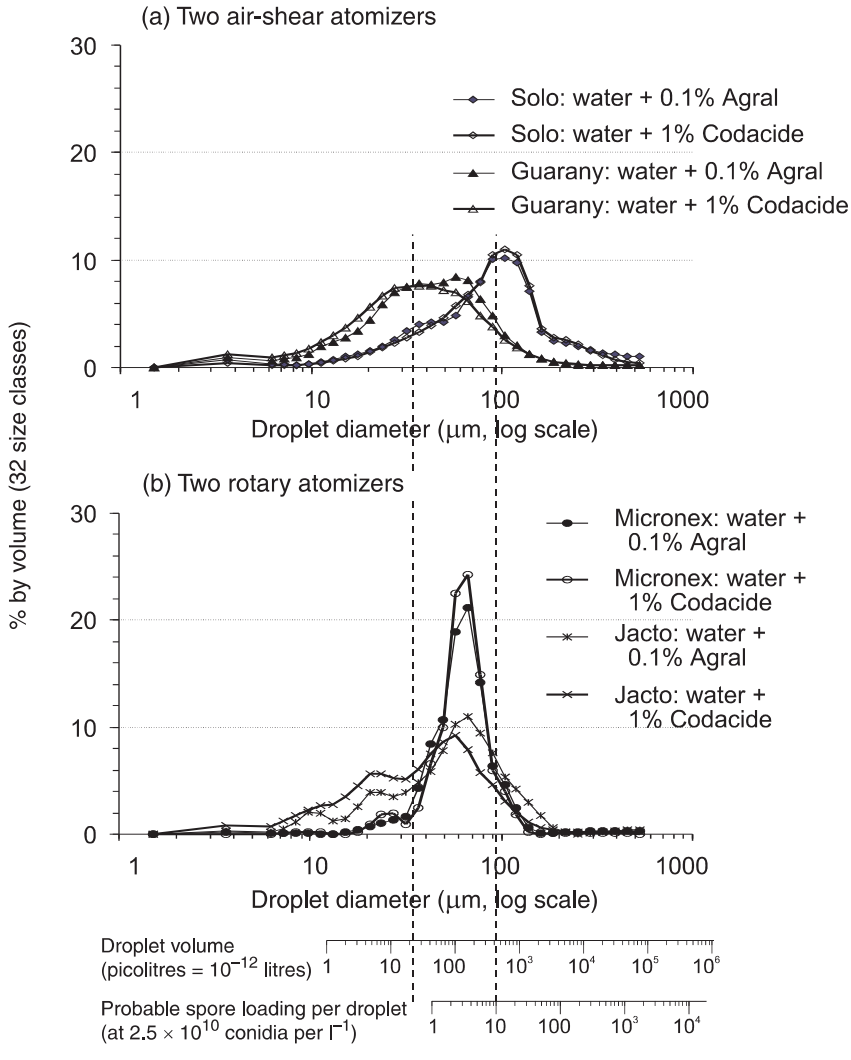

Fig. 11.7. Droplet size spectra of four motorized mist-blower nozzles, operating at 200 ml min^{-1}, comparing 1% 'Codacide' with a water + 0.1% Agral standard. The vertical hatched lines demarcate the approximate droplet sizes at which (on the left) there would be a < 50% probability of droplets containing conidia and (on the right) more than ten conidia per droplet. (From Bateman and Alves, 2000.) NB: equivalent axes.

nominal concentration would be appropriate for a VAR of 200 l ha^{-1} with a pathogen application rate of 5×10^{12} conidia ha^{-1}. Using these parameters, the proportion (by volume) of spray droplets with a < 50% chance of containing a single spore is high with sprayers, such as the 'Guarany 3.5HP', which produce a very fine spray spectrum. Not only do these very small droplets have a greatly reduced chance of impaction on leaf surfaces (because of their aerodynamic properties (May and Clifford, 1967)), but also the adjuvants contained in this spray volume will effectively be wasted (Chapple and Bateman, 1997). Droplets > 91.5 μm will probably contain more than 10 spores, and this has been used as an (arbitrary) upper limit for an 'optimal' droplet

size range covering an order of magnitude of dose variation. By using a rotary atomizer, such as the 'Micronex', the volume of spray liquid contained in droplets that contain one to ten spores (effectively dispersing and impacting them on leaves) may be doubled in comparison with a simple air-shear nozzle.

Mycoherbicides

One of the top priorities in herbicide spraying is the accurate placement of larger (> 150 μm) droplets, with minimal drift to non-target plants. If the ground is the target (e.g. pre-emergence or systemic herbicides), control of droplet size may be relatively unimportant provided there are no small driftable (< 100 μm) droplets: a range of low-pressure hydraulic nozzles is available that produce drop spectra with the majority of the spray volume as larger droplets, and small droplet production can be eliminated with rotary or 'kinetic' nozzles (e.g. the dribble bar or watering-can). If weed leaves are the target, there is a need to balance the low-drift requirement with a droplet size that is small enough to be retained by the leaf surface.

Lake (1977) showed that small (100–200 μm) droplets are substantially more easily retained on water-repellent leaves (such as those of *Avena fatua*) than larger (300–400 μm) droplets, with water-based formulations of high surface tension. The interactions between droplet size and other application variables on the biological performance of foliage-applied chemical herbicides was examined by Merritt (1980). He also showed that only gross differences in droplet size (400 μm vs. 100 μm) affect performance: the need for small droplets can be reduced with suitable formulations containing surfactants to improve spray retention. In general, greater amounts of a.i. are retained as VAR is decreased; however, formulations applied at very high concentration may cause leaf scorch, reduced uptake of herbicide and thus a reduction in biological performance. In fact, the relationship is more complex. The literature has been reviewed recently by Knoche (1994).

For mycoherbicides, the production of excessively large droplets effectively 'wastes' a large proportion of particles. Since many mycoherbicidal fungi can only be produced economically for low rates of application, large droplets may be very inefficient, and a narrow-spectrum CDA sprayer, such as the 'Herbi', might be appropriate. This was recognised by Lawrie *et al.* (1997), who applied conidia of *Stagonospora* sp. and *Micocentrospora acerina* at 2×10^9 and 5×10^8 conidia l^{-1} using a Micron 'Micromax 84' (a tractor-mounted rotary atomizer).

A commonly adopted technique to maintain a moist environment around propagules is the use of humectants (such as guar gum) and other adjuvants (including oils). The spray analyses of the 'Herbi' shown in Fig. 11.8 indicate that a gum humectant is detrimental to both the size and the quality of the droplet spectrum; on the other hand, the use of an emulsified oil, such as 'Codacide', reduces the VMD, but maintains a narrow droplet spectrum. Using a hypothetical formulation containing 1×10^9 particles l^{-1}, the secondary *X* axis indicates that there is considerable potential for wasting inoculum with the production of excessively large drops of > 500 μm, each containing > 100 propagules.

There is currently much interest in minimizing spray drift, and low-drift nozzles, such as the 'Turbo Teejet', may be useful for certain biopesticides that are prone to cause nozzle blockages (Bateman *et al.*, 2000). However, as indicated in Fig. 11.8, such nozzles may also distribute inoculum inefficiently.

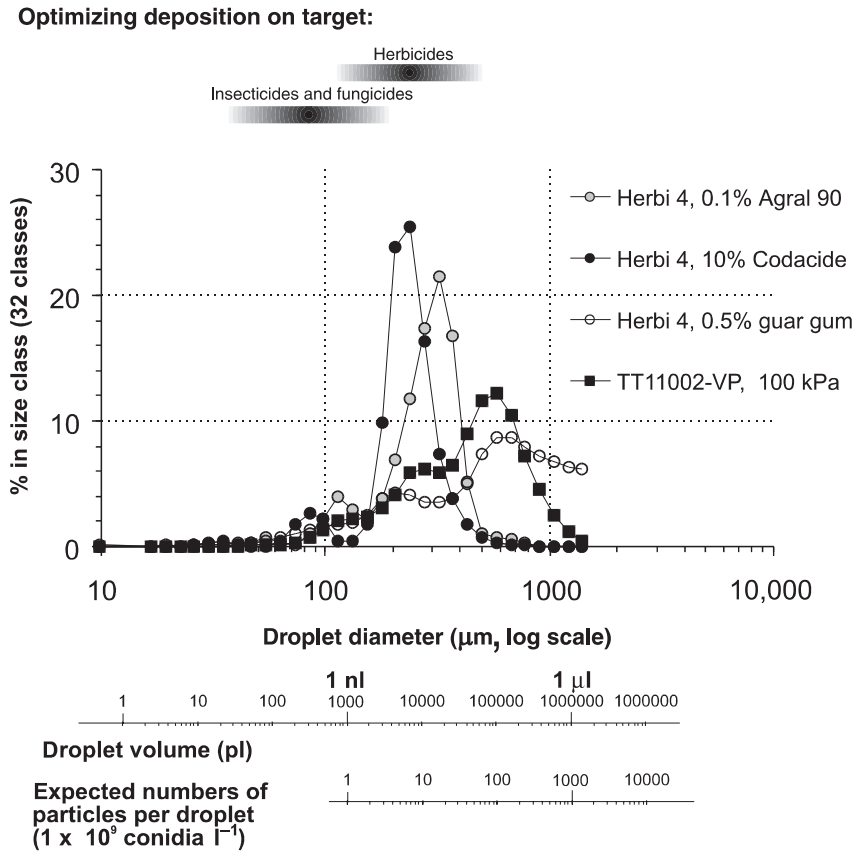

Fig. 11.8. Possible examples of herbicide application, including three formulations atomized by the Micron 'Herbi 4' and water + 0.1% Agral atomized by a Spraying Systems 'Turbo Teejet' with a 10^6 conidia ml^{-1} formulation.

It is interesting that similar techniques and problems have been encountered with studies on entomopathogenic nematodes. Mason *et al.* (1998) examined the numbers of infective juveniles (IJs) in the output of spinning-disc atomizers and found that, at lower concentrations (the highest being 1.2×10^7 IJs l^{-1}), many droplets contained no nematodes. Chapple *et al.* (2000) point out that large particles (such as nematodes, and thus presumably macroconidia or hyphae) can influence droplet production.

Conclusions

Conventional hydraulic nozzles are the mainstay of pesticide application techniques. However, from a theoretical point of view, reliance on them may severely reduce the potential for environmentally benign biological agent activity – hence the references to certain 'novel' techniques. However, changes in policy and practice to limit pesticide drift (especially in Europe and North America) effectively encourage the use of larger droplet sizes in ordinary spray nozzles. Not only has 'exo-drift' been reduced at the expense of 'endo-drift', but this also imposes substantial burdens on the develop-

ment of these environmentally advantageous microbial agents. Clearly, fundamental questions must be answered about the future role of biopesticides in farming systems, including 'Is drift an issue at all with biopesticides?'

In general, the only available spray application system for mycopesticides is that system in widespread use in the target crop in the geographical area of interest or local market. This also applies where a mycopesticide is being included with other pesticides (e.g. as part of an integrated pest management (IPM) system). Alternative application systems (e.g. CDA, mist-blower, etc.) often have substantial advantages over the hydraulic nozzle so must not be ignored, especially for niche markets where either no application system exists or where other application systems are ineffective. It has long been recognized that there is considerable grower resistance to investing in specialized equipment (Parish, 1970); although it not impossible to change application practice, the benefits have to be clearly demonstrated. A grower can be asked to change the nozzle, the VAR and other parameters, such as nozzle pressure and forward speed. With biological control agents such as mycopesticides, it may be possible to ask more of growers than would normally be asked when applying conventional agrochemicals. However, there are limits, and these must be explored properly before making label recommendations.

The use of oil formulations and adjuvants has been shown to enhance the activity of several mycopesticides. Since it would be impractical and uneconomical to use oils in conventional medium- to high-volume application equipment, the use of emulsified oils may provide a technical solution. However, trials with *Beauveria bassiana* products in North America have produced indifferent results (Wraight and Carruthers, 1999) and further research is needed.

Substantial work has been carried out to increase spray coverage with air-assisted sprayers and has been reviewed in a symposium by Lavers *et al.* (1991). Motorized knapsack mist-blowers produce relatively small droplets and are usually operated at lower VARs than the other types of hydraulic sprayer. They can achieve very good coverage, but, when used for medium- rather than low- or very-low-volume applications, they may use (expensive) adjuvants inefficiently in tank mixtures containing microbial pesticides (Chapple and Bateman, 1997).

On a larger scale, various tractor-boom-mounted air-assist nozzles have been developed, often with the aim of reducing drift with chemical pesticides, but also achieving increased canopy penetration and under-leaf coverage by air entrainment into field-crop canopies. Taylor and Andersen (1997) describe the benefits of spray booms where a perforated sleeve provided a curtain of air alongside the hydraulic nozzles and projected the spray into the crop. Less draconian modifications to spray booms include twin fluid nozzles, such as Cleanacres 'Airtec' deflector nozzles (which also substantially reduce drift), or vehicle-mounted booms with drop legs that pass in between crop rows (e.g. as produced by Benest Engineering). These nozzles also require a compressor and their use with biopesticides is discussed further in Bateman *et al.* (2000). Instead of using air to entrain fine droplets into crop canopies, the use of coarse sprays from conventional hydraulic tips in a 'double nozzle' system has been proposed by Chapple *et al.* (1996); this might also avoid the need for surfactants in oil-based mycopesticide formulations, but this device is only just becoming available commercially.

The development of other techniques for enhancing biological pesticides has been advocated, but requires further research and awaits the availability of commercial equipment. Electrostatic charging of sprays may improve under-leaf coverage and ED nozzles

offered benefits of low maintenance and power requirements in comparison with other CDA systems (Coffee, 1981). However, the ED is no longer available commercially and, in any case, the polar solvents used in ED formulations are toxic to biological agents.

Failure to consider the numerical aspects of dose transfer of particles during application may be catastrophic during the testing of new microbial control agents. Although there is potential for substantially improving the efficacy of biopesticides with better delivery systems, this is limited (probably to less than ten times) and must be constrained by practical considerations, such as acceptability to growers.

Note

[1]Lutte Biologique contre les Locustes et les Sauteriaux: a collaborative, multidisciplinary research and development programme funded by the governments of Canada (CIDA), the Netherlands (DGIS), Switzerland (SDC) and the UK (DfID).

References

Adams, A.J., Chapple, A.C. and Hall, F.R. (1990) Droplet spectra for some agricultural fan nozzles, with respect to drift and biological efficiency. In: Bode, E., Hazan, J.L. and Chasin, D.G. (eds) *Pesticide Formulations and Application Systems: Tenth Symposium.* American Society for Testing and Materials, Philadelphia, STP 1078, pp. 156–169.

Agudelo, F. and Falcon, L.A. (1983) Mass production, infectivity, and field application studies with the entomophagous fungus *Paecilomyces farinosus. Journal of Invertebrate Pathology* 42, 124–132.

Amsellem, Z., Sharon, A., Gressel, J. and Quimby, P.C. (1990) Complete abolition of high inoculum threshold of two mycoherbicides (*Alternaria cassiae* and *A. crassa*) when applied in invert emulsion. *Phytopathology* 80, 925–929.

Bache, D.H. and Johnstone, D.R. (1992) *Microclimate and Spray Dispersion.* Ellis Horwood, Chichester, UK, 238 pp.

Bals, E.J. (1969) The principles of and new developments in ultra low volume spraying. *Proceedings of the 5th British Insecticide and Fungicide Conference.* British Crop Protection Council, Farnham, UK, pp. 189–193.

Bateman, R.P. (1993) Simple, standardised methods for recording droplet measurements and estimation of deposits from controlled droplet applications. *Crop Protection* 12, 201–206.

Bateman, R.P. (1997) Methods of application of microbial pesticide formulations for the control of grasshoppers and locusts. *Memoirs of the Entomological Society of Canada* 171, 67–79.

Bateman, R.P. and Alves, R.T. (2000) Delivery systems for mycoinsecticides using oil-based formulations. *Aspects of Applied Biology* 57, 163–170.

Bateman, R.P., Douro-Kpindu, O.K., Kooyman, C., Lomer, C. and Oambama, Z. (1998) Some observations on the dose transfer of mycoinsecticide sprays to desert locusts. *Crop Protection* 17 (2), 151–158.

Bateman, R.P., Matthews, G.A. and Hall, F.R. (2000) Ground-based application equipment. In: Lacey, L. and Kaya, H. (eds) *Field Manual of Techniques in Invertebrate Pathology.* Kluwer, Dordrecht, the Netherlands, pp. 77–112.

Blanford, S., Thomas, M.B. and Langewald, J. (1998) Behavioural fever in a population of the Senegalese grasshopper, *Oedaleus senegalensis*, and its implications for biological control using pathogens, *Ecological Entomology* 23, 9–14.

Brent, K.J. and Atkin, R.K. (eds) (1987) *Rational Pesticide Use.* Cambridge University Press, Cambridge, 348 pp.

Burges, H.D. (ed.) (1998) *Formulation of Microbial Biopesticides, Beneficial Microorganisms, Nematodes and Seed Treatments.* Kluwer Academic, Dordrecht, 412 pp.

Butler Ellis, M.C., Tuck, C.R. and Miller, P.C.H. (1997) The effect of some adjuvants on sprays produced by agricultural flat fan nozzles. *Crop Protection* 16, 41–50.

Chapple, A.C. and Bateman, R.P. (1997) Application systems for microbial pesticides: necessity not novelty. In: *Microbial Insecticides: Novelty or Necessity?* British Crop Protection Council Proceedings/Monograph Series No. 68, pp. 181–190.

Chapple, A.C., Downer, R.A., Wolf, T.M., Taylor, R.A.J. and Hall, F.R. (1996) The application of biological pesticides: limitations and a practical solution. *Entomophaga* 41 (3/4), 465–474.

Chapple, A.C., Downer, R.A. and Bateman, R.P. (2000) Theory and practice of microbial insecticide application. In: Lacey, L. and Kaya, H. (eds) *Field Manual of Techniques for the Evaluation of Entomopathogens*. Kluwer, Dordrecht, the Netherlands, pp. I-2, 5–37.

Cherry, A., Jenkins, N., Heviefo, G., Bateman, R. and Lomer, C. (1999) A West African pilot scale production plant for aerial conidia of *Metarhizium* spp. for use as a mycoinsecticide against locusts and grasshoppers. *Biocontrol Science and Technology* 9, 35–51.

Clarkson, J.M. and Charnley, A.K. (1996) New insights into the mechanisms of fungal pathogenesis in insects. *Trends in Microbiology* 4, 197–204.

Clayphon, J.E. (1971) Comparison trials of various motorised knapsack mistblowers at the Cocoa Research Institute of Ghana. *PANS* 17(2), 209–224.

Coffee, R.A. (1981) Electrodynamic crop spraying. *Outlook on Agriculture* 10, 350–356.

Copping, L.G. (1998) *The Biopesticide Manual.* British Crop Protection Council, Farnham, UK, 333 pp.

Courshee, R.J. (1959) Drift spraying for vegetation baiting. *Bulletin of Entomological Research* 50 (2), 355–370.

Craymer, H.E. and Boyle, D.G. (1973) *The Micrometeorology and Physics of Spray Particle Behaviour.* Pesticide Spray Technology Workshop, Emeryville, California.

Food and Agriculture Organization of the United Nations (FAO) (1992). *The Desert Locust Guidelines, IV: Control.* Food and Agriculture Organization Booklet (ed. P. Symmons), 64 pp.

Food and Agriculture Organization of the United Nations (FAO) (1997) *Evaluation of Field Trial Data on the Efficacy and Selectivity of Insecticides on Locusts and Grasshoppers. Report to FAO by the Pesticide Referee Group, 6th meeting, Rome, 10–12 December 1996*. 21 pp.

Food and Agriculture Organization of the United Nations (FAO) (1998) *Agricultural Pesticide Sprayers,* Vol. 2: *FAO Technical Standards: Sprayer Specifications and Test Procedures.* FAO-AGSE, Rome, 63 pp.

Furness, G.O., Magarey, P.A., Miller, P.H. and Drew, H.J. (1998) Fruit tree and vine sprayer calibration based on canopy size and length of row: unit canopy row (UCR) method. *Crop Protection* 17, 639–644.

Georgis, R. (1997) Commercial prospects of microbial insecticides in agriculture. In: *Microbial Insecticides: Novelty or Necessity?* British Crop Protection Council Proceedings/Monograph Series No. 68, BCPC, Farnham, pp. 243–252.

Global Crop Protection Federation (GCPF, formerly GIFAP) (1999) *Catalogue of Pesticide Formulation Types and International Coding System.* GCPF Technical Monograph, No. 2, 4th edn, GCP, Brussels, Belgium, 5 pp.

Graham-Bryce, I.J. (1977) Crop protection: a consideration of the effectiveness and disadvantages of current methods and of the scope for improvement. *Philosophical Transactions of the Royal Society, London B* 281, 163–179.

Hall, F.R., Chapple, A.C., Downer, R.A., Kirchner, L.M. and Thacker. J.R.M. (1993) Pesticide application as affected by spray modifiers. *Pesticide Science* 38, 123–133.

Hall, R.A. and Papierok, B. (1982) Fungi as biological control agents of arthropods of agricultual and medical importance. *Parasitology* 84, 205–240.

Helyer, N., Gill, G. and Bywater, A. (1992) Elevated humidities for control of chrysanthemum pests with *Verticillium lecanii. Pesticide Science* 36, 373–378.

Himel C.M. (1969) The optimum drop size for insecticide spray droplets. *Journal of Economic Entomology* 62, 919–925.

Himel, C.M. (1974) Analytical methodology in ULV. In: *Pesticide Application by ULV Methods.* British Crop Protection Council Monograph No. 11, pp. 112–119.

Hislop E.C. (1987) Can we define and achieve optimum pesticide deposits? *Aspects of Applied Biology* 14, 153–165.

Hofstein, R. and Chapple, A. (1998) Commercial development of biofungicides. In: Hall, F.R. and Menn, J.J. (eds) *Biopesticides: Use and Delivery.* Humana Press, Totowa, New Jersey, pp. 77–102.

Jeffree, C.E. (1986) The cuticle, epicuticular waxes and trichomes of plants, with reference to their structure, functions and evolution. In: Juniper, B. and Southwood, R. (eds) *Insects and the Plant Surface.* Edward Arnold, London, pp. 23–64.

Knoche, M. (1994) Effect of droplet size and carrier volume on performance of foliage-applied herbicides. *Crop Protection* 13, 163–178.

Lake, J.R. (1977) The effect of drop size and velocity on the performance of agricultural sprays. *Pesticide Science* 8, 515–520.

Langewald, J., Thomas, M.B., Douro-Kpindou, O.-K. and Lomer, C.J. (1997) Use of *Metarhizium flavoviride* for control of *Zonocerus variegatus*: a model linking dispersal and secondary infection from the spray residue with mortality in caged field samples. *Entomologia Experimentalis et Applicata* 82, 1–8.

Langewald, J., Ouambama, Z., Mamadou, A., Peveling, R., Stolz, I., Bateman, R., Attignon, S., Blanford, S., Arthurs, S. and Lomer, C. (1999) Comparison of an organophosphate insecticide with a mycoinsecticide for the control of *Oedaleus senegalensis* (Orthoptera: Acrididae) and other Sahelian grasshoppers at an operational scale. *Biocontrol Science and Technology* 9, 199–214.

Lavers, A., Herrington, P. and Southcombe, E.S.E. (eds) (1991) *Air-assisted Spraying in Crop Protection.* British Crop Protection Council Monograph No. 46, BCPC, Farnham, 308 pp.

Lawrie, J., Greaves, M.P., Down, V.M. and Chassot, A. (1997) Some effects of spray droplet size on distribution, germination of and infection by mycoherbicide spores. *Aspects of Applied Biology* 48, 175–182.

LeFebvre, A.H. (1989) *Atomization and Sprays.* Hemisphere, New York, 421 pp.

Lisansky (1997) Microbial biopesticides. In: Evans, H.F. (ed.) *Microbial Insecticides: Novelty or Necessity?* British Crop Protection Council Proceedings/Monograph 68, BCPC, Farnham, pp. 3–10.

Lomer, C.J., Bateman, R.P., Dent, D., De Groote, H., Douro-Kpindou, O.-K., Kooyman, C., Langewald, J., Ouambama, Z., Peveling, R. and Thomas, M. (1999) Development of strategies for the incorporation of biological pesticides into the integrated management of locusts and grasshoppers. *Agricultural and Forest Entomology* 1, 71–88.

Mabbett, T.H. (1985) A biological appraisal of fungicide application in the tropics. In: British Crop Protection Council Monograph 28, pp. 265–287.

Mabbett, T.H. and Phelps, R.H. (1972) Heavy alkylate – a new carrier liquid for ultra low volume spraying. *Tropical Agriculture (University of the West Indies, Trinidad)* 49 (4), 363–364.

Mabbett, T.H. and Phelps, R.H. (1983) The behaviour of copper deposits from reduced volume sprays and their control of citrus greasy spot. *International Journal of Pest Management* 29 (2), 137–144.

Mason, J.M., Matthews, G.A. and Wright, D.J. (1998) Appraisal of spinning disc technology for the application of entomopathogenic nematodes. *Crop Protection* 17, 453–461.

Matthews, G.A. (1992) *Pesticide Application Methods*, 2nd edn. Longman Scientific and Technical, Harlow, UK, 405 pp.

May, K.R. and Clifford, R. (1967) The impaction of aerosol particles on cylinders, spheres, ribbons and discs. *Annals of Occupational Hygiene* 10, 83–95.

Merritt, C.R. (1980) The influence of application variables on the biological performance of

foliage applied herbicides. In: *Spraying Systems for the 1980s.* British Crop Protection Council Monograph 24, BCPC, Farnham, UK, pp. 35–43.

Parish, K.P. (1970) Economic aspects of application involving specialised equipment. In: *Symposium for Research Workers on Application.* British Crop Protection Council Monograph No. 2, BCPC, Farnham, pp. 43–48.

Parkin, C.S. (1992) Methods for measuring spray droplet sizes. In: Matthews, G.A. and Hislop, E.C. (eds) (1993) *Application Technology for Crop Protection.* CAB International, Wallingford, UK, pp. 57–84.

Prior, C., Jollands, P. and Le Patourel, G. (1988) Infectivity of oil and water formulations of *Beauveria bassiana* (Deuteromycotina; Hyphomycetes) to the cocoa weevil pest *Pantorhytes plutus* (Coleoptera: Curculionidae). *Journal of Invertebrate Patholog* 52, 66–72.

Taylor, W.A. and Anderson, P.G. (1997) A review of benefits of air assisted spraying trials in arable crops. *Aspects of Applied Biology* 48, 163–173

Thomas, M.B., Wood, S.N. and Lomer, C.J. (1995) Biological control of locusts and grasshoppers using a fungal pathogen: the importance of secondary cycling. *Transactions of the Royal Society* 259, 265–270

Wraight, S.P. and Carruthers, R.I. (1999) Production, delivery and use of mycoinsecticides for control of insect pests on field crops. In: Hall, F.R. and Menn, J.J. (eds) *Biopesticides: Use and Delivery.* Humana Press, Totowa, New Jersey, pp. 233–270.

Wrigley, G. (1973) Mineral oils as carriers for ultra-low-volume (ULV) spraying. *PANS* 19 (1), 54–61.

Young, B.W. (1986) The need for greater understanding in the application of pesticides. *Outlook on Agriculture* 15, 80–87.

12 Toxic Metabolites of Fungal Biocontrol Agents

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Introduction

One of the major hurdles in the registration and subsequent commercialization of fungal biological control agents (BCAs) is risk assessment. This is a costly, controversial and contentious area, which may have deterred many entrepreneurs from investing in the development of fungal BCAs. Risk assessment and related issues are dealt with in more detail in Chapter 13. Of particular interest (and the focus of this chapter) are the biological properties of toxic metabolites secreted by fungal BCAs. Plant pathologists have generally defined toxins as low-molecular-weight products of microorganisms active in low concentrations (Graniti, 1972).

Fungi secrete a wide array of compounds with biological activity against other organisms, mostly products of secondary metabolism (Table 12.1). Secondary metabolites originate as derivatives from various intermediates in primary metabolism (Fig. 12.1). Most of these compounds arise from five main metabolic sources: (i) amino acids; (ii) the shikimic acid pathway for the biosynthesis of aromatic amino acids; (iii) the polyketide biosynthetic pathway from acetyl coenzyme A (CoA); (iv) the mevalonic acid pathway from acetyl CoA; and (v) polysaccharides and peptidopolysaccharides. Biosynthesis of fungal secondary metabolites is beyond the scope of this book, but has been briefly reviewed by Griffin (1994).

Fungal metabolites serve different functions, depending on the ecological niche of the fungus (Fig. 12.2). Some metabolites may be antibiotics to protect the BCA against antagonistic microorganisms, or may prevent growth of saprophytic microbes on the host after it is killed and thus improve the survival of the BCA. Mycoparasites, in particular, may exploit this strategy to displace plant pathogens or postharvest diseases. Some bioactive metabolites are also important pathogenicity determinants (Strasser *et al.*, 2000) and others have antifeedant/repellent properties that presumably deter mycophagous organisms. This chapter reviews the production and biological activity of selected metabolites of fungal BCAs. It is impossible to cover all metabolites,

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Table 12.1. Selected metabolites of some important fungal biological control agents (BCAs).

Fungal BCA	Main target	Metabolites produced *in vitro* and/or *in vivo*
Metarhizium anisopliae	Insects	Destruxins (> 27 types), swainsinone, cytochalasin C
Beauveria bassiana	Insects	Bassianin, beauvericin, bassianolide, beauverolides, tenellin
Beauveria brongniartii	Insects	Oosporein
Paecilomyces fumosoroseus	Insects	Beauvericin, beauverolides, pyridine-2,6-dicarboxylic acid
Verticillium lecanii	Insects	Dipcolonic acid, hydroxycarboxylic acid, cyclosporin
Tolypocladium spp.	Insects	Cyclosporin, efrapeptins (five types)
Hirsutella thompsonii	Insects and mites	Hirsutellin A, hirsutellin B, phomalactone
Trichoderma spp.	Fungi	Harzianic acid, alamethicins, tricholin, peptaibols, antibiotics, 6-pentyl-α-pyrone, massoilactone
Gliocladium spp.	Fungi	Viridin, gliovirin, glisoprenins, heptelidic acid
Fusarium spp.	Fungi, insects, and weeds	Trichothecenes, beauvericin, naphthazarins (e.g. fusarubin and anhydrofusarubin), fusaric acid,
Colletotrichum	Weeds	Colletotrichin

since the discovery of new compounds is an ongoing process, with many remaining to be discovered. A few selected examples will illustrate concepts or patterns that may apply to other fungal BCAs.

Toxins of Entomogenous Fungi

The most studied species of entomogenous fungi are *Metarhizium anisopliae* and *Beauveria bassiana*. Less studied, but equally important species of commercial importance are *Paecilomyces fumosoroseus*, *Tolypocladium* spp., *Verticillium lecanii* and *Hirsutella* spp. These fungi secrete an array of secondary metabolites, some of which are restricted to specific genera, while others are more ubiquitous. Here, attention is focused on a few representative examples from selected genera.

Destruxins

The first systematic study of toxin production by fungal entomopathogens *in vitro* was conducted on *M. anisopliae* and led to the discovery of destruxins A and B (Kodaira, 1961). Since then, many other compounds have been isolated from *M. anisopliae* (Table 12.2). Suzuki *et al.* (1971) first demonstrated the production of destruxins *in vivo*. Destruxins are quite disparate compounds, which may occur as isomers or congeners. Their basic structural backbone consists of five amino acids and an α-hydroxy acid. To date, 28 structurally different, related destruxins have been identified from three different sources; most congeners are produced by *M. anisopliae* (Strasser *et al.*, 2000). Other natural analogues of destruxins have been described, including roseotoxin (Engstrom *et al.*, 1975) and bursephalocids A and B (Kawazu *et al.*, 1993).

Recently, destruxins A4, A1 and A5 and homodestruxin B were isolated from the entomopathogenic fungus *Aschersonia* sp. (Krasnoff and Gibson, 1996). Destruxin B,

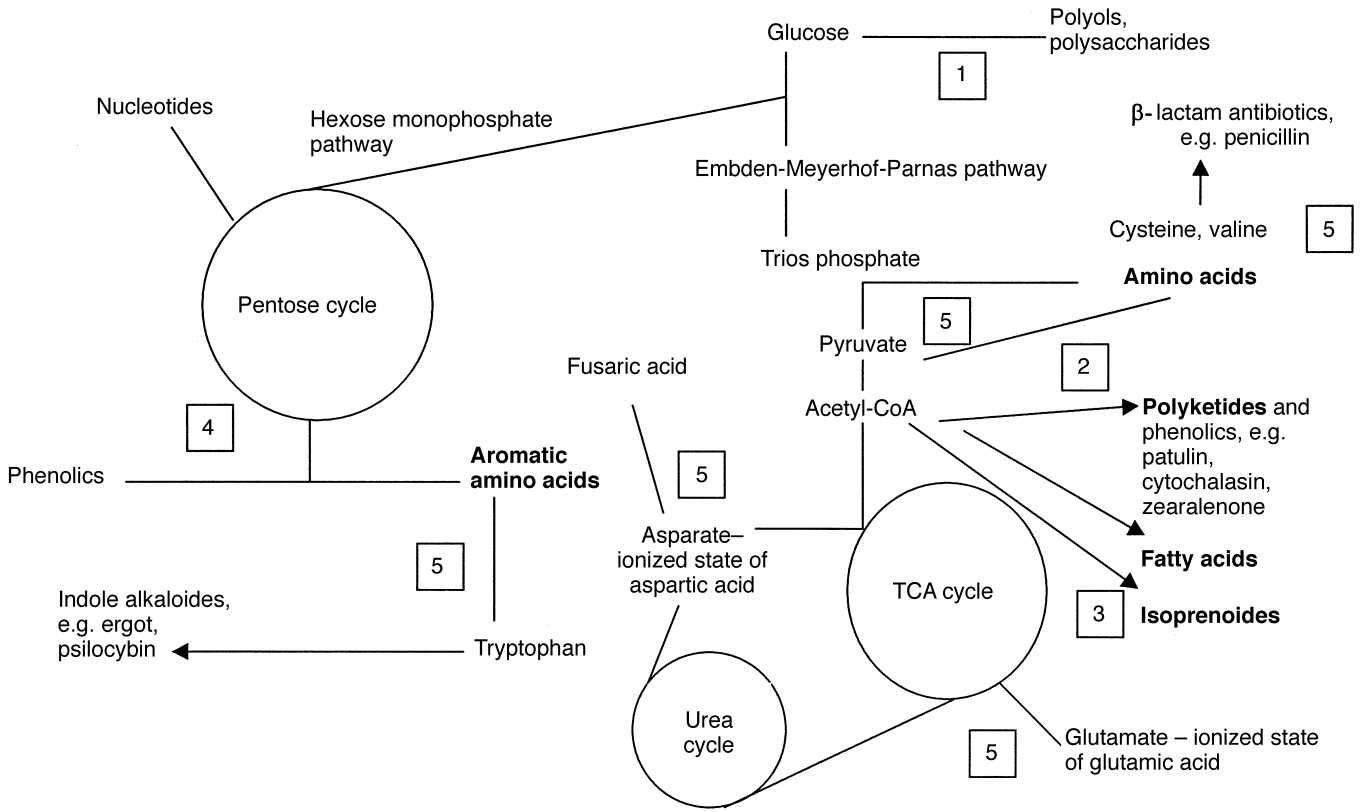

Fig. 12.1. Interrelationships of metabolic pathways in primary and secondary metabolism. The principal pathways of secondary metabolism are numbered as follows: (1) glucose-derived metabolites; (2) acetate–malonate pathway; (3) mevalonic acid pathway; (4) shikimic acid pathway; and (5) amino acid-derived pathways. TCA, tricarboxylic acid. (Adapted from Griffin 1994.)

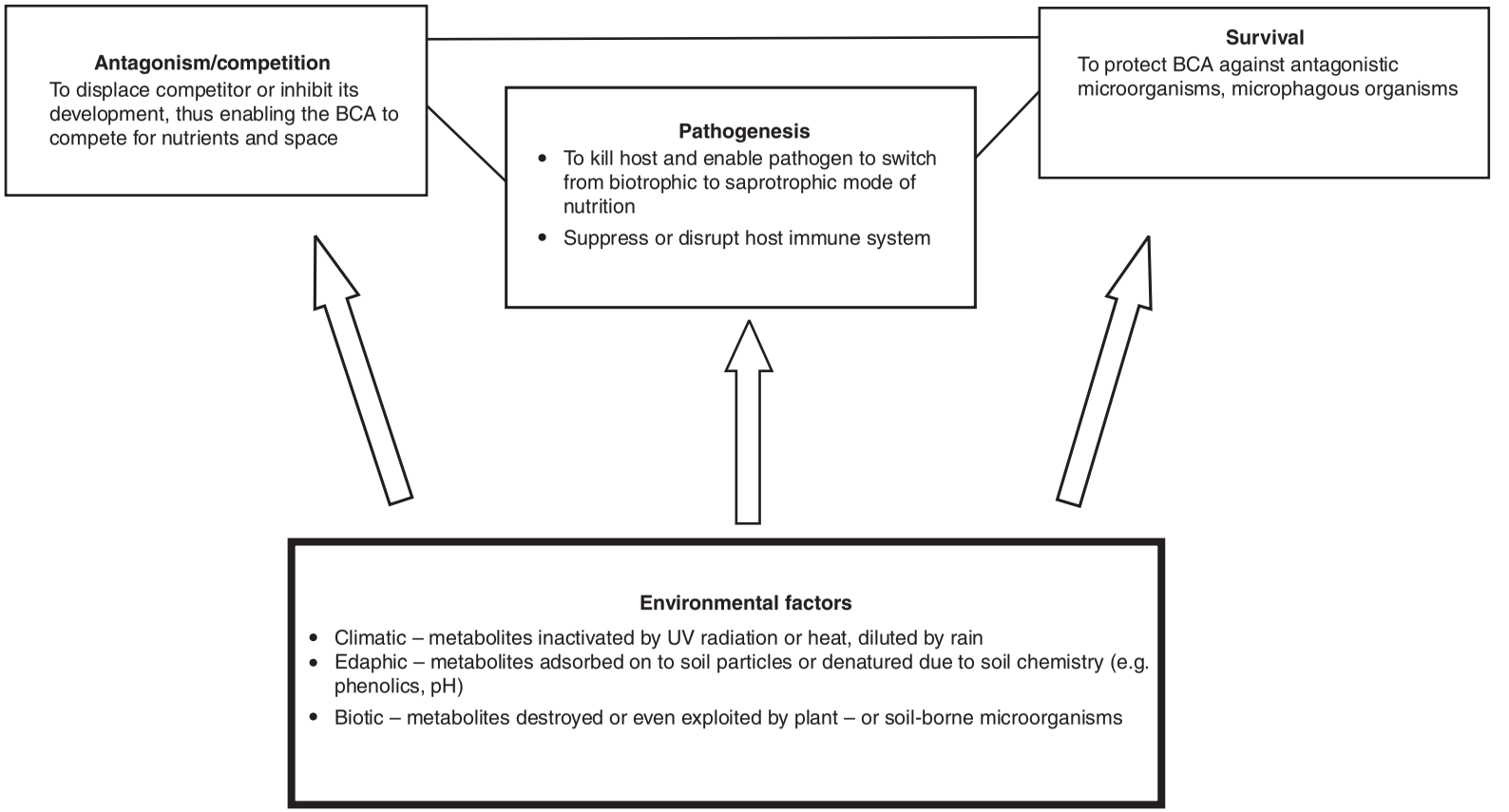

Fig. 12.2. Role of fungal metabolites and factors affecting their persistence. UV, ultraviolet.

Table 12.2. Destruxin analogues isolated from *Metarhizium anisopliae*.

Analogue	Reference
Desmethyl destruxins B, D and C	Suzuki *et al.*, 1970
Destruxins E, A1, A2, B1, B2, C2, D1, D2 and E1	Païs *et al.*, 1981
Destruxin E2 and chlorhydrin	Gupta *et al.*, 1989, 1991
Destruxins A3 and F and desmethyl destruxins A and C	Wahlman and Davidson, 1993

homodestruxin B and desmethyl destruxin B were also isolated from the plant-pathogenic fungi *Alternaria brassicae, Trichothecium roseum* and *Ophiosphaerella herpotricha* (Ayer and Pena-Rodriguez, 1987; Bains and Tewari, 1987; Gupta *et al.*, 1989; Buchwaldt and Jensen, 1991).

Insects vary in their susceptibility to destruxins, with some Lepidoptera being highly susceptible (Roberts, 1981; Charnley, 1984; Fargues and Robert, 1986; Samuels *et al.*, 1988a; Brousseau *et al.*, 1996; Thomsen *et al.*, 1996; Kershaw *et al.*, 1999). The median lethal dose (LD_{50}) of destruxin A and B injected into silkworm larvae was 0.015–0.030 mg g^{-1}, 24 h post-injection (Kodaira, 1961; Suzuki *et al.*, 1971; Tamura and Takahashi, 1971), but these compounds were ten- to 30-fold less active in waxmoth (*Galleria*) larvae (Roberts, 1966).

The effects of destruxins also vary between the species and specific developmental stages of the test organism and of the producing organism. For example, insects injected with low doses exhibit tetanus within 3 min and, at higher doses, tetanus paralysis is brief or absent and a flaccid paralysis occurs (Roberts, 1981). Toxins also affect insect growth. Larvae of the mustard beetle (*Phaedon cochleariae*) and potato lady beetle (*Epilachna sparsa*) grow slowly when exposed to leaves treated with destruxins compared with those fed on untreated leaves (Kodaira, 1961; Amiri *et al.*, 1999). The influence of destruxins on the larval growth, pupal weight and emergence rate of females was also observed in the eastern spruce bud-worm, *Choristoneura fumiferana* (Brousseau *et al.*, 1996).

Relationships between the chemical structure of destruxins and their biological activity have been investigated. Most workers note significant differences in the insecticidal activity of the compounds of this family, while others report no difference. Destruxins A and E appear to be the most toxic molecules and destruxin D the least toxic toward *Galleria* larvae (Fargues *et al.*, 1986; Vey and Quiot, 1989; Dumas *et al.*, 1994). Fargues and co-workers found that destruxins A and E were equally toxic, but more toxic than destruxin B when injected into or ingested by *Galleria* larvae. Housefly maggots appear to be more sensitive to destruxin E than to destruxin A or B (Robert and Fargues, 1986). Destruxin E was more toxic for *Galleria* larvae than for *Musca domestica*, which, in turn, was more susceptible to this toxin than the onion maggot fly, *Delia antiqua* (Roberts, 1981; Proprawski *et al.*, 1985; Fargues *et al.*, 1986; Robert and Fargues, 1986).

Some workers report that, depending on the type and host species involved, destruxin has no contact toxicity when applied to the integument (Fargues *et al.*, 1986), while others report contact toxicity (Poprawski *et al.*, 1994; Amiri *et al.*, 1999). Exactly how these compounds cross the insect cuticle remains unclear.

There is some evidence that destruxin E is systemic in plants, because the crucifer pest *Brevicoryne brassicae* (cabbage aphid) is repelled by cabbage leaves soaked in an 8.8 p.p.m. solution of destruxin E. *Myzus persicae* is also susceptible to destruxin

E (LD_{50} = 0.4 mg cm^{-2}), but not to the same degree as *B. brassicae*. In contrast, the cereal aphid *Rhopalosiphum padi* continued to feed on cereal leaves treated with destruxin E, even at relatively high doses, e.g. 6.6 mg cm^{-2} (Robert and Riba, 1989).

Little is known about the functionally active groups in the different destruxin molecules. It has been suggested that an epoxy group in destruxin E increases potency, while a COOH group may decrease potency, such as in destruxin D (Dumas *et al.*, 1994). It appears that destruxins have a spectrum of other biological activities, such as disruption of the calcium balance in cells (Dumas *et al.*, 1996a) and inhibition of vacuolar adenosine triphosphatases (ATPases) (Muroi *et al.*, 1994; Bandani *et al.*, 2001); some of these activities are summarized in Table 12.3.

There is some evidence that insects can remove injected destruxins from circulation (Samuels *et al.*, 1988b). Insects differ in their ability to detoxify destruxins (Fargues *et al.*, 1985). The fat body seems to possess a greater affinity for this cyclic peptide than any other tissue or organ and is a more important detoxification system than the pericardial tissue (Lange *et al.*, 1991, 1992). When destruxin E is injected into *Locusta migratoria*, it is converted by the fat body into destruxin E-diol (which is inactive when injected in *Galleria mellonella*) and E-diol is secreted by the Malpighian tubules. Likewise destruxin A is transformed into the corresponding linear peptide (Lange *et al.*, 1991, 1992; Loutelier *et al.*, 1994, 1995).

The induction of paralysis by injection of haemolymph from insects infected by *M. anisopliae* suggested that destruxins are secreted during fungal infection (Kodaira, 1961; Roberts, 1966). The destruxin content amounted to 240 ng per silkworm larva 4 days after inoculation with spores. Sufficient amounts of destruxins are secreted into the haemolymph to induce a cytotoxic effect on host tissues (Vey *et al.*, 1986).

Al-Aïdroos and Roberts (1978) reported a link between the amount of destruxin produced by mutants of *M. anisopliae in vitro* and virulence against mosquito larvae. Recent studies by Amiri-Besheli *et al.* (2000) reveal inter- and intra-specific variation in destruxin production by the genus *Metarhizium*, both *in vivo* and *in vitro*. Specialized species, such as *Metarhizium album*, which is restricted to hemipteran insects, produce very little destruxin, while generalist species, such as *M. anisopliae* var. *anisopliae*, produce destruxins A, B and E, often in significant quantities. These observations suggest that destruxin may be significant in determining host specificity. The authors also argued that destruxins were possibly important pathogenicity determinants for some strains of *Metarhizium.*

Destruxins have antifeedant properties and are toxic to insects following absorption through the cuticle (Amiri *et al.*, 1999). They are also toxic to small mammals. The LD_{50} of destruxins A and B following intraperitoneal injection in mice was 1–1.35 mg kg^{-1} and 13.2–16.9 mg kg^{-1} within 1 h, respectively (Kodaira, 1961). In contrast, destruxins are less toxic to fish and amphibians. No lethal or teratogenic effect or postponement of emergence of the embryos was observed in the teleostean fish *Brachydanio rerio* H.B. (Debeaupuis and Lafont, 1985). The acute toxicity of destruxins on the amphibians *Xenopus laevis* Daudin and *Rana temporaria* L. is low, i.e. as a reference point, chemical pesticides, such as the fungicide thiram, show a stronger (lethal) effect (Fargues and Robert, 1986).

Recently, a neutral lipophilic extract (methylene chloride, pH 7.2) derived from *M. anisopliae* cultures was evaluated for toxicity and mutagenicity, using aquatic animal bioassays and the Ames test (Genthner *et al.*, 1998). The average LC_{50} of the extract obtained in static, acute 96 h tests conducted with 24-h-old *Mysidopsis bahia* was 2.41 mg l^{-1}. However, by partially purifying destruxins from the neutral extract,

Table 12.3. Some activities of destruxins (Dtx).

Effect/property	Reference
Dtx A, B and E cause *Gromphadorhina laevigata* (Dictyoptera) and *Bombyx mori* cell lines (Lepidoptera) to contract, become granulated and stop dividing. The cell line of *B. mori* was more susceptible to these destruxins than cells of *G. laevigata,* even at relatively low doses, i.e. 0.05 p.p.m. versus 1 p.p.m.	Quiot *et al.*, 1985
Dtx E effects on invertebrate cells include: aggregation of chromatin, deformation of nuclei, degradation of mitochondria and rough endoplasmic reticulum and impaired functioning of the ribosomes	
Interfere with haemocyte function and can prevent nodulation	Vey *et al.*, 1985; Huxham *et al.*, 1989
Trigger degranulation of isolated haemocytes of the crayfish (*Pacifastacus leniusculus*)	Cerenius *et al.*, 1990
Inhibit phagocytosis in plasmatocytes *in vitro* and in infected larvae	Vilcinskas *et al.*, 1997
Inhibit synthesis of DNA, RNA and proteins even at low doses, e.g. nucleotide synthesis of mouse P388 leukaemic cell lines.	Odier *et al.*, 1992
Dtx E acts on the midgut, Malpighian tubules and circulating haemocytes	Vey and Quiot, 1989; Dumas *et al.*, 1996 b
Block H^+-ATPase activity	Muroi *et al.*, 1994
Trigger phosphorylation of unidentified cellular proteins	Dumas *et al.*, 1996a
Alter enzyme activity, i.e. phenoloxidase, which is involved in melanin synthesis	Vey *et al.*, 1985; Huxham *et al.*, 1989; Cerenius *et al.*, 1990
Antiviral effects	Quiot *et al.*, 1980, 1985; Kopecky *et al.*, 1992; Sun *et al.*, 1994; Yeh *et al.*, 1996; Chen *et al.*, 1997
Cause rapid decrease in the transmembrane resting potential; directly or indirectly open endogenous Ca^{2+} channels within the muscle membrane of *Manduca sexta*	Bradfisch and Harmer, 1990
Prevent secretion of ecdysteroids by prothoracic glands of *Manduca*	Sloman and Reynolds, 1993
Inhibit fluid secretion by *Schistocerca gregaria* Malpighian tubules	James *et al.*, 1993

it was shown that destruxins alone were not responsible for the observed toxicity in mysids. After 3 months, no mortalities or adverse effects were observed in adult *Gambrusia affinis* fed a diet partially composed of a freeze-dried *M. anisopliae* culture. The same extract showed no mutagenicity in the Ames test using strains TA98 and TA100, with and without metabolic activation by rat liver S9. However, the extract was toxic to developing grass shrimp, *Palaemonetes pugio*, and frog, *X. laevis*, embryos; the LC_{50} values were 52 and 32 mg l^{-1}, respectively. The extract was toxic to juvenile mosquito fish, *G. affinis*, at an LC_{50} value of 141 mg l^{-1}. However, adult female

G. affinis surviving a 24 h exposure to 200 μg ml^{-1} of the neutral extract produced healthy broods.

In the case of the plant pathogen *A. brassicae*, destruxins play an important role in the infection of several *Brassica* species. These toxins induce chlorosis and have been isolated from infected leaf tissue (Ayer and Pena-Rodriguez, 1987; Bains and Tewari, 1987; Buchwaldt and Jensen, 1991; Buchwaldt and Green, 1992). Bains and Tewari (1987) suggested that the level of sensitivity of *Brassica* species to destruxin B was related to their degree of susceptibility to *A. brassicae*, with non-host plants being least affected. Likewise, Venkatasubbaiah *et al.* (1994) reported that destruxin B from *O. herpotricha* induced necrotic/chlorotic reactions in host but not in non-host plants.

Efrapeptins

The linear peptidic efrapeptins (types C to G) have been isolated only from *Tolypocladium* species (Weiser and Matha, 1988; Krasnoff and Gupta, 1991). Efrapeptins are inhibitors of intracellular protein transport and mitochondrial ATPases (Fricaud *et al.*, 1992; Krasnoff *et al.*, 1991). They arrest syncytium formation (SF) and have cytopathic effects (CPE) in Newcastle disease virus (NDV)- and vesicular stomatitis virus (VSV)-infected BHK cells, respectively, without profoundly affecting glycoprotein synthesis (Muroi *et al.*, 1996). Efrapeptins blocked cell-surface expression of NDV-HN and VSV-G glycoproteins, but did not suppress intoxication by ricin or diphtheria toxin even after prolonged pretreatment. Efrapeptins inhibit F-ATPase or ATP synthase, but their inhibitory effect on SF and CPE was independent of the intracellular ATP concentration (Muroi *et al.*, 1996).

Efrapeptins show insecticidal and miticidal effects against arthropod species such as spider mites, potato beetle, tobacco bud-worm and diamondback moth (Matha *et al.*, 1988; Krasnoff *et al.*, 1991). Dose-related antifeedant and insect growth inhibitory properties have also been reported by Bandani and Butt (1999). Furthermore, efrapeptins have limited antifungal and antibacterial activity. There is no information concerning the phytotoxicity and antiviral properties of the efrapeptins.

As with destruxin, there was inter- and intraspecific variation in efrapeptin production. In *Tolypocladium cylindrosporum, Tolypocladium niveum* and *Tolypocladium parasiticum*, efrapeptin production reached 116 mg l^{-1}, 80 mg l^{-1} and 2 mg l^{-1} in respective culture filtrates after 14 days' incubation. *Tolypocladium* species secreted low amounts of efrapeptins in the insect haemocoel during the infection process. Few fungal hyphae were detected in dead, infected *G. mellonella* and *Calliophora* sp., suggesting that death was to due to toxicosis. The LD_{50} values of the purified efrapeptin mixture for final-instar larvae of the Lepidoptera *G. mellonella* and *Manduca sexta* in injection assays were, on average, 30 and 47 ng, respectively (Bandani *et al.*, 2000).

Oosporein

The red-coloured dibenzoquinone oosporein is produced by a large number of soil fungi and the entomogenous fungi belonging to the genus *Beauveria* (Eyal *et al.*, 1994; Strasser *et al.*, 2000a, b). Oosporein is considered to react with proteins and amino acids through redox reactions by altering SH groups, resulting in enzyme malfunction (Wilson, 1971). Oosporein, like tenellin and bassianin, will inhibit erythrocyte mem-

brane ATPase activity in a dose-dependent manner by as much as 50% at 200 μg ml^{-1}. These pigments inhibited Ca^{2+}-ATPases to a greater extent than Na^{+}/K^{+}-ATPase activity. The ATPase-inhibitory activity of these pigments was not specific, but was probably a consequence of membrane disruption, since they all caused alterations in erythrocyte morphology and promoted varying degrees of cell lysis (Jeffs and Khachatourians, 1997). Oosporein is an antiviral compound, preferentially inhibiting herpes simplex virus-I DNA-polymerase (Terry *et al.*, 1992). The authors found that oosporein was a competitive inhibitor of dGTP or dCTP incorporation into DNA.

Oosporein is an effective antibiotic against Gram-positive bacteria, but has little effect on Gram-negative bacteria (Vining *et al.*, 1962; Brewer *et al.*, 1984; Taniguchi *et al.*, 1984; Wainwright *et al.*, 1986). It has no obvious antifungal properties but there are mixed reports on its phytotoxicity; some workers report plant growth-inhibiting and phytotoxic effects, while others report the contrary (Cole *et al.*, 1974; Brewer *et al.*, 1977; Strasser *et al.*, 2000). However, oosporein has been reported to cause avian gout in broiler chicks and turkeys (Cole *et al.*, 1974; Pegram and Wyatt, 1981; Pegram *et al.*, 1982; Manning and Wyatt, 1984; Brown *et al.*, 1987). Furthermore, oosporein has been found to be toxic to 1-day-old male chickens (LD_{50} = 6 mg kg^{-1}; Manning and Wyatt, 1984)). Toxicity studies of oosporein in mice and hamsters indicated an LD_{50} value of 0.5 mg kg^{-1} body weight, when injected intraperitoneally (Wainwright *et al.*, 1986). However, a daily oral administration of 7 mg kg^{-1} oosporein to mice over 47 days was non-lethal. Cytotoxicity tests on two different mammalian cell lines revealed that oosporein, at 600 ng ml^{-1}, had no adverse effect (Abendstein and Strasser, 2000). In addition, oosporein at 100 μg ml^{-1} had no effects on *in vitro* cell cultures of hamster tumour cells and baby hamster kidney cells (Wainwright *et al.*, 1986). Aleo *et al.* (1991) studied the nephrotoxic potential of oosporein using rat renal proximal tubules. The authors reported that tubule viability was altered, but there was no evidence to support a direct inhibitory effect on mitochondrial respiration at a maximum oosporein concentration of 306 μg ml^{-1}.

Strasser *et al.* (2000) found that oosporein was the only major secondary metabolite produced by three commercial strains of the entomopathogenic fungus *Beauveria brongniartii* in submerged cultures and on sterilized barley kernels. None of the other major toxins (bassianin, beauvericin and tenellin) normally produced by *Beauveria* species were detected by sensitive high-performance liquid chromatography (HPLC) and mass spectrometry (MS) techniques (Strasser *et al.*, 2000b). Both *in vitro* and *in vivo* studies on the distribution of oosporein revealed negligible amounts in the environment, suggesting that these particular strains of *B. brongniartii* pose no risk to humans and animals (Strasser *et al.*, 2000a). Laboratory experiments have shown that the maximum amount of oosporein produced in liquid batch reactors was 270 mg l^{-1}, after 4 days' incubation, while that produced on sterilized barley kernels ranged between 2.0 and 3.2 mg kg^{-1}, after 14 days' incubation (production time). The maximum amount of oosporein detected in cockchafer (*Melolontha melolontha*) larvae infected with *B. brongniartii* was 0.23 mg $larva^{-1}$. Melocont®-Pilzgerste, a commercial product based on *B. brongniartii*, was not phytotoxic to garden cress (*Lepidium sativum*), Hurd's grass (*Phleum pratense*) and potatoes (*Solanum tuberosum*), nor were fungal metabolites detected in these indicator plants and potato tubers (Strasser *et al.*, 2000; H. Strasser, unpublished data). No systemic effects (e.g. chlorosis, necrosis and stunting) of oosporein were observed in pasture turf treated with oosporein-enriched *B. brongniartii* culture broth, even several months after treatment (Strasser *et al.*, 2000).

Based on the results of laboratory and field experiments, a theoretical oosporein

concentration of 4.8–6.4 mg m^{-2} can be expected to be detected in the soil. This calculation is based on an average infestation density of 80 *M. melolontha* larvae m^{-2} and an average infection rate of 30–40%.

Beauvericin, bassianolide and beauveriolide

Beauvericin is a hexadepsipeptide, previously isolated from the entomopathogenic fungi *Beauveria* spp. and *Paecilomyces* spp. and the plant-pathogenic fungi *Fusarium* spp. and *Polyporus fumosoroseus* (Grove and Pople, 1980; Gupta *et al.*, 1991; Plattner and Nelson, 1994; Logrieco *et al.*, 1998; Munkvold *et al.*, 1998), and two analogues, A and B, were described by Gupta *et al.* (1995).

Beauvericin is structurally and functionally similar to the membrane-damaging antibiotics enniatins A, B and C and differs from these compounds with respect to the *N*-methylamino acids (Steinrauf, 1985). Beauvericin forms Na^+ and K^+ complexes, leading to increased permeability of natural and artificial membranes (Ovchinnikov *et al.*, 1971). Beauvericin shows antibiotic activity against several bacteria, such as *Bacillus subtilis*, *Escherichia coli*, *Mycobacterium phlei*, *Sarcinea lutea*, *Staphylococcus aureus* and *Streptococcus faecalis* (Ovchinnikov *et al.*, 1971). Furthermore, beauvericin has moderate insecticidal properties (Suzuki *et al.*, 1977; Kanaoka *et al.*, 1978; Champlin and Grula, 1979; Qadri *et al.*, 1989; Zizka and Weiser, 1993; Gupta *et al.*, 1995). However, some workers report that it has no toxicity for certain insects (Champlin and Grula, 1979).

Beauvericin is a specific cholesterol acyltransferase inhibitor of certain cell lines, induces programmed cell death similar to apoptosis and causes cytolysis, accompanied by internucleosomal DNA fragmentation into multiples of 200 base pairs (Ojcious *et al.*, 1991). It is toxic to brine shrimp (*Artemia salina* L.) with an LD_{50} = 2.8 μg ml^{-1} water (Moretti *et al.*, 1995), and to *M. bahia*, at an LD_{50} of 0.56 mg l^{-1}. The toxicity of beauvericin persists in sterile sea water for at least 3 but not 8 weeks (Genthner *et al.*, 1994). A non-polar extract of mycelia from *B. bassiana* containing beauvericin was toxic at an LC_{50} of 84.2 mg l^{-1}. Beauvericin toxicity was mostly investigated using insects, but recent reports show high *in vitro* toxicity towards murine (Ojcious *et al.*, 1991) and human cell lines (Di Paola *et al.*, 1994).

Another toxin secreted by *B. bassiana* is the cyclo-octadepsipeptide called bassianolide (Suzuki *et al.*, 1977). This chemical induces atonic symptoms in silkworm larvae fed on an artificial diet containing small amounts of this compound, but was lethal at higher doses (13 p.p.m.). Bassianolide, like beauvericin, is an ionophore antibiotic, but differs in its reaction to different cations (Suzuki *et al.*, 1977; Kanaoka *et al.*, 1978). Neither beauvericin nor bassianolide has been shown to exhibit mammalian or plant toxicity, but there may possibly be a synergistic effect with the structurally related mycotoxin moniliformin (Cole *et al.*, 1973). *Fusarium* species under certain conditions produce beauvericin, moniliformin, fumonisins (b1, b2) and fusaproliferin (Gupta *et al.*, 1991; Logrieco *et al.*, 1998; Munkvold *et al.*, 1998). Ingestion of *Fusarium*-contaminated grain by mammals and the subsequent uptake of these metabolites causes cancer of the oesophagus and heart problems (Marasas *et al.*, 1981). Whether beauvericin should be classified as a food toxin has not yet been determined.

Beauveria species also produce beauveriolides and beauverolides, which are peptides structurally related to beauvericin and bassianolide (Namatame *et al.*, 1999). Their toxicity to animals, plants and insects remains generally unknown. Where tested, they

give negative results, with the exception of beauveriolide I (Mochizuki *et al.*, 1993).

Bassianin and tenellin (two non-peptide toxins) have also been isolated from *Beauveria* species. These yellow-coloured secondary metabolites inhibit the erythrocyte membrane ATPases (Jeffs and Khachatourians, 1997). There is very little information on the effect of these toxins on target pests.

Hirsutellin

The hyphomycete *Hirsutella thompsonii* produces an extracellular insecticidal protein, hirsutellin A, which has been purified from culture filtrates during liquid fermentation (Mazet, 1992; Vey *et al.*, 1993). Liu *et al.* (1995) monitored the production of hirsutellin A by *H. thompsonii* during submerged fermentation. The peak level of exocellular production of hirsutellin A (13–14 μg ml^{-1}) occurred at the late exponential growth phase (39–45 h), as determined by densitometric analysis of the 16.3 kDa bands on SDS-PAGE gels and enzyme-linked immunosorbent assay (ELISA). Hirsutellin A production was directly correlated with mycelial growth. Twenty-one-hour culture filtrates were highly toxic to larvae of the greater wax-moth. Pure hirsutellin A at a concentration of 40 pmol was highly toxic to *G. mellonella* larvae.

The amino acid composition and the N-terminal sequence of hirsutellin A have been determined by Mazet and Vey (1995). The toxin appears to be distinct from other known proteins. It is not glycosylated and does not show proteolytic activity. The toxin is also antigenic, thermostable and not inactivated by treatments with proteolytic enzymes.

The hirsutellin A gene codes for a precursor of 164 amino acids, which includes a 34-amino-acid leader sequence, which, like those found in ribosomal-inhibiting proteins (RIPs), contains a signal and a pro sequence. The mature 130-amino-acid hirsutellin A, with a calculated M_r= 14,159 and pI = 9.21, is considered to be a stable hydrophilic protein. The sequence of hirsutellin A is unique and does not produce the secondary or tertiary structures characteristic of other fungal RIPs (Boucias *et al.*, 1998).

Hirsutellin A was tested using contact/residual leaf bioassay methodologies at concentrations of 0, 10, 32, 56 and 100 μg ml^{-1} against adult citrus rust mite, *Phyllocoptruta oleivora*, the natural host of the parasitic fungus, *H. thompsonii*. Mite mortality increased with an increase in hirsutellin A concentration, reaching virtually 100% at 100 μg ml^{-1}, using both leaf assay methods. The number of eggs found on leaf discs within a 3-day period decreased significantly with increasing concentrations of the toxin, suggesting that fecundity was affected prior to host death (Omoto and McCoy, 1998). Toxicity bioassays showed that wax-moth larvae injected with hirsutellin A at 1 mg toxin g^{-1} body weight caused a high mortality rate. Hirsutellin A was also toxic per os to neonatal mosquito (*Aedes aegypti*) larvae (Mazet and Vey, 1995). It is also capable of inhibiting protein translation, and possesses biological features similar to the well-characterized RIPs sarcin, mitogellin and restrictocin. Liu *et al.* (1996) reported that at 0.5 and 5.0 μM concentrations, hirsutellin A caused detectable cytopathic effects on *Spodoptera frugiperda* (Sf-9) cells within 2–4 h and completely inhibited Sf-9 cell growth 4 days after treatment. Electron-microscopic data showed that hirsutellin A-treated Sf-9 cells became hypotrophied, with disrupted internal organelles and cell membranes. At the same concentration, it effectively inhibited Brome mosaic virus protein synthesis of both rabbit reticulocyte and wheat germ in *in vitro* translation systems. The ribosomal RNA extracted from hirsutellin A-treated Sf-9 cells produced a

smaller RNA (about 528 bases) in addition to larger bands present in control and treated ribosomal preparations (Liu *et al.*, 1996).

Organic acids

Among organic acids, oxalic, kojic, cyclopyazonic, fusaric and 4′-hydroxymethylazoxybenzene-4-carboxylic acids have been isolated from fungi pathogenic to invertebrates, and are considered toxic to lepidopterans or dipterans (Roberts, 1981; Bidochka and Khachatourians, 1991; Khachatourians, 1996). Oxalic acid is an important pathogenicity determinant of some plant pathogens (Godoy *et al.*, 1990). It is also produced by *B. bassiana* (Kodaira, 1961) and is considered to be an important pathogenicity determinant because it can solubilize specific cuticular proteins. Bidochka and Khachatourians (1991) described this metabolite as a synergistic factor that enhances the hydrolytic activity of proteases and chitinase. The zygomycete *Entomophthora virulenta* produces azoxybenzene-4,4′-dicarboxylic acid and 4′-hydroxymethylazoxybenzene-4-carboxylic acid. The hydroxyacid is toxic for *Calliphora erythrocephala* when applied by injection, and is responsible for the insecticidal activity in culture filtrates (Claydon, 1978). This compound has structural similarities to the dichlorodiphenyltrichloroethane (DDT) group of insecticides (Roberts, 1981).

Toxins of Mycoparasites

Two of the most important commercial mycoparasites belong to the related genera *Trichoderma* and *Gliocladium* (Tomlin, 1997). These fungi produce a large variety of metabolites with diverse functions. However, the success of these BCAs as disease control agents can be partly attributed to the production of antifungal metabolites, aggressive growth habits and high competition for nutrients. This is also one of the reasons for the success of the naturally occurring mutant strain Fo47 of *Fusarium oxysporum*. This isolate protects plants from pathogenic strains of *F. oxysporum* and *Fusarium moniliforme* due to its rhizosphere competency (i.e. ability to colonize roots quickly) and competition for nutrients. It may displace plant-pathogenic strains through competitive exclusion, using antibiotics without harming the host plant.

Gliocladium virens strain GL-21 has been developed by Grace Biopesticides in collaboration with the US Department of Agriculture Agricultural Research Service (USDA-ARS) Biocontrol of Plant Diseases Laboratory in Beltsville, Maryland. The commercial product, SoilGard™, is an entirely biorational product, consisting of spores of this fungus, and is registered with the US Environmental Protection Agency (EPA) for control of damping-off and root-rot pathogens of ornamental and food-crop plants in greenhouses, nurseries and interior gardens. *G. virens* can parasitize some soil pathogens, such as *Rhizoctonia solani.* The *Gliocladium* will actually wrap itself around the pathogen and release enzymes that destroy the pathogen's wall, leaving the pathogen susceptible to attack. It also produces a broad-spectrum antibiotic, called gliotoxin, which kills many soil pathogens (see Chapter 2). Gliotoxin is sensitive to oxidation and probably poses no health risk because of rapid degradation. This is supported by the demonstrated lack of toxicity in oral and pulmonary studies conducted on rats (Anon, 1990).

Gliotoxin is not found in the SoilGard™ formulations, but, when the spores of

strain GL-21 begin to grow in the soil, they produce the antibiotic. SoilGard™ has a 'Caution' label, which is given to products which are least harmful and which may cause only slight irritation after normal exposures. It is exempt from tolerance for use on all food crops. SoilGard™ has the minimum re-entry interval allowed by the EPA, and has a 'zero day' preharvest interval.

Trichoderma harzianum is produced by several companies for the control of a wide range of plant-pathogenic fungi including *Botrytis cinerea* (Chapters 1 and 2). Several formulations based on selected strains of this fungus have been registered with the EPA. Toxicity testing on vertebrate species indicated no pathogenic or toxic effects, and the EPA has granted an exemption from tolerance for selected commercial strains.

In this review we focus on two important, controversial metabolites, peptaibols and gliotoxin. Other metabolites produced by these fungi will also be briefly reviewed, since they usually work in concert with peptaibols and gliotoxin.

Peptaibols

These are a family of short-chain polypeptides consisting of 15–20 residues or fewer. A high proportion of the amino acid residues are atypical, such as isovaline, hydroxyproline, ethylnorvaline and aminoisobutyric acid. There is a particularly high proportion of aminoisobutyric acid, which has a high tendency to form helices. This is borne out by the helical structures of the peptaibols. The chain has an alkyl N-terminus (usually acetyl) and a hydroxy-amino acid at the C-terminus.

Peptaibols generally exhibit antimicrobial activity and are referred to as antibiotic peptides. The main sources of the peptaibols known to date are fungi of the genera *Trichoderma* and *Emericellopsis*. The antimicrobial activity is thought to arise from their membrane activity and their ability to form pores in lipid membranes. The pores so formed cause leakage of ionic species across membranes, leading to loss of osmotic balance and cell death.

Peptaibols are usually secreted as microheterogeneous mixtures of peptides. Those secreted by *T. harzianum* include trichorzianins (El Hajji *et al.*, 1987; Rebuffat *et al.*, 1989), trichokindins (Iida *et al.*, 1994), trichorzins and harzianins (Rebuffat *et al.*, 1992; Goulard *et al.*, 1995; Duval *et al.*, 1998; Leclerc *et al.*, 1998). The trichokindins, which are 18-residue peptides containing one to three isovaline residues, induced Ca^{2+}-dependent catecholamine secretion from bovine adrenal medullary chromaffin cells (Iida *et al.*, 1994). *Trichoderma viridae* produces alamethicins (Kleinkauf and Rindfleisch, 1975; Brewer *et al.*, 1987) and trichotoxin (Irmscher *et al.*, 1978). Paracelsin, originally described as a metabolite of *Trichoderma resei*, is, in fact, produced by many *Trichoderma* species (Bruckner and Graf, 1983; Solfrizzo *et al.*, 1994). Paracelsin is highly toxic to *A. salina* larvae (calculated LD_{50} = 2.2 μM) (Solfrizzo *et al.*, 1994).

Lorito *et al.* (1996) reported synergism between peptaibols and cell-wall hydrolytic enzymes in the antagonism of phytopathogenic fungi by *T. harzianum*. β-Glucan synthase activity on isolated plasma membranes of *B. cinerea* was inhibited *in vitro* by the peptaibols trichorzianin TA and TB, but inhibition was reversed by the addition of phosphatidylcholine. β-Glucan synthesis *in vivo* (assayed by incorporation of [2-(3)*H*]glucose into cell-wall material) was inhibited by peptaibols, and this inhibition was synergized by exogenous *T. harzianum* β-1,3–glucanase. This synergism is, therefore, explained by an inhibition of the membrane-bound β-1,3-glucan synthase

of the host by the peptaibols, which inhibit the resynthesis of cell-wall β-glucans, sustain the disruptive action of β-glucanases and altogether enhance the fungicidal activity. Therefore cell-wall turnover is a major target of mycoparasitic antagonism.

Gliotoxin

The epidithiodiketopiperazine antibiotic gliotoxin was discovered in 1934 as an antifungal agent (Weindling, 1934) and then various biological activities were reported. It has subsequently been shown to have antimicrobial, antiviral and immunomodulating activities (Taylor, 1986). Furthermore, it is known to be an inhibitor of the platelet activating factor (PAF), which induces platelet aggregation (Okamoto *et al.*, 1986a, b; Yoshida *et al.*, 1988). The antifungal properties of gliotoxin are synergistically enhanced by the cell wall-degrading enzymes of *T. harzianum* and *G. virens* (Lorito *et al.*, 1994).

Gliotoxin is a toxic product of several moulds that cause a serious respiratory disease of poultry and humans. This toxin affects the immune system and inhibits many functional aspects of this system, and renders the host prone to disease-causing agents.

The relatively short period of bioactivity limits the use of this agent in certain applications. Wilhite and Straney (1966) examined the apparent transient accumulation of gliotoxin, a potential limitation in biocontrol activity. ^{35}S-pulse labelling of gliotoxin indicated that *G. virens* strain G20-4VIB synthesizes this compound only within a short 16 h period, during replicative growth. An apparent lack of gliotoxin production in later growth phases was due to the cessation of synthesis, rather than to increased gliotoxin catabolism. Media-transfer experiments indicated that cessation of gliotoxin synthesis could not be explained by gliotoxin feedback inhibition, a diffusible inhibitor or a change in the nutritional status of the medium over a 2 h period. These results demonstrate that the regulation of gliotoxin biosynthesis is a major determinant in the kinetics of gliotoxin appearance and points out the need for further study on the regulation of gene expression (Wilhite and Straney, 1966).

Other important metabolites of Trichoderma and Gliocladium *species*

Trichoderma and *Gliocladium* secrete diverse secondary metabolites with antibiotic properties, including polyketides, terpenoids, polypeptides and metabolites derived from α-amino acids (Taylor, 1986). Thus *T. harzianum* produces harzianic acid (Sawa *et al.*, 1994), the terpenoid cyclonerodiol and the corresponding octaketide keto-diol (Ghisalberti and Rowland, 1993), 6-pentyl-α-pyrone antibiotics (Graeme-Cook and Faull, 1991), and a new sesquiterpene antibiotic, heptelidic acid, also produced by *G. virens* (Itoh *et al.*, 1980). Heptelidic acid has antibiotic activity against Gram-positive and Gram-negative bacteria and inhibits the growth of some anaerobic bacteria (Itoh *et al.*, 1980).

T. harzianum produces unidentified volatile metabolites with fungistatic effects on *Agaricus bisporus* (Mumpuni *et al.*, 1998). T-2 toxin and related trichothecenes are secreted by *Trichoderma* species (Ueno, 1984). The production by *T. harzianum* of a novel trichothecene, harzianum A, which exhibits modest antifungal activity, has been reported (Corley *et al.*, 1994). Furthermore, a novel antifungal protein from *T. viride*, tricholin, has been described (Lin *et al.*, 1994). This ribosome-inactivating protein causes cessa-

tion of growth and uptake of amino acids, and is active against *R. solani*. Cyclonerodiol and koninginins have been purified and characterized from strains of *Trichoderma koningii* (e.g. Ghisalberti and Rowland, 1993). Some of these compounds have been purified from culture filtrates of a *T. harzianum* strain isolated from wheat roots and were active against the take-all fungus, *Gaeumannomyces graminis* var. *tritici*.

Antifungal antibiotics produced by *T. harzianum* are not sufficient to explain its mycoparasitic activity. Ultraviolet light-induced mutants with altered antibiotic production revealed that those strains with elevated antibiotic production did increase inhibition of hyphal growth of phytopathogenic fungi, but there was no correlation between this factor and colonization ability (Graeme-Cook and Faull, 1991).

In addition to secondary metabolites, *Trichoderma* and *Gliocladium* spp. also produce different classes of fungal cell-wall-hydrolytic enzymes such as chitinases, β1,3-glucanases and proteases, which play an important role in mycoparasitism (Schirmbock *et al.*, 1994). Cell-wall-degrading enzymes produced by *T. harzianum* and *G. virens* inhibit spore germination of *B. cinerea in vitro* (Lorito *et al.*, 1994). The role of *T. harzianum* endochitinase has been studied, using constructed strains carrying multiple copies of the gene encoding for this enzyme and by using gene disrupters. The level of chitinase activity increased strongly in multi-copy strains, while gene disrupters had practically no activity. However, comparative observations regarding the efficacy of the strains generated as BCAs revealed no major differences (Carsolio *et al.*, 1999).

Hydrolytic enzymes and peptaibol antibiotics are produced in parallel by *T. harzianum* in the same cultural conditions when cell walls of *B. cinerea* are introduced in fresh medium. When enzymes and peptaibols were tested together, an antifungal synergistic action on spore germination and hyphal elongation, measured by the reduction of 50% of the effective dose value, was noted. These data revealed that parallel formation and synergism of hydrolytic enzymes and antibiotics may have an important role in the antagonistic action of *T. harzianum* (Schirmbock *et al.*, 1994). Investigations on the molecular bases for this synergism showed that it is peptaibol inhibition of the membrane-bound β-1,3-glucan synthase of *B. cinerea* that strengthens the disruptive activity of β-glucanases (Lorito *et al.*, 1996).

In conclusion, the biocontrol efficacy of *Trichoderma* spp. seems to be a combination of antibiosis, lysis, competition, mycoparasitism and promotion of plant growth (Ghisalberti and Sivasithamparam, 1991).

Besides the metabolites already mentioned, *Gliocladium* spp. are able to produce other antibiotics such as the fungistatic compound viridin (Jones and Hancock, 1987) and gliovirin (Stipanovic and Howell, 1982). *Gliocladium* species also produce a chitinase inhibitor in liquid culture called argifin. The IC_{50} value of argifin against *Lucilia cuprina* chitinase was 3.7 μM. It arrested the moult of cockroach larvae upon injection into the ventral abdominal part (Omura *et al.*, 2000). Various other metabolites have also been discovered in culture broths, including verticillin, glisoprenin (Joshi *et al.*, 1999) and polyketide antibiotics (Kohno *et al.*, 1999). Glisoprenins inhibit the formation of appressoria by the phytopathogenic fungus *Magnaporthe grisea* on inductive surfaces (Thines *et al.*, 1998).

Toxins of Mycoherbicides

Some microbes (phytopathogenic and non-phytopathogenic bacteria and fungi) and secondary microbial products (phytotoxins) exhibit potential as biological weed control agents (see Chapter 6; Lax *et al.*, 1988). Collectively these organisms and natural products are called bioherbicides. Fungi with potential bioherbicidal activity are termed mycoherbicides. These organisms are generally applied to weeds in a similar manner to synthetic herbicides, i.e. spray applications to weed surfaces. This use of pathogens for weed control differs from that of 'classical biological control', where organisms are released and allowed to spread to host plants via natural dispersion in the environment. Mycoherbicidal organisms are usually applied directly to host weeds or narcotic plants (Chapters 1 and 6) at relatively high fungal spore and/or propagule concentrations. Since many of the fungi evaluated as mycoherbicides have little or no potential to propagate to epidemic levels during the next season following the first application, re-application of the same organism is required for each growing season.

The initiative for using phytopathogens and phytotoxins and other microorganisms as biological weed control agents (bioherbicides) began about three decades ago. Since then, numerous fungi have been screened for phytotoxic potential and several dozen fungi have been more closely examined as mycoherbicides. These concepts and organisms and their phytotoxins have been reviewed in books (Hoagland, 1990; TeBeest, 1991). It is apparent, from the vast amount of research currently being conducted in this area, that many more fungal and bacterial weed pathogens and phytotoxins from pathogenic and non-pathogenic microorganisms will be discovered that possess useful bioherbicidal activity. Although most bioherbicidal products have been targeted at agronomic weeds, they may also be useful for weed control in non-agronomic areas (recreational areas, forests, rights of way, lawns, gardens, etc.), where synthetic herbicides are either not registered or their use is cost-prohibitive.

Interest in these organisms (either directly or as sources of naturally occurring phytotoxins) has also increased recently, due to the search for less persistent, more selective and more environmentally benign herbicides. Pathogens also have potential for use in integrated weed management programmes, if the organisms can tolerate various agricultural chemicals. Genetic engineering and microbial strain selection can be used to increase pathogen virulence, alter host range and enhance interactions with other chemical regulators or synergists. Although many fungi have been discovered that have potential as mycoherbicides for the control of many weed species (see Table 12.4), there has been little research aimed at risk assessment of these microbes or their chemical products with regard to living targets other than weeds and soil and water quality. Futhermore, to date, only a very few organisms possessing mycoherbicidal activity have been patented for use as weed control agents. Some plant pathogens that have been or are being developed for commercial use include: *Phytophthora palmivora* (DeVine) for the control of strangler vine in citrus groves, *Colletotrichum gloeosporioides* f. sp. *aeschynomene* (Collego) for the control of northern joint-vetch in rice and soybean fields (Templeton and Heiny, 1989), *C. gloeosporioides* f. sp. *malvae* (BioMal), a foliar pathogen for round-leaved mallow (*Malva pusilla*) control and *Alternaria cassiae* for control of sicklepod (*Cassia obtusifolia*) (Hoagland, 2000).

Overall, a few major points are evident. Most of these organisms with mycoherbicidal potential have not been exhaustively examined for their phytotoxic mode of action, i.e. digestive enzymes, identification of specific phytotoxins, elucidation of interactions of multiple phytotoxins, etc. Furthermore, the toxicity of specific phytotoxins

Table 12.4. Selected examples of mycoherbicides for economically important terrestrial and aquatic weeds. (Adapted from Charudattan, 1990.)

Weed	Pathogen	Reference
Velvetleaf (*Abutilon theophrasti*)	*Colletotrichum coccodes*	Hodgson *et al.*, 1988
	Fusarium lateritium	Walker, 1981
Giant ragweed (*Ambrosia trifida*)	*F. lateritium*	Anon., 1989
Wild oat (*Avena fatua*)	*Septoria tritici* Desm. f. sp. *avenae*	Madariaga and Scharen, 1985
Common lambsquarters	*Ascochyta caulina* Sacc.	Scheepens and van Zon, 1982
(*Chenopodium album*)	*Cercospora chenopodii* Fres.	Scheepens and van Zon, 1982
	Cercospora dubia (Riess) Wint.	Scheepens and van Zon, 1982
Field bindweed	*Phomopsis convolvulus*	Anon., 1989,
(*Convolvulus arvensis)*		Vogelgsang *et al.*, 1998
Yellow nutsedge	*Cercospora caricis* Oud.	Anon., 1989
(*Cyperus esculentus)*		
Purple nutsedge (*C. rotundus*)	*Phyllachora cyperi* Rehm.	Anon., 1989
Large crabgrass	*Pyricularia grisea* (Cke.) Sacc.	Anon., 1989
(*Digitaria sanguinalis*)		
Barnyard-grass	*Cochliobolus lunatus* Nelson &	Scheepens, 1987
(*Echinochloa crus-galli*)	Haasis	
Water hyacinth	*Alternaria eichhorniae* Nag Raj	Shabana, 1987
(*Eichhornia crassipes*)	& Ponnappa	
Goosegrass (*Eleusine indica*)	*Bipolaris setariae* (Saw.)	Anon., 1989
	Shoemaker	
Common purslane	*Dichotomophthora indica* Rao	Baudoin, 1986
(*Portulaca oleracea*)	*Dichotomophthora portulacae*	Klisiewicz, 1985
	Mehrlich & Fitzpatrick ex	
	M.B. Ellis	
Itchgrass (*Rottboellia*	*Curvularia* sp.	Evans and Ellison, 1988
cochinchinensis)	*Phaeoseptoria* sp.	Evans and Ellison, 1988
Johnsongrass	*Sphacelotheca holci* Jack.	Massion and Lindow, 1986
(*Sorghum halepense*)	*Bipolaris halepense* Chiang,	Chiang *et al.*, 1989
	Leonard & Van Dyke	Winder and van Dyke, 1989
	Bipolaris sorghicola (Lefebvre	Anon., 1989
	& Sherwin) Alcorn	Anon., 1989
	Colletotrichum graminicola	
	(Ces.) G.W. Wils.	
	Gloeocercospora sorghi D. Hain	
	& Edg.	
Sicklepod (*Cassia obtusifolia*)	*Pseudocercospora nigricans*	Hofmeister and Charudattan, 1987

to non-target organisms has only rarely been investigated and most of these organisms have not been examined for other potentially harmful effects or the production of harmful non-phytotoxic chemicals. Mass production of mycoherbicides and their distribution and use on a wide agricultural scale could pose a significant exposure problem to humans, animals and the environment. Whether this also results in elevated amounts of secondary metabolites has still to be determined. Risk assessment will increase as more mycoherbicides are discovered, evaluated, patented for use and used to control various weeds.

Phytopathogenic fungi

Plant-pathogenic fungi produce a range of phytotoxins that interfere with plant metabolism and produce results ranging from whole-plant death to subtle effects on gene expression. Fungal phytotoxins must interact with a plant component (e.g. enzyme or membrane receptor), but, if that component is missing or altered, the compound will have no effect. Thus, fungal phytotoxins and/or their targets are important determinants of pathogen host range. Indeed, some phytotoxins are non-specific while others are host-specific. Non-specific phytotoxins produced by pathogenic fungi have not been critically evaluated for possible roles in plant disease; however, several of the host-specific phytotoxins have undergone extensive analysis, such as the maculosins (Bobylev *et al.*, 1996; Table 12.5). Some of these compounds will be discussed below, but, ironically, very little information is available on the production and properties of phytotoxins from commercialized mycoherbicides.

Host-specific phytotoxins

Chemically, the host-specific toxins are a diverse group of low-molecular-weight secondary metabolites. For example, HC- and AM-toxins are cyclic tetrapeptides of M_r 436 and 445, respectively. HS-toxin is a sesquiterpene galactofuranoside (M_r 884), T-toxin is a linear polyketol (M_r 768), AK-toxin is an ester of epoxy-decatrienoic acid (M_r 413), and AAL-toxin is a dimethylheptadecapentol ester of propane tricarboxylic acid (M_r 508). Several of these phytotoxins are found in culture filtrates as families of isomers or congeners.

Table 12.5. Examples of host-specific toxins produced by phytopathogenic fungi. (Adapted from Yoder and Turgeon, 1985; Hoagland, 1990.)

Fungus[a]	Toxin	Host and some properties of the toxin
[b]*Cochliobolus victoriae*	HV	Specific for oats. Pathogenicity determinant. Induces tissue leakage
Cochliobolus carbonum	HC	Specific for maize. Targets include plasma membrane
Cochliobolus nicotianae	Colletotrichin	Toxic to tobacco
Cochliobolus heterostrophus	T	Toxic to maize. Mitochondria of susceptible maize cultivars become leaky
Alternaria alternata f. sp. *lycopersici*	AAL	Disrupts pyrimidine synthesis in tomato by inhibiting aspartate carbamoyl transferase. Structural similarity to fumonisin B_1 suggests possible inhibition of ceramide synthase
A. alternata f. sp. *fragariae*	AF	Toxic to strawberry
A. alternata f. sp. *mali*	AM	Induces necrotic spots on leaves and apple fruit
A. alternata f. sp. *kikuchiana*	AK	Causes necrosis of Japanese pear
A. alternata f. sp. *terreus*	Acetylaranotin	Inhibits plant growth
A. alternata f. sp. *citri*	ACTG	Toxic to mandarine oranges

[a]Each phytotoxin affects only susceptible genotypes of the plant and is an important pathogenicity determinant.
[b]*Cochliobolus* designates the teleomorph of *Helminthosporium, Biolaris* and *Drechslera*.

Host-specific phytotoxins cause the visible and physiological changes that are characterisitic of infected plants. Gross physiological effects include changes in respiration, cell permeability, protein synthesis and CO_2 fixation. Most of the changes appear to be secondary, relative to the primary or initial biochemical lesions, as indicated by the single-gene control of sensitivity and by experiments with isolated organelles (Scheffer and Livingston, 1984). Host-specific phytotoxins are, for the most part, those that significantly affect only one plant species, the species to which the producing microorganism is a pathogen. Some host-specific phytotoxins are only toxic to certain cultivars of the host-plant species. For example, tomato plants homozygous for the *Asc* locus are resistant (*Asc1*^{1}/*Asc1*^{1} and *Asc1*^{2}/*Asc1*^{2}) or sensitive (*asc*/*asc*) to the fungal pathogen *Alternaria alternata* f. sp. *lycopersici* and its host-specific AAL-toxins (Abbas *et al.*, 1995b). Heterozygous plants are resistant to the fungus, but resistant (*Asc1*^{2}/*asc*) or sensitive (*Asc1*^{1}/*asc*) to high toxin concentrations. In susceptible plant tissues, AAL toxins and the structurally related fumonisins of *F. moniliforme* lead to the accumulation of free sphingoid bases by inhibition of ceramide synthase, a key enzyme in *de novo* sphingolipid biosynthesis, and cause apoptosis-like symptoms. Recently, free sphingoid bases and their metabolites have been shown to function as second messengers that regulate cell death (Merrill *et al.*, 1997).

All known host-specific phytotoxins are derived from fungal pathogens. Fewer than 20 host-specific phytotoxins that affect crops have been reported (Scheffer and Livingston, 1984). These phytotoxins have been reported to possess the same host range as the pathogens producing them. Only one of the host-specific phytotoxins affecting crops that has been tested on weed species is AAL-toxin. Other studies have demonstrated that it is not a host-specific phytotoxin and that it is highly phytotoxic to a wide range of weed species (Abbas *et al.*, 1992b, 1993b; Tanaka *et al.*, 1993). Destruxin B (discussed above) was reported to be host-specific (Bains and Tewari, 1987); however, it was later demonstrated to be non-host-specific (Buchwalt and Green, 1992). Colletotrichin, a product of several *Colletotrichum* species, is toxic to cucumber, tobacco and solanaceous weed species, including nightshades and horsenettle (*Solanum* spp.) (Gobhara *et al.*, 1978; Duke *et al.*, 1992). The first ultrastructural symptom of phytotoxicity is the loss of structural integrity of the plasma membrane. Lipid peroxidation is associated with the membrane damage; however, radical-quenching agents do not protect the plant cells from the toxin. Colletotrichin reduces or prevents the phytotoxicity of the synthetic herbicide acifluorfen (Gobhara *et al.*, 1978). Acifluorfen requires the activity of a plasma membrane-associated redox enzyme for its activity (Jacobs *et al.*, 1991). Thus, colletotrichin may interfere with the function of this plasma membrane and other membranes.

Non-specific phytotoxins

These compounds are produced by both specialist and generalist plant pathogens. For example, *A. alternata* f. sp *citri*, causal agent of brown spot disease of tangerines and mandarins, simultaneously produces host-specific toxins and non-specific phytotoxins (e.g. tentoxin and tenuazonic acid) in culture broth (Kono *et al.*, 1986). In this section most attention will focus on toxins of *Fusarium* because of the different niches this fungus occupies, principally as a saprophyte, plant pathogen, insect pathogen and fungal BCA (Teetor-Barsch and Roberts, 1983; Desjardins, 1992). There has been interest in exploiting different *Fusarium* species for use as mycoparasites, mycoinsecticides

and mycoherbicides. One benign BCA strain, Fusaclean™, has been commercialized by NPP (France) to promote plant growth by displacing plant-pathogenic strains of *Fusarium* (see Chapter 1). Other strains are being developed for the control of gorse (Morin *et al.*, 2000) and narcotics (see http://mycoherbicide.net).

The phytopathogenic species of *Fusarium* produce a wide variety of chemical contaminants of plant tissues including trichothecenes (T2-toxin and others), fumonisins, naphthazarins, fusaric acid and related pyridine derivatives (Desjardins, 1992). The phytotoxicity of *Fusarium* spp. and some of their natural products has been reviewed (Hoagland and Abbas, 1995). The exact role of some of these compounds remains unclear, but some are insecticidal (Grove and Pople, 1980), suggesting that they may offer some protection to the plants against phytophagous insects (Miller *et al.*, 1985; Strongman *et al.*, 1988).

Trichothecenes

These are produced by several common moulds, including species in the genera *Acremonium* (*Cephalosporium*), *Cylindrocarpon, Dendrodochium, Myrothecium, Trichoderma, Trichothecium* and, most numerously, *Fusarium*. Trichothecenes are composed of a tetracyclic sesquiterpene skeleton containing a six-membered oxane ring, a stable 12–13-epoxide group and a 9,10-olefinic bond. They have been classified into four groups. *Fusarium* spp. contain several well-known trichothecenes, including two members of group A with high mammalian toxicity, diacetoxyscirpenol (DAS) and T2-toxin, and toxins in group B, including deoxynivalenol (DON) and nivalenol. DON is the most common and possibly least toxic of these. Trichothecenes cause diarrhoea, severe haemorhages and immunotoxic effects and are also strong inhibitors of protein synthesis in mammalian cells. However, DON received its common name, vomitoxin, from the vomiting that generally accompanies trichothecene poisoning (D'Mello, 1997). DON is not mutagenic, but has clastogenic neurotoxic and immunotoxic effects (van Egmond and Speijers, 1990; Eriksen and Alexander, 1998). One typical feature of the toxicity of T2 is that it causes adverse cardiovascular effects in some experimental animal species, including pigs and monkeys. However, insufficient data exist on whether it is a carcinogen. Immunotoxic effects have also been reported in humans (Eriksen and Alexander, 1998).

Lambs will consume a wheat diet containing DON at 15.6 mg kg^{-1} of body weight for 28 days. DON does not appear to alter their feed consumption, weight gain or feed efficiency. Oral administration of DON showed that it was rapidly and primarily excreted in urine, essentially unchanged (95%). Incubation of DON with ruminal microorganisms *in vitro* for 48 h resulted in partial conversion to de-epoxy DON. These results indicate that the impact of DON on ruminants is lower than initially suspected. DON caused no organ damage to animals. Extremely low amounts of DON (< 4 ng ml^{-1}) were transmitted to milk after a single oral dose of 920 mg to a dairy cow (Diekman and Green, 1992). For the USA, the Food and Drug Admininstration (FDA) have suggested guidelines as to tolerable levels of DON in wheat for milling and human/animal consumption (Wood, 1992).

Macrocyclic trichothecenes also possess a wide range of phytotoxic specificity. Verrucarin A and J and trichoverrin B show phytotoxicity in wheat coleoptile bioassays at concentrations of 10^{-7} M (Cutler and Jarvis, 1985). Roridin A exhibited phytotoxicity on tobacco, maize and bean seedlings (Cutler and Jarvis, 1985). Simple

trichothecenes, such as neosoliniol monoacetate produced by *Fusarium tricinctum* showed phytotoxicity at 10^{-6} M, and diacetoxy-scripenol severely injured pea seedlings at 2.7×10^{-5} M, but was not toxic to wheat (Brain *et al.*, 1961).

Zearalenone (ZEN)

Zearalenone and zearalenol are both oestrogenic resorcylic acid lactones produced by *Fusarium* spp. (Diekman and Green, 1992). Despite their structural dissimilarity to the steroidal oestrogens, ZEN and several of its derivatives possess oestrogenic activity. ZEN undergoes a folding such that hydroxyl or potential hydroxyl groups become appropriately orientated to facilitate binding to tissue receptors that normally bind oestrogens. Similar binding affinities for ZEN have been determined for the oestrogen receptor in uteruses of sheep and calves (Diekman and Green, 1992).

Poultry show little reaction to ZEN ingestion. However, pigs are strongly affected, with symptoms in prepubertal gilts including enlarged mammae, swelling of the uterus and vulva and atrophy of the ovaries. In severe cases, prolapse of the vulva and rectum may occur. Boars exhibit enlarged mammae and atrophied testes (Flannigan, 1991). *In vivo* studies have revealed that ZEN was rapidly metabolized in animals and humans and eliminated mainly as water-soluble glucuronides. Free and conjugated forms of ZEN have been found in the milk of lactating cows under experimental conditions. That high oral doses of the toxin are required to elicit such a response indicates that consumption of contaminated feed by dairy cows would not result in a health hazard to humans (Wood, 1992).

Zearalenone has been shown to be genotoxic and carcinogenic in mice but not rats, affects reproduction in low doses and produces hormonal effects in mice and rats. Hormonal effects were produced in monkeys by α-zearalanol a compound closely related to α-zearalenol, which is a major metabolite of zearalenone (van Egmond and Speijers, 1990; Eriksen and Alexander, 1998).

Enniatins

Enniatins are cyclohexadepsipeptides produced by various strains of *Fusarium*. They consist of three residues of D-2–hydroxy-isovaleric acid (D-HIV) and an *N*-methyl-L-branched-chain amino acid, which are arranged in an alternated fashion. They are synthesized by enniatin synthetase, a 350 kDa multifunctional enzyme. Enniatins exhibit antibiotic properties towards a number of bacteria and fungi due to their ionophoretic properties. Furthermore, they are reported to act as inhibitors of cholesterol acyl transferase in mammalians and act as phytotoxins during infection of plants by *Fusaria*. Enniatins A/A1 extracted from *Fusarium avenaceum* also appear to have insecticidal properties (Strongman *et al.*, 1988).

Fumonisins

Fumonisin B_1 is both a toxin and a phytotoxin produced by both saprophytic *Fusarium* species (Abbas *et al.*, 1991, 1992b) and an *A. alternata* strain known to cause stem canker on certain tomato varieties (Chen *et al.*, 1992). Fumonisin B_1 (FB_1), first isolated from

F. moniliforme MRC 826 (Bezuidenhout *et al.*, 1988), is a hydroxylated, long-chain alkylamine with two tricarboxylic acid moieties attached. It is highly phytotoxic to most weed and crop species tested (Abbas *et al.*, 1992a, b; van Asch *et al.*, 1992; Tanaka *et al.*, 1993). Ultrastructural and physiological studies of jimson-weed leaves with FB_1 revealed that it caused light-dependent plasma membrane and tonoplast disruption through an unknown mechanism (Abbas *et al.*, 1992a; Tanaka *et al.*, 1993). Although the fumonisins and AAL-toxin have similar activity, AAL-toxin is generally more potent than FB_1 (Tanaka *et al.*, 1993). Aminoalcohols, hydrolysis products of the fumonisins, have very low phytotoxicity. FB_1 is also a potent mammalian toxin (Shier *et al.*, 1991; Abbas *et al.*, 1993a), and in animal systems the mechanism of action is apparently altered sphingolipid synthesis (Wang *et al.*, 1991). The mode of action of FB_1 in plants and mammals is inhibition of ceramide synthase (Hoagland and Abbas, 1995). Sphingolipids themselves, at relatively high concentrations, can cause phytotoxicity symptoms similar to those of fumonisins and AAL-toxin (Vesonder *et al.*, 1992a, b; Tanaka *et al.*, 1993). For example, sphingosine and phytosphingosine required micromolar concentrations to cause effects similar to those of nanomolar levels of FB_1 and AAL-toxin.

Other phytotoxins from Fusarium *spp.*

Fusaric acid is produced by both the virulent plant pathogen *F. oxysporum*, the cause of wilt in several species, and by non-pathogenic *Fusarium* species that grow saprophytically on maize kernels (Abbas *et al.*, 1989). Recently, fusaric acid has been demonstrated to be herbicidal against several weed species, including jimson-weed (Abbas *et al.*, 1991, 1995a) and duckweed (Vesonder *et al.*, 1992a).

Moniliformin can be obtained from isolates of saprophytic *Fusarium* species, as well as pathogenic isolates of *F. oxysporum* (Abbas *et al.*, 1989). Moniliformin causes growth inhibition, necrosis and chlorosis in many weed species. (Cole *et al.*, 1974; Abbas *et al.*, 1991, 1995a; Vesonder *et al.*, 1992a). However, it lacks selectivity and is highly toxic to mammals. This compound was used as a chemical structure template in the design and synthesis of many compounds for herbicide discovery (Fischer and Bellus, 1983).

Other fungal phytotoxins

AAL-toxin is a hydroxylated long-chain alkylamine containing one tricarboxylic acid moiety. It is a close analogue of the fumonisins and is produced by *A. alternata* f. sp. *lycopersici*. In susceptible varieties of tomatoes, it causes rapid wilting and necrosis (Abbas *et al.*, 1995b). Its physiological effects appear to be identical to those of the fumonisins, and it is thus suspect for mammalian toxicity.

Cercosporin is a red compound produced by several species of *Cercospora* (Nasini *et al.*, 1977). Isocercosporin, a closely related compound, is produced by *Scolecotrichum graminis*, the agent of leaf streak in orchardgrass (*Dactylis glomerata* L.) (Tabuchi *et al.*, 1991). These are photoactivated, active-oxygen-producing toxins (Hartman *et al.*, 1988). Similar compounds, such as hypericin (a plant product) (Knox and Dodge, 1985), have been suggested as herbicides; however, such compounds may have high risk factors since they are toxic to all life forms that exist in sunlight and an oxygen atmosphere.

As mentioned earlier, destruxins are produced by *A. brassicae* and a few other plant pathogens (Buchwaldt and Jenson, 1991; Buchwaldt and Green, 1992). These compounds cause necrotic and chlorotic symptoms in susceptible species (Buchwaldt and Green, 1992).

Aflatoxin

A last example of a natural product with multiple biological activities (phytotoxic and carcinogenic) to be presented is aflatoxin. Aflatoxins are a group of mycotoxins produced by certain strains of *Aspergillus flavus, Aspergillus parasiticus* and *Aspergillus nominius*. Although these fungi are ubiquitous, not all strains produce aflatoxin. Toxin titre is also dependent on environmental conditions related to fungal growth, such as moisture, temperature and nutrient availability. These mycotoxins occur naturally on commodities, including groundnuts and groundnut meal, cotton-seed meal, maize, dried chilli peppers, etc. There are numerous members of this mycotoxin group, with B_1 being one of the most potent mutagens and carcinogens yet discovered. Other analogues or derivatives are less toxic (Merck Index, 1996). Aflatoxin is currently recognized as an extremely toxic contaminant in various food and feed products and as a risk factor for liver cancer in humans (Wogan, 1992).

Biological testing of aflatoxins on plants has shown that these compounds also possess phytotoxic activity. In 1965, AB_1 was shown to have phytotoxic effects on plant tissues (Lilley, 1965; Schoental and White, 1965). Since then, many other reports have demonstrated the phytotoxicity of these compounds in a variety of plant and plant-tissue bioassays (McLean, 1994). These compounds are absorbed by plants, translocated to various plant parts and distributed within specific cellular compartments (McLean, 1993; McLean *et al.*, 1994). Studies on the metabolism of these compounds in plants has been controversial, i.e. some researchers report a lack of metabolism by plants (Mertz *et al.*, 1980; Reiss, 1984), while others have detected metabolic products in aflatoxin-treated plants (Howes *et al.*, 1991; McLean, 1993). As is the case with many other chemicals, it is highly probable that the uptake, translocation and metabolism of aflatoxins are dependent upon the test-plant species.

Toxins and Risk Assessment – Conclusions

Most fungal BCAs are widespread soil inhabitants. We believe that epizootics/epidemics induced naturally or artificially through inundative introductions of BCAs do not pose a risk to human or animal health. This is partly because the BCAs are subject to a wide range of regulatory (climatic and biotic) controls, so inoculum levels generally subside over time (see Chapters 3 and 4). As far as we are aware, there are no documented accounts of toxin levels rising as a result of artificially induced or natural epizootics/epidemics, nor are there any reports of BCA metabolites entering the food-chain. These observations should not lead to complacency, but to rationalization of risk assessment (see Chapters 13 and 14). For example, it should not be assumed that all strains produce similar metabolites, because recent studies clearly show inter- and intraspecific variation in quantity and types of toxin produced (Amiri-Besheli *et al.*, 2000; Bandani *et al.*, 2000). Furthermore, what is detected in liquid culture may have no link to what is produced in the target host or released into the environment.

In our opinion, both the efficacy of the fungal BCA and data on the fate of both inoculum and any major metabolites secreted are essential. The fact that some strains produce few, if any, metabolites may encourage some companies to select these organisms, except when these compounds play an important role in pathogenesis/antagonism. More information also needs to be generated on the fate of bioactive metabolites in the environment. We know that gliotoxins are inactivated by oxidation, but is this true for other metabolites?

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References

Abbas, H.K., Mirocha, C.J., Kommedahl, T., Vesonder, R.F. and Golinski, P. (1989) Production of trichothecene and non-trichothecene mycotoxins by *Fusarium* species isolated from maize in Minnesota. *Mycopathologia* 108, 55–58.

Abbas, H.K., Boyette, C.D., Hoagland, R.E. and Vesonder, R.F. (1991) Bioherbicidal activity of *Fusarium moniliforme* (Sheldon) and its phytotoxin fumonisin. *Weed Science* 39, 673–677.

Abbas, H.K., Paul, R.N., Boyette, C.D., Duke, S.O. and Vesonder, R.F. (1992a) Physiological and ultrastructural effects of fumonisin on jimsonweed leaves. *Canadian Journal of Botany* 70, 1824–1833.

Abbas, H.K., Vesonder, R.F., Boyette, C.D., Hoagland, R.E. and Krick, T. (1992b) Production of fumonisins by *Fusarium moniliforme* cultures isolated from jimsonweed in Mississippi. *Journal of Phytopathology* 136, 199–203.

Abbas, H.K., Gelderbloom, W.C.A., Cawood, M.F. and Shier, W.T. (1993a) Biological activities of fumonisins, mycotoxins from *Fusarium moniliforme*, in jimsonweed (*Datura stramonium* L.) and mammalian cell cultures. *Toxicon* 31, 345–353.

Abbas, H.K., Vesonder, R.F., Boyette, C.D. and Petterson, S.W. (1993b) Phytotoxicity of AAL-toxin and other compounds produced by *Alternaria alternata* to jimsonweed (*Datura stramonium*). *Canadian Journal of Botany* 71, 155–160.

Abbas, H.K., Boyette, C.D. and Hoagland, R.E. (1995a) Phytotoxicity of *Fusarium* isolates and their phytotoxins fumonisin, fusaric acid and moniliformin on jimsonweed. *Phytoprotection* 76, 17–25.

Abbas, H.K., Tanaka, T. and Duke, S.O. (1995b) Pathogenicity and/or phytotoxicity of *Alternaria alternata* and its AAL-toxin, *Fusarium moniliforme* and its fumonisin B1 on tomato varieties. *Journal of Phytopathology* 143, 329–334.

Abendstein, D. and Strasser, H. (2000) Considerations on toxic metabolites produced by *Beauveria brongniartii*. In: Keller, S. (ed.) *Integrated Control of Soil Pests*. IOBC Bulletin, Avignon, France, pp. 789–796.

Al-Aïdroos, K. and Roberts, D.W. (1978) Mutants of *Metarhizium anisopliae* with increased virulence toward mosquito larvae. *Canadian Journal of Genetics and Cytology* 20, 211–219.

Aleo, M.D., Wyatt, R.D. and Schnellmann, R.G. (1991) Mitochondrial dysfunction is an early event in ochratoxin A but not oosporein toxicity to rat renal proximal tubules. *Toxicology and Applied Pharmacology* 107, 73–80.

Amiri, B., Ibrahim, L. and Butt, T.M. (1999) Antifeedant properties of destruxins and their use with the entomogenous fungus *Metarhizium anisopliae* for improved control of crucifer pests. *Biocontrol Science and Technology* 9, 487–498.

Amiri-Besheli, B., Khambay, B., Cameron, S., Deadman, M. and Butt, T.M. (2000) Inter- and intra-specific variation in destruxin production by the insect pathogenic *Metarhizium*, and its significance to pathogenesis. *Mycological Research* 104, 447–452.

Anon. (1989) Discovery and development of plant pathogens for biological control of weeds. In: Reg. Res. Proj. SRCS 8801 (S-136), Plant Pathology Department, University of Florida, Gainesville, p. 44.

Anon. (1990) Pesticide Fact Sheet No. 217: '*Gliocladium virens*' *GL-21*. Environmental Protection Agency, Washington, DC. Office of Pesticide Programs Report No. EPA/540/FS-91/118.

van Asch, M.A.J., Rijkenberg, F.H.J. and Coutinho, T.A. (1992) Phytotoxicity of fumonisin B1, moniliformin, and T-2 toxin to corn callus cultures. *Phytopathology* 82, 1330–1332.

Ayer, W.A. and Pena-Rodriguez, M. (1987) Metabolites products by *Alternaria brassicae*, the black spot pathogen of canola. Part 1, the phythotoxic components. *Journal of Natural Products* 50, 400–407.

Bains, P.S. and Tewari, J.P. (1987) Purification, chemical characterisation and host-specificity of the toxin produced by *Alternaria brassicae*. *Physiological Molecular Plant Pathology* 30, 259–271.

Bandani, A.R. and Butt, T.M. (1999) Insecticidal, antifeedant, growth inhibitory activities of efrapeptins, metabolites of the entomogenous fungus *Tolypocladium*. *Biocontrol Science and Technology* 9, 499–506.

Bandani, A.R., Khambay, B.P.S., Faull, J., Newton, R., Deadman, M. and Butt, T.M. (2000) Production of efrapeptins by *Tolypocladium* species and evaluation of their insecticidal and antimicrobial properties. *Mycological Research* 104, 537–544.

Bandani, A.R., Amiri, B., Butt, T.M. and Gordon-Weekes, R. (2001) Effects of efrapeptin and destruxin, metabolites of entomogenous fungi, on the hydrolytic activity of a vacuolar type ATPase from the brush border membrane vesicles of *Galleria mellonella* midgut and on plant membrane bound hydrolytic enzymes. *Biochemica et Biophysica Acta* 1510, 367–377.

Baudoin, A.B.A.M. (1986) First report on *Dichotomophthora indica* on common purslane in Virginia. *Plant Disease* 70, 352.

Bezuidenhout, S.C., Gelderblom, W.C.A., Gorst-Allman, C.P., Horak, R.M., Marasas, W.E.O., Spiteller, G. and Vleggaar, R. (1988) Structure elucidation of the fumonisins, mycotoxins from *Fusarium moniliforme*. *Journal of the Chemical Society. Chemical Communications* 11, 743–745.

Bidochka, M.J. and Khachatourians, G.G. (1991) The implication of metabolic acids produced by *Beauveria bassiana* in pathogenesis of the migratory grasshopper, *Melanoplus sanguinipes*. *Journal of Insect Pathology* 58, 106–117.

Bobylev, M.M., Bobyleva, L.I. and Strobel, G.A. (1996) Synthesis and bioactivity of analogs of maculosin, a host-specific phytotoxin produced by *Alternaria alternata* on spotted knapweed (*Centaurea maculosa*). *Journal of Agricultural Food Chemistry* 44, 3960–3964.

Boucias, D.G., Farmerie, W.G. and Pendland, J.C. (1998) Cloning and sequencing of cDNA of the insecticidal toxin Hirsutellin A. *Journal Invertebrate Pathology* 72, 258–261.

Bradfisch, G.A. and Harmer, S.L. (1990) Omega conotoxin GVIIA and nifedipine inhibit the depolarizing action of the fungal metabolite destruxin B on muscle from the tobacco budworm *Heliothis virescens*. *Toxicon* 28, 1249–1254.

Brain, B., Dawkins, A.W., Grove, J.F., Henning, H.G., Lowe, D. and Norris, G.L.F. (1961) Phytotoxic compounds produced by *Fusarium equeseti*. *Journal of Experimental Botany* 12, 1–7.

Brewer, D., Maass, W.S.G. and Taylor, A. (1977) The effect on fungal growth of some 2,5-dihydroxy-1,4–benzoquinones. *Canadian Journal of Microbiology* 23, 845–851.

Brewer, D., Jen, W.C., Jones, G.A. and Taylor, A. (1984) The antibacterial activity of some naturally occurring 2,5-dihydroxy-1,4-benzoquinones. *Canadian Journal of Microbiology* 30, 1068–1072.

Brewer, D., Mason, F.G. and Taylor, A. (1987) The production of alamethicins by *Trichoderma* spp. *Canadian Journal of Microbiology* 33, 619–625.

Brousseau, C., Charpentier, G. and Belloncik, (1996) Susceptibility of spruce budworm, *Choristoneura fumiferana* Clemens, to destruxins, cyclodepsipeptidic mycotoxins of *Metarhizium anisopliae*. *Journal of Invertebrate Pathology* 68, 180–182.

Brown, T.P., Fletcher, O.J., Osuna, O. and Wyatt, R.D. (1987) Microscopic and ultrastructural renal pathology of oosporein-induced toxicosis in broiler chicks. *Avian Diseases* 31, 868–877.

Bruckner, H. and Graf, H. (1983) Paracelsin, a peptide antibiotic containing alpha-aminoisobutyric acid, isolated from *Trichoderma reesei* Simmons. Part A. *Experientia* 39, 528–530.

Buchwaldt, L. and Green, H. (1992) Phytotoxicity of destruxin B and its possible role in the pathogenesis of *Alternaria brassicae. Plant Pathololgy* 4, 55–63.

Buchwaldt, L. and Jensen, J.S. (1991) HPLC purification of destruxin produced by *Alternaria brassicae* in culture and leaves of *Brassica napus. Phytochemistry* 30, 2311–2316.

Carsolio, C., Benhamou, N., Haran, S., Cortès, C., Gutièrrez, A., Chet, I. and Herrera-Estrella, A. (1999) Role of *Trichoderma harzanium* endochitinase gene, ech 42, in mycoparasitism. *Applied Environmental Microbiology* 65, 929–935.

Cerenius, L., Thorn Quist, P.O., Vey, A., Johanson, M.W. and Soderhall, K. (1990) The effect of the fungal toxin destruxin E on isolated crayfish haemocytes. *Journal of Insect Physiology* 36, 785–789.

Champlin, F.R. and Grula, E.A. (1979) Noninvolvement of beauvericin in the entomopathogenicity of *Beauveria bassiana. Applied and Environmental Microbiology* 37, 1122–1125.

Charnley, A.K. (1984) Physiological aspects of destructive pathogenesis in insects by fungi: a speculative view. In: Anderson, J.M., Rayner, A.D.M. and Walton, D.W.H. (eds) *Invertebrate Microbial Interactions*. Cambridge University Press, Cambridge, pp. 229–270.

Charudattan, R. (1990) Pathogens with potential for weed control. In: Hoagland, R.E. (ed.) *Microbes and Microbial Products as Herbicides.* American Chemical Society Symposium Series No. 439, ACS Books, Washington, DC, pp. 132–154.

Chen, H.C., Chou, C.K., Sun, C.M. and Yeh, S.F. (1997) Suppressive effect of destruxin B on hepatitis B virus surface antigen gene expression in human hepatoma cells. *Antiviral Research* 34, 137–144.

Chen, J., Miroscha, C.J., Xie, W., Hogge, L. and Olson, D. (1992) Production of the mycotoxin fumonisin B1 by *Alternaria alternata* f. sp. *lycopersici*. *Environmental Microbiology* 58, 3928–3931.

Chiang, M.Y., Leonard, K.J. and van Dyke, C.G. (1989) *Bipolaris halepense*: a new species from *Sorghum halepense* (Johnsongrass). *Mycologia* 81, 532–538.

Claydon, N. (1978) Insecticidal secondary metabolites from the entomogenous fungi: *Entomophthora virulenta. Journal of Invertebrate Pathology* 32, 319–324.

Cole, R.J., Kirksey, J.W., Cutler, H.G., Doupnik, B.L. and Peckham, J.C. (1973) A toxin from *Fusarium moniliforme*: effects on plants and animals. *Science* 179, 3124–1326.

Cole, R.J., Kirksey, J.W., Cutler, H.G. and Davis, E.E. (1974) Toxic effects of oosporein from *Chaetomium trilaterale. Journal of Agricultural and Food Chemistry* 22, 517–520.

Corley, D.G., Miller-Wideman, M. and Durley, R.C. (1994) Isolation and structure of harzianum A: a new trichothecene from *Trichoderma harzianum*. *Journal of Natural Products* 57, 422–425.

Cutler, H.G. and Jarvis, B.B. (1985) Preliminary observations on the effects of macrocylic trichothecens on plant growth. *Environmental and Experimental Botany* 25, 115–128.

Debeaupuis, J.P and Lafont, P. (1985) Effet de quelques mycotoxines et substances génotoxiques de synthèse sur le développement de l'embryon et de la larve de *Brachydanio rerio. Comptes Rendus de l'Académie des Sciences, Série 3 (Paris)* 300, 167–170.

Desjardins, A.E. (1992) Genetic approaches to the chemical ecology of phytopathogenic *Fusarium* species. In: Bhatnagar, D., Lillehoj, E.B. and Arora, D. (eds) *Handbook of Applied Mycology*, Vol.5, *Mycotoxins in Ecological Systems.* Marcel Dekker, New York, pp. 333–357.

Diekman, M.A. and Green, M.L. (1992) Mycotoxins and reproduction in domestic livestock. *Journal of Animal Science* 70, 1615–1627.

Di Paola, R., Nenna, S., Fornelli, F., Moretti, A., Logrieco, A., Caiaffa, M.F., Bottalico, A., Tursi, A. and Macchia, L. (1994) Cytotoxicity of beauvericin on human B-lymphocyte cell lines. *Allergy and Clinical Immunology News* 2, p. 256.

D'Mello, F.J.P. (ed.) (1997) *Handbook of Plant and Fungal Toxicants.* CRC Press, Boca Raton, Florida.

Duke, S.O., Gobhara, M., Paul, R.N. and Duke, M.V. (1992) Colletotrichin causes rapid membrane damage to plants. *Journal of Phytopathology* 134, 289–305.

Dumas, C., Robert, P., Pais, M., Vey, A. and Quiot, J.-M. (1994) Insecticidal and cytotoxic effects of natural and hemisynthetic destruxins. *Comparative Biochemistry and Physiology* 108C, 195–203.

Dumas, C., Matha, V., Quiot, J.-M. and Vey, A. (1996a) Effect of destruxins, cyclic depsipeptide mycotoxins, on calcium balance and phosphorylation of intracellular proteins in lepidopteran cell lines. *Comparative Biochemistry and Physiology* 114C, 213–219.

Dumas, C., Ravallec, M., Matha, V. and Vey, A. (1996b) Comparative study of the cytological aspects of the mode of action of destruxins and other peptidic fungal metabolites on target epithelial cells. *Journal of Invertebrate Pathology* 67, 137–146.

Duval, D., Riddell, F.G., Rebuffat, S., Platzer, N. and Bodo, B. (1998) Ionophoric activity of the antibiotic peptaibol trichorzin PA VI: a 23Na- and 35Cl-NMR study. *Biochimica et Biophysica Acta* 1372, 370–378.

van Egmond, H.P. and Speijers, G.J.A. (1990) Food contaminants, naturally occurring toxicants in foodstuffs. 1. Mycotoxins. *Food Laboratory News*, 20, 38–45.

El Hajji, M., Rebuffat, S., Lecommandeur, D. and Dodo, B. (1987) Isolation and sequence determination of trichorzianines A antifungal peptides from *Trichoderma harzianum*. *International Journal of Peptide and Protein Research*. 29, 207–215.

Engstrom, G., Delance, J., Richard, A.L. and Baetz, J. (1975) Purification and characterization of roseotoxin B, a toxic cyclodepsipeptide from *Trichothecium roseum. Journal of Agriculture and Food Chemistry* 23, 244–253.

Eriksen, G.S. and Alexander, J. (1998) Fusarium *Toxins in Cereals – A Risk Assessment.* Nordic Council of Ministers, TemaNord Food 502, Copenhagen, Denmark.

Evans, H. and Ellison, C. (1988) Preliminary work on the development of a mycoherbicide to control *Rottboellia cochinchinensis*. In: DelFosse, E.S. (ed.) *Proceedings VII International Symposium on Biological Control of Weeds.* 1st Sper. Patologia Vegetale (MAF), Rome, p. 76.

Eyal, J., Mabud, A., Fischbein, K.L., Walter, J.F., Osborne, L.S. and Landa, Z. (1994) Assessment of *Beauveria bassiana* Nov. EO-1 strain, which produces a red pigment for microbial control. *Applied Biochemistry and Biotechnology* 44, 65–80.

Fargues, J. and Robert, P.-H. (1986) Potentialités insecticides des destruxines, mycotoxines produites par l'hyphomycete entomopathogéne *Metarhizium anisopliae* (Metsch.) Sorok. In: *Proceedings 4ème Congrès Protection cultures et santé en milieu tropical, Marseille, France,* pp. 357–362.

Fargues, J., Robert, P.-H. and Vey, A. (1985) Effet des destruxines A, B et E dans la pathogénèse de *Metarhizium anisopliae* chez les larves de Coléoptères Scarabeidae. *Entomophaga* 30, 353–364.

Fargues, J., Robert, P.-H., Vey, A. and Païs, M. (1986) Toxicité relative de la destruxine E pour le Lépidoptère *Galleria mellonella. Comptes Rendus de l'Académie des Sciences, Paris Série III* 303, 83–86.

Fischer, H.-P. and Bellus, D. (1983) Phytotoxicants from microorganisms and related compounds. *Pesticide Science* 14, 334–346.

Flannigan, B. (1991) Mycotoxins. In: *Toxic Substances in Crop Plants.* Royal Society of Chemists, pp. 226–257.

Fricaud, A.C., Walters, A.J., Whitehouse, D.G. and Moore, A. (1992) The role(s) of adenylate kinase and the adenylate carrier in the regulation of plant mitochondrial respiratory activity. *Biochimica et Biophysica Acta* 1099, 253–261.

Genthner, F.J., Cripe, G.M. and Crosby, D.J. (1994) Effect of *Beauveria bassiana* and its toxins on *Mysidopsis bahia* (Mysidacea). *Archives of Environmental Contamination and Toxicology* 26, 90–94.

Genthner, F.J., Chancy, C.A., Couch, J.A., Foss, S.S., Middaugh, D.P., George, S.E., Warren, M.A. and Bantle, J.A. (1998) Toxicity and pathogenicity testing of the insect pest control fungus, *Metarhizium anisopliae*. *Archives of Environmental Contamination and Toxicology* 35, 317–324.

Ghisalberti, E.L. and Rowland, C.Y. (1993) Antifungal metabolites from *Trichoderma harzianum*. *Journal of Natural Products* 56, 1799–1804.

Ghisalberti, E.L. and Sivasithamparam, K. (1991) Antifungal antibiotics produced by *Trichoderma* spp. *Soil Biology and Biochemistry* 23, 1011–1020.

Gobhara, M., Kosuge, Y., Yamaaki, S., Kimura, Y., Suzuki, A. and Tamura, S. (1978) Isolation, structures and biological activities of colletotrichins, phytotoxic substances from *Colletotrichum nicotianae. Agricultural and Biological Chemistry* 42, 1037–1043.

Godoy, G., Steadman, J.R., Dickman, M.B. and Dam, R. (1990) Use of mutants to demonstrate the role of oxalic acid in pathogenicity of *Sclerotinia sclerotiorum* on *Phaseolus vulgaris. Physiological and Molecular Plant Pathology* 37, 179–191.

Goulard, C., Hlimi, S., Rebuffat, S. and Bodo, B. (1995) Trichorzins HA and MA, antibiotic peptides from *Trichoderma harzianum*. I. Fermentation, isolation and biological properties. *Journal of Antibiotics* (*Tokyo*) 48, 1248–1253.

Graeme-Cook, K.A. and Faull, J.L. (1991) Effect of ultraviolet-induced mutants of *Trichoderma harzianum* with altered antibiotic production on selected pathogens *in vitro*. *Canadian Journal of Microbiology* 37, 659–664.

Graniti, A. (1972) The evolution of the toxin concept in plant pathology. In: Woods, R.K.S., Ballio, A. and Graniti, A. (eds) *Phytotoxins in Plant Diseases.* Academic Press, New York, pp. 1–18.

Griffin, D.H. (1994) *Fungal Physiology.* Wiley-Liss, New York.

Grove, J.F. and Pople, M. (1980) The insecticidal activity of beauvericin and the enniatin complex. *Mycopathologia* 70, 103–105.

Gupta, S., Roberts, D.W. and Renwick, J.A.A. (1989) Preparative isolation of destruxins from *Metarhizium anisopliae* by high performance liquid chromatography. *Journal of Liquid Chromatography* 12, 383–395.

Gupta, S., Krasnoff, S.B., Underwood, N.L., Renwick, J.A. and Roberts, D.W. (1991) Isolation of beauvericin as an insect toxin from *Fusarium semitectum* and *Fusarium moniliforme* var. *subglutinans*. *Mycopathologia* 115, 185–189.

Gupta, S., Montillor, C. and Hwang, Y.-S. (1995) Isolation of novel beauvericin analogues from the fungus *Beauveria bassiana. Journal of Natural Products* 58, 733–738.

Hartman, P.E., Dixon, W.J., Dahl, T.A. and Daub, M.E. (1988) Multiple modes of photodynamic action by cercosporin. *Phytochemistry and Photobiology* 44, 699–703.

Hoagland, R.E. (ed.) (1990) *Microbes and Microbial Products as Herbicides.* ACS Books, American Chemical Society, Washington, DC.

Hoagland, R.E. (2000) Plant pathogens and microbial products as agents for biological weed control. In: Tewari, J.P., Lakhanpal, T.N., Singh, J., Gupta, R. and Chamola, B.P. (eds) *Advances in Microbial Biotechnology.* APH, New Delhi, India.

Hoagland, R.E. and Abbas, H.K. (1995) Phytotoxicity of *Fusarium* spp. and their natural products. In: Greene, D.W. (ed.) *Proceedings of the Plant Growth Regulator Society of America.* PGRSA Press, La Grange, Georgia, pp. 109–114.

Hodgson, R.H., Wymore, L.A., Watson, A.K., Snyder, R.H. and Collette, A. (1988) Efficacy

of *Colletotrichum coccodes* and thidiazuron for velvetleaf (*Abutilon theophrasti*) control in soybean (*Glycine max*). *Weed Technology* 2, 473–480.

Hofmeister, F.M. and Charudattan, R. (1987) *Pseudocercospora nigricans*, a pathogen of sicklepod (*Cassia obtusifolia*) with biocontrol potential. *Plant Disease* 71, 44–46.

Howes, A.W., Dutton, M.F. and Chuturgoon, A.A. (1991) Metabolism of aflatoxin B1 by *Petroselinum crispum* (parsley). *Mycopathologia* 113, 25–29.

Huxham, I.M., Lackie, A.M. and McCorkindale, N.J. (1989) Inhibitory effect of cyclodepsipeptides, destruxins, from the fungus *Metarhizium anisopliae*, on cellular immunity in insects. *Journal of Insect Physiology* 35, 97–105.

Iida, A., Sanekata, M., Fujita, T., Tanaka, H., Enoki, A., Fuse, G., Kanai, M., Rudewicz, P.J. and Tachikawa, E. (1994) Fungal metabolites. XVI. Structure of new peptaibols, trichokindins I–VII, from the fungus *Trichoderma harzianum*. *Chemical and Pharmaceutical Bulletin* (*Tokyo*) 42, 1070–1075.

Irmscher, G., Bovermann, G., Boheim, G. and Jung, G. (1978) Trichotoxin A 40, a new membrane-exciting peptide. Part A. Isolation, characterization and conformation. *Biochimica et Biophysica Acta* 507, 470–484.

Itoh,Y., Kodama, K., Furuya, K., Takahashi, S., Haneishi, T., Takiguchi, Y. and Arai, M. (1980) A new sesquiterpene antibiotic, heptelidic acid: producing organisms, fermentation, isolation and characterization. *Journal of Antibiotics* (*Tokyo*) 33, 468–473.

Jacobs, J.M., Jacobs, N.J., Sherman, T.D. and Duke, S.O. (1991) Effect of diphenyl ether herbicides on oxidation of protoporphyrinogen to protoporphyrin in organellar and plasma membrane-enriched fractions of barley. *Plant Physiology* 97, 197–203.

James, P.J., Kershaw, M.J., Reynolds, S.E. and Charnley, A.K. (1993) Inhibition of desert locust (*Shistocerca gregaria*) Malpighian tubule fluid secretion by destruxins, cyclic peptide toxins from the insect pathogenic fungus *Metarhizium anisopliae*. *Journal of Insect Physiology* 39, 797–804.

Jeffs, L.B. and Khachatourians, G.G. (1997) Toxic properties of *Beauveria* pigments on erythrocyte membranes. *Toxicon* 35, 1351–1356.

Jones, R.W. and Hancock, J.G. (1987) Conversion of viridin to viridiol by viridin producing fungi. *Canadian Journal of Microbiology* 33, 963–966.

Joshi B.K., Gloer J.B. and Wicklow D.T. (1999). New verticillin and glisoprenin analogues from *Gliocladium catenulatum*, a mycoparasite of *Aspergillus flavus* sclerotia. *Journal of Natural Products* 62, 730–733.

Kanaoka, M., Isogai, A., Murakoshi, S., Ichione, M., Suzuki, A. and Tamura, S. (1978) Bassianolide, a new insecticidal cylcodepsipeptide from *Beauveria bassiana* and *Verticillium lecanii*. *Agricultural and Biological Chemistry* 42, 629–635.

Kawazu, K., Murakami, T., Ono, Y., Kanzaki, H., Kobayashi, A., Mikawa, T. and Yoshikawa, N. (1993) Isolation and characterization of two novel nematicidal depsipeptides from an imperfect fungus, strain D1084. *Bioscience, Biotechnology and Biochemistry* 57, 98–101.

Kershaw, M.J., Moorhouse, E.R., Bateman, R., Reynolds, S.E. and Charnley, A.K. (1999) The role of destruxins in the pathogenicity of *Metarhizium anisopliae* for three species of insects. *Journal of Invertebrate Pathology* 74, 213–223.

Khachatourians, G.G. (1996) Biochemistry and molecular biology of entomopathogenic fungi. In: Howard, D.H. and Miller, J.D. (eds) *The Mycota VI. Human and Animal Relationships.* Springer Verlag, Berlin, pp. 331–363.

Kleinkauf, H. and Rindfleisch, H. (1975) Non-ribosomal biosynthesis of the cyclic octadecapeptide alamethicin. *Acta Microbiol. Acad. Sci. Hung.* 22, 411–418.

Klisiewicz, J.M. (1985) Growth and reproduction of *Dichotomophthora portulacae* and its biological activity on purslane. *Plant Disease* 69, 761–762.

Knox, J.P. and Dodge, A.D. (1985) Isolation and activity of the photodynamic pigment hypericin. *Plant Cell Environment* 8, 19–25.

Kodaira, Y. (1961) Biochemical studies on the muscardine fungi in the silkworms, *Bombyx mori.*

Journal of the Faculty of Textile Science and Technology, Sinshu University, Sericult. 5, 1–68.

Kohno, J., Asai, Y., Nishio, M., Sakurai, M., Kawano, K., Hiramatsu, H., Kameda, N., Kishi, N., Okuda, T. and Komatsubara, S. (1999) TMC-171A, B, C and TMC-154, novel polyketide antibiotics produced by *Gliocladium* sp. TC 1304 and TC 1282. *Journal of Antibiotics* (*Tokyo*) 52 (12), 1114–1123.

Kono, Y., Gardner, J.M. and Takeuchi, S. (1986) Nonselective phytotoxins simultaneously produced with host-selective ACTG-toxins by a pathotype of *Alternaria citri* causing brown spot disease of mandarins. *Agricultural and Biological Chemistry* 50, 2401–2403.

Kopecky, J., Matha, V. and Jegorov, A. (1992) The inhibitory effect of destruxin A on the arboviruses in *Aedes albopictus* C6/36 cell line. *Comparative Biochemistry and Physiology* 103C, 23–25.

Krasnoff, S. and Gibson, D.M. (1996) New destruxins from the entomopathogenic fungus *Aschersonia* sp. *Journal of Natural Products* 59, 485–489.

Krasnoff, B., Gupta, S.B., St Leger, R.J., Renwick, J.A. and Roberts, D.W. (1991) Antifungal and insecticidal properties of efrapeptins: metabolites of the fungus *Tolypocladium niveum. Journal of Invertebrate Pathology* 58, 180–188.

Krasnoff, S.B. and Gupta, S. (1991) Identification and biosynthesis of efrapeptins in the fungus *Tolypocladium geodes* Gams (Deuteromycotina: Hyphomycetes). *Journal of Chemical Ecology* 17, 1953–1960.

Lange, C., Mulheim, C., Cherton, J.C., Cassier, P., Vey, A. and Pais, M. (1991) Desorption of ions from locust tissue – behaviour of E-destruxin using positive-ion fast atom bombardment mass spectrometry. *Rapid Communications in Mass Spectrometry* 5, 169–174.

Lange, C., Loutelier, C., Cherton, J.-C., Cassier, P., Vey, A. and Pais, M. (1992) Desorption of ions from locust tissues. II. Metabolites of E-destruxin using negative-ion fast-atom bombardment mass spectrometry. *Rapid Communications in Mass Spectrometry* 6, 28–33.

Lax, A.R., Sheperd, H.S. and Edwards, J.V. (1988) Tentoxin, a chlorosis-inducing toxin from *Alternaria* as a potential herbicide. *Weed Technology* 2, 540–544.

Leclerc, G., Rebuffat, S. and Bodo, B. (1998) Directed biosynthesis of peptaibol antibiotics in two *Trichoderma* strains. II. Structure elucidation. *Journal of Antibiotics* (*Tokyo*) 51, 178–183.

Lilley, L.J. (1965) Induction of chromosome aberrations by aflatoxins. *Nature* 207, 433–434.

Lin, A., Lee, T.M. and Rern, J.C. (1994) Tricholin, a new antifungal agent from *Trichoderma viride* and its action in biological control of *Rhizoctonia solani. Journal of Antibiotics* (*Tokyo*) 47, 799–805.

Liu, J.-C., Boucias, D.G., Pendland, J.C., Liu, W.-Z. and Maruniak, J. (1996) The mode of action of Hirsutellin A on eukaryotic cells. *Journal of Invertebrate Pathology* 67, 224–228.

Liu, W.-Z., Boucias, D.G. and McCoy, C.W. (1995) Extraction and characterization of the insecticidal toxin hirsutellin A produced by *Hirsutella thompsonii* var. *thompsonii. Experimental Mycology* 19, 254–262.

Logrieco, A., Moretti, A., Castella, G., Kostecki, M., Golinski, P., Ritieni, A. and Chelkowski, J. (1998) Beauvericin production by *Fusarium* species. *Applied Environmental Microbiology* 64, 3084–3088.

Lorito, M., Peterbauer, C., Hayes, C.K. and Harman, G.E. (1994) Synergistic action between fungal cell wall degrading enzymes and different antifungal compounds enhances inhibition of spore germination. *Microbiology* 140, 623–629.

Lorito, M., Farkas, V., Rebuffat, S., Bodo, B. and Kubicek, C.P. (1996) Cell wall synthesis is a major target of mycoparasitic antagonism by *Trichoderma harzianum*. *Journal of Bacteriology* 178, 6382–6385.

Loutelier, C., Lange, C., Cassier, P., Vey, A. and Cherton, J.C. (1994) Non-extractive study of E- and A-destruxines in the locust, *Locusta migratoria* L. III: Direct high performance liquid chromatography analysis and parallel FAB-MS monitoring. *Journal of Chromatography B, Biomedical Applications* 656, 281–292.

Loutelier, C., Marcual, A., Cassier, P., Cherton, J.-C. and Lange, C. (1995) Desorption of ions from locust tissues. III. Study of a metabolite of A-destruxin using fast-atom bombardment linked-scan mass spectrometry. *Rapid Communications Mass Spectrometry* 9, 408–413.

Madariaga, R.B. and Scharen, A.L. (1985) *Septoria tritici* blotch in Chilean wild oat. *Plant Disease* 69, 126–127.

Manning, R.O. and Wyatt, R.D. (1984) Comparative toxicity of *Chaetomium* contaminated corn and various chemical forms of oosporein in broiler chickens. *Poultry Science* 63, 251–259.

Marasas, W.F.O., Wehner, F.C., van Rensburg, S.J. and van Schalkwyk, D.J. (1981) Mycoflora of corn produced in human esophageal cancer areas in Transkei, Southern Africa. *Phytopathology* 71, 792–796.

Massion, C.L. and Lindow, S.E. (1986) Effects of *Sphacelotheca holci* infection on morphology and competitiveness of johnsongrass (*Sorghum halepense*). *Weed Science* 34, 883–888.

Matha, V., Weiser, J. and Olejnicek, J. (1988) The effect of tolypin in *Tolypocladium niveum* crude extract against mosquito and blackfly larvae in the laboratory. *Folia Parasitologia* 35, 381–383.

Mazet, I. (1992) Recherches sur les hirsutellines, toxines protéiques produites par *Hirsutella thompsonii* Fisher, champignon parasite d'acariens phytophages. Doctoral dissertation, Université de Montpellier 2, France (cited in Liu *et al.*, 1996).

Mazet, I. and Vey, A. (1995) Hirsutellin A, a toxic protein produced *in vitro* by *Hirsutella thompsonii*. *Microbiology* 141, 1343–1348.

McLean, M. (1993) Towards an understanding of the effects of aflatoxin B1 on plant tissue. PhD, thesis, University of Natal, Durban, South Africa.

McLean, M. (1994) The phytotoxic effects of aflatoxin B1: a review (1984–1994) *South African Journal of Science* 90, 385–390.

McLean, M., Watt, M.P., Berjak, P., Dutton, M.F. and Snyman, C. (1994) Effects of aflatoxin B1 on *in vitro* cultures of *Nicotiana tabacum* var. Samsun. II: Root and shoot development in tobacco plantlets. *Mycopathologia* 125, 107–117.

Merck Index (1996) *Aflatoxins B*, ed. S. Budavari. Merck and Co., Inc. Whitehouse Station, New Jersey, p. 33.

Merrill, A.H. Jr, Schmelz, E.-M., Dillehay, D.L., Spiegel, A., Shayman, J.A., Schroeder, J.J., Riley, R.T. and Wang, E. (1997) Sphingolipids. The enigmatic lipid class: biochemistry, physiology and pathophysiology. *Toxicology and Applied Pharmacology* 142, 208–225.

Mertz, D., Lee, D., Zuber, M. and Lillehoj, E.B. (1980) Uptake and metabolism of aflatoxin by *Zea mays*. *Journal of Agricultural and Food Chemistry* 28, 963–966.

Miller, J.D., Strongman, D. and Whitney, N.J. (1985) Observations on fungi associated with spruce budworm infested balsam fir needles. *Canadian Journal of Forestry Research* 15, 896–901.

Mochizuki, K., Ohmori, K., Tamura, H., Shizuri, Y., Nishiyama, S., Miyoshi, E. and Yamamura, S. (1993) The structures of bioactive cyclodepsipeptides, Beauveriollides I and II, metabolites of entomopathogenic fungi *Beauveria* sp. *Bulletin of the Chemical Society of Japan* 66, 3041–3046.

Moretti, A., Logrieco, A., Bottalico, A., Ritienti, A., Randazzo, G. and Corda, P. (1995) Beauvericin production by *Fusarium subglutinans* from different geographical areas. *Mycological Research* 99, 282–286.

Morin, L., Gianotti, A.F. and Lauren, D.R. (2000) Trichothecene production and pathogenicity of *Fusarium tumidum*, a candidate bioherbicide for gorse and broom in New Zealand. *Mycological Research* 104, 993–999.

Mumpuni, A., Sharma, H.S.S. and Brown, A. (1998) Effect of metabolites produced by *Trichoderma harzianum* biotypes and *Agaricus bisporus* on their respective growth radii in culture. *Applied Environmental Microbiology* 64, 5053–5056.

Munkvold, G., Stahr, H.M., Logrieco, A., Moretti, A. and Ritieni, A. (1998) Occurrence of

fusaproliferin and beauvericin in *Fusarium*-contaminated livestock feed in Iowa. *Applied Environmental Microbiology* 64, 3923–3926.

Muroi, M., Shiragami, N. and Takatsuki, A. (1994) Destruxin B, a specific and readily reversible inhibitor of vacuolar type H+ translocating ATPase. *Biochemical and Biophysical Research Communications* 205, 1358–1365.

Muroi, M., Kaneko, N., Suzuki, K., Nishio, T., Oku, T., Sato, T. and Takatsuki, A. (1996) Efrapeptins block exocytic but not endocytic trafficking of proteins. *Biochemical and Biophysical Research Communications* 227, 800–809.

Namatame, I., Tomoda, H., Tabata, N., Si, S.Y. and Omura, S. (1999) Structure elucidation of fungal Beauveriolide-III, a novel inhibitor of lipid droplet formation in mouse macrophages. *Journal of Antibiotics* 52, 7–12.

Nasini, G., Locci, L., Camarda, L., Merlini, R. and Nasini, G. (1977) Screening of the genus *Cercospora* for secondary metabolites. *Phytochemistry* 16, 243–247.

Odier, F., Vey, A. and Bureau, J.P. (1992) *In vitro* effects of fungal cyclodepsipeptides on leukemic cells: study of destruxins A, B and E. *Biology of the Cell* 74, 267–271.

Ojcious, D.M., Zychlinsky, L., Zheng, M. and Yong, D.-E. (1991) Ionophore-induced apoptosis: role of DNA fragmentation and calcium fluxes. *Experimental Cell Research* 197, 43–49.

Okamoto, M., Yoshida, K., Uchida, I., Nishikawa, M., Kohsaka, M. and Aoki, H. (1986a) Studies of platelet activating factor (PAF) antagonists from microbial products. I. Bisdethio bis(methylthio)gliotoxin and its derivatives. *Chemical and Pharmaceutical Bulletin* (*Tokyo*) 34, 340–344.

Okamoto, M., Yoshida, K., Uchida, I., Kohsaka, M. and Aoki, H. (1986b) Studies of platelet factor (PAF) antagonists from microbial products. II. Pharmacological studies of FR-49175 in animal models. *Chemical and Pharmaceutical Bulletin* (*Tokyo*) 34, 345–348.

Omoto, C. and McCoy, C.W. (1998) Toxicity of purified fungal toxin hirsutellin A to the citrus rust mite *Phyllocoptruta oleivora. Journal of Invertebrate Pathology* 72, 319–322.

Omura S., Arai, N., Yamaguchi, Y., Masuma, R., Iwai, Y., Namikoshi, M., Turberg, A., Kolbl, H. and Shiomi, K. (2000) Argifin, a new chitinase inhibitor, produced by *Gliocladium* sp. FTD-0668. I. Taxonomy, fermentation, and biological activities. *Journal of Antibiotics* (*Tokyo*) 53 (6), 603–608.

Ovchinnikov, Y.A., Ivanov, V.T. and Mikhaleva, I.I. (1971) The synthesis and some properties of beauvericin. *Tetrahedron Letters* 2, 159–162.

Païs, M., Das, B.C. and Ferron, P. (1981) Depsipeptides from *Metarhizium anisopliae*. *Phytochemistry* 20, 715–723.

Pegram, R.A. and Wyatt, R.D. (1981) Avian gout caused by oosporein, a mycotoxin produced by *Chaetomium trilaterale. Poultry Science* 60, 2429–2440.

Pegram, R.A., Wyatt, R.D. and Smith, T.L. (1982) Oosporein toxicosis in turkey poult. *Avian Diseases* 26, 47–49.

Plattner, R.D. and Nelson, P.E. (1994) Production of beauvericin by a strain of *Fusarium proliferatum* isolated from corn fodder for swine. *Applied Environmental Microbiology* 60, 3894–3896.

Poprawski, T.J., Robert, P.H. and Maniana, N.K. (1985) Susceptibility of the onion maggot *Delia antiqua* (Diptera Antomyiidae) to the mycotoxin destruxin E. *Canadian Entomologist* 117, 801–802.

Poprawski, T.J., Robert, P.H. and Maniania, N.K. (1994) Contact toxicity of the mycotoxin destruxin E to *Empoasca vitis. Journal of Applied Entomology* 117, 135–143.

Qadri, S., Mohiuddin, S., Anwar, N., Rizki, Y.M., Qureshi, S.A. and Anwarullah, M. (1989) Larvicidal activity of β-exotoxin and beauvericin against two dipterous species. *Pakistan Journal of Scientific and Industrial Research* 32, 467–470.

Quiot, J.-M., Vey, A., Vago, C. and Pais, M. (1980) Action antivirale d'une mycotoxine. Etude d'une toxine de l'hyphomycète *Metarhizium anisopliae* (Metsch.) Sorok. en culture cellulaire. *Comptes Rendus de l'Academie des Sciences, Série D* (*Paris*) 291, 763–766.

Quiot, J.-M., Vey, A. and Vago, C. (1985) Effects of mycotoxins on invertebrate cells *in vitro*. *Advances in Cell Culture* 4, 199–212.

Rebuffat, S., El Hajji, M., Hennig, P., Davoust, D. and Bodo, B. (1989) Isolation, sequence, and conformation of seven trichorzianines B from *Trichoderma harzianum*. *International Journal of Peptide and Protein Research* 34, 200–210.

Rebuffat, S., Duclohier, H., Auvin-Guette, C., Molle, G., Spach, G. and Bodo, B. (1992) Membrane-modifying properties of the pore-forming peptaibols saturnisporin SA IV and harzianin HA V. *FEMS Microbiology Immunology* 5, 151–160.

Reiss, J. (1984) Failure of plant tissue to metabolize aflatoxin B1? *Mycopathologia* 85, 43–44.

Robert, P.-H. and Fargues, J. (1986) Toxicité par ingestion des destruxines A, B et E pour les larves de *Musca domestica* L. *Comptes Rendus de l'Académie des Sciences, Paris, Serie III* 303, 641–643.

Robert, P.H. and Riba, G. (1989) Toxic and repulsive effect of spray, *per os* and systemic application of destruxin E to aphids. *Mycopathologia* 108, 170–183.

Roberts, D.W. (1966) Toxins from the entomogenous fungus *Metarhizium anisopliae*. II. Symptoms and detection in moribund hosts. *Journal of Invertebrate Pathology* 8, 222–227.

Roberts, D.W. (1981) Toxins of entomopathogenic fungi. In: Burges H.D. (ed.) *Microbial Control of Pests and Plant Diseases 1970–1980*. Academic Press, New York, pp. 441–464.

Samuels, R.I., Reynolds, S.E. and Charnley, A.K. (1988a) Calcium channel activation of insect muscle by destruxins, insecticidal compounds produced by the entomopathogenic fungus, *Metarhizium anisopliae*. *Comparative Biochemistry and Physiology* 90C, 403–412.

Samuels, R.I., Charnley, K. and Reynolds, S.E. (1988b) Application of reversed-phase HPLC in separation and detection of the cyclodepsipeptide toxins produced by the entomopathogenic fungus *Metarhizium anisopliae*. *Journal of Chromatographic Science* 26, 15–19.

Sawa, R., Mori, Y., Inuma, H., Naganawa, H., Kamada, M., Yoshida, S., Furutani, H., Kajimura, Y., Fuwa, T. and Takeuci, T. (1994) Harzianic acid, a new microbial antibiotic from a fungus. *Journal of Antibiotics* (*Tokyo*) 47, 731–732.

Scheepens, P.C. (1987) Joint action of *Cochliobolus lunatus* and atrazine on *Echinochloa crus-galli* (L.) Beauv. *Weed Research* 27, 43–47.

Scheepens, P.C. and van Zon, H.C.J. (1982) Microbial herbicides. In: Kurstak, E. (ed.) *Microbial and Viral Pesticides.* Marcel Dekker, New York, pp. 623–641.

Scheffer, R.P. and Livingston, R.S. (1984) Host-selective toxins and their role in plant diseases. *Science* 223, 17–21.

Schirmbock, M., Lorito, M., Wang, Y.L., Hayes, C.K., Arisan-Atac, I., Scala, F., Harman, G.E. and Kubicek, C.P. (1994) Parallel formation and synergism of hydrolytic enzymes and peptaibol antibiotics, molecular mechanisms involved in the antagonistic action of *Trichoderma harzianum* against phytopathogenic fungi. *Applied Environmental Microbiology* 60, 4364–4370.

Schoental, R. and White, A.F. (1965) Aflatoxins and 'albinism' in plants. *Nature* 205, 57–58.

Shabana, Y.M.N.E. (1987) Biological control of water weeds by using plant pathogens. Dissertation, Mansoura University, El-Mansoura, Egypt.

Shier, W.T., Abbas, H.K. and Mirocha, C.J. (1991) Toxicity of the mycotoxins fumonisins B1 and B2 and *Alternaria alternata* f. sp. *lycopersici* toxin (AAL) in cultured mammalian cells. *Mycopathologia* 116, 97–104.

Sloman, I.S. and Reynolds, S.E. (1993) Inhibition of ecdysteroid secretion from *Manduca sexta* prothoracic glands *in vitro* by destruxins – cyclic depsipeptide toxins from the insect pathogenic fungi *Metarhizium anisopliae*. *Insect Biochemistry and Molecular Biology* 23, 43–46.

Solfrizzo, M., Altomare, C., Visconti, A., Bottalico, A. and Perrone, G. (1994) Detection of peptaibols and their hydrolysis products in cultures of *Trichoderma* species. *Natural Toxins* 2, 360–365.

Steinrauf, L.K. (1985) Beauvericin and the other enniatins. In: Sigel, H. and Sigel, A. (eds)

Metal Ions in Biological Systems – Antibiotics and their Complexes. Marcel Dekker, New York, pp. 139–171.

Stipanovic, R.D. and Howell, C.R. (1982) The structure of gliovirin a new antibiotic from *Gliocladium virens. Journal of Antibiotics* (*Tokyo*) 35, 1326–1330.

Strasser, H., Vey, A. and Butt, T.M. (2000) Are there any risks in using entomopathogenic fungi for pest control, with particular reference to the bioactive metabolites of *Metarhizium, Tolypocladium* and *Beauveria* species? *Biocontrol Science and Technology* 10, 717–735.

Strongman, D.B., Strunz, G.M., Giguere, P., Yu, C.-M. and Calhoun, L. (1988) Enniatins from *Fusarium avenaceum* isolated from balsam fir foliage and their toxicity to spruce budworm larvae, *Choristoneura fumiferana* Clem. *Journal of Chemical Ecology* 14, 753–764.

Sun, C., Chen, H. and Yeh, S.F. (1994) Suppressive effects of metabolites from *Alternaria brassicae* on the hepatitis B surface antigen. *Planta Medica* 60, 87–88.

Suzuki, A., Taguchi, H. and Tamura, S. (1970) Isolation and structure elucidation of three new insecticidal cyclodepsipeptides, destruxins C and D and desmethyldestruxin B produced by *Metarhizium anisopliae*. *Agricultural and Biological Chemistry* 34, 813–816.

Suzuki, A., Kawakami, K. and Tamura, S. (1971) Detection of destruxins in silkworm larvae infected with *Metarhizium anisopliae*. *Agricultural and Biological Chemistry* 35, 1641–1643.

Suzuki, A., Kanaoka, M., Isogai, A., Murakoshi, S., Ichinoe, M. and Tamura, S. (1977) Bassianolide, a new insecticidal cyclodepsipeptide from *Beauveria bassiana* and *Verticillium lecanii*. *Tetrahedron Letters* 25, 2167–2170.

Tabuchi, H., Tajimi, A. and Ichihara, A. (1991) (+)-Isocerosporin, a phytotoxic compound isolated from *Scolecotrichum graminis* Fuckel. *Agricultural and Biological Chemistry* 55, 2675–2676.

Tamura, S. and Takahashi, N. (1971) Destruxins and piercidens. In: Jacobson, M. and Grosby, D.G. (eds) *Naturally Occurring Insecticides.* Marcel Dekker, New York, pp. 499–539.

Tanaka, T.H., Abbas, H.K. and Duke, S.O. (1993) Phytotoxin structure–activity relationships of fumonisins, aminopentals, sphingolipids and AAL-toxin in a duckweed (*Lemna pausiocostata* L.) bioassay. *Phytochemistry* 33, 779–785.

Taniguchi, M., Kawaguchi, T., Tanaka, T. and Oi, S. (1984) Antimicrobial and respiration inhibitory activities of oosporein. *Agricultural and Biological Chemistry* 48, 1065–1067.

Taylor, A. (1986) Some aspects of the chemistry and biology of the genus *Hypocrea* and its anamorphs, *Trichoderma* and *Gliocladium*. *Proceedings of the Nova Scotia Institute of Science* 36, 27–58.

TeBeest, D.O. (ed.) (1991) *Microbial Control of Weeds.* Chapman & Hall, New York.

Teetor-Barsch, G. and Roberts, D.W. (1983) Entomogenous *Fusarium* species. *Mycopathologia* 84, 3–16.

Templeton, G.E. and Heiny, D.K. (1989) Improvement of fungi to enhance mycoherbicide potential. In: Whipps, J.M. and Lumsden, R.D. (eds) *Biotechnology of Fungi for Improving Plant Growth.* Cambridge University Press, Cambridge, pp. 127–151.

Terry, B.J., Liu, W.-C., Cianci, C.W., Proszynski, E., Fernandes, P. and Meyers, E. (1992) Inhibition of herpes simplex virus type I DNA polymerase by the natural product oosporein. *Journal of Antibiotics* 2, 286–288.

Thines, E., Eilbert, F., Anke, H. and Sterner, O. (1998) Glisoprenins C, D and E, new inhibitors of appressorium formation in *Magnaporthe grisea*, from cultures of *Gliocladium roseum*. 1. Production and biological activities. *Journal of Antibiotics* (*Tokyo*) 51, 117–122.

Thomsen, L., Eilenberg, J. and Esberg, P. (1996) Effects of destruxins on *Pieris brassicae* and *Agrotis segetum*. *IOBC Bulletin* 19, 190–195.

Tomlin, C.D. (1997) *The Pesticide Manual.* BCPC Publications, Bracknell, UK, 1606 pp.

Ueno,Y. (1984) Toxicological features of T-2 toxin and related trichothecenes. *Fundamental. Applied Toxicology* 4, 124–132.

Venkatsubbaiah, P., Tisserat, N.A. and Chilton, W.S. (1994) Metabolites of *Ophiosphaerella herpotricha. Mycopathologia* 128, 155–159.

Vesonder, R.F., Labeda, D.P. and Peterson, R.E. (1992a) Phytotoxic activity of selected water-

soluble metabolites of *Fusarium* against *Lemna minor* L. (duckweed). *Mycopathologia* 118, 185–189.

Vesonder, R.F., Peterson, R.E., Lebeda, D. and Abbas, H.K. (1992b) Comparative phytotoxicity of fumonisins, AAL-toxin and yeast sphingolipids in *Lemna minor* L. (duckweed). *Archives of Environmental Contamination and Toxicology* 23, 464–467.

Vey, A. and Quiot, J.-M. (1989) Etude *in vitro* et chez l'insecte hôte des destruxines, toxines cyclodepsipeptidiques produites par le champignon entomopathogène *Metarhizium anisopliae*. *Canadian Journal of Microbiology* 35, 1000–1008.

Vey, A., Quiot, J.-M., Vago, C. and Fargues, J. (1985) Effet immunodépresseur de toxines fongiques: inhibition de la réaction d'encapsulement multicellulaire par les destruxines. *Comptes Rendus de l'Académie des Sciences, Série 3* (*Paris*) 300, 647–651.

Vey, A., Quiot, J.-M. and Païs, M. (1986) Toxémie d'origine fongique chez les invertébrés et ses conséquences cytotoxiques: étude sur l'infection à *Metarhizium anisopliae* (Hyphomycète, Moniliales) chez les lépidoptères et les coléoptères. *Comptes Rendus de la Société de Biologie* 180, 105–112.

Vey, A., Quiot, J.-M., Mazet, I. and McCoy, C.W. (1993) Toxicity and pathology of crude broth filtrate produced by *Hirsutella thompsonii* var. *thompsonii* in shake culture. *Journal of Invertebrate Pathology* 61, 131–137.

Vilcinskas, A., Matha, V. and Gotz, P. (1997) Inhibition of phagocytic activity of plasmatocytes isolated from *Galleria mellonella* by entomogenous fungi and their secondary metabolites. *Journal of Insect Physiology* 43, 475–483.

Vining, L.C., Kelleher, W.J. and Schwarting, A.E. (1962) Oosporein production by a strain of *Beauveria bassiana* originally identified as *Amanita muscaria*. *Canadian Journal of Microbiology* 8, 931–933.

Vogelgsang, S., Watson, A.K., Ditommaso, A. and Hurle, K. (1998) Effect of the pre-emergence bioherbicide *Phomopsis convolvulus* on seedling and established plant growth of *Convolvulus arvensis. Weed Research* 38, 175–182.

Wahlman, M. and Davidson, B.S. (1993) New destruxins from the entomopathgenic fungus *Metarhizium anisopliae*. *Journal of Natural Products* 56, 643–647.

Wainwright, M., Betts, R.P. and Teale, D.M. (1986). Antibiotic activity of oosporein from *Verticillium psalliotae*. *Transactions of the British Mycological Society* 86, 168–170.

Walker, H.L. (1981) *Fusarium lateritium*: a pathogen of spurred anoda (*Anoda cristata*), prickly sida (*Sida spinosa*), and velvetleaf (*Abutilon theophrasti*). *Weed Science* 29, 629–631.

Wang, E., Norred, W.P., Bacon, C.W., Riley, R.T. and Merrill, A.H. (1991) Inhibition of sphingolipid biosynthesis by fumonisins. *Journal of Biological Chemistry* 266, 14486–14490.

Weindling, R. (1934) Studies in a toxic principle effect in the parasitic association of *Trichoderma lignorum* on *Rhizoctonia solani* and other soil fungi. *Phytopathology* 24, 1153–1179.

Weiser, J. and Matha, V. (1988) Tolypin, a new insecticidal metabolite of fungi of the genus *Tolypocladium*. *Journal of Invertebrate Pathology* 51, 94–96.

Wilhite, S.E. and Straney, D.C. (1966) Timing of gliotoxin biosynthesis in the fungal biological control agent *Gliocladium virens* (*Trichoderma virens*). *Applied Microbiology and Biotechnology* 45, 513–518.

Wilson, B.J. (1971) Miscellaneous *Aspergillus* toxins. In: Ciegler, A., Kadis, S. and Aje, S.J. (eds) *Microbial Toxins: a Comprehensive Treatise,* Vol. 6. Academic Press, New York, pp. 288–289.

Winder, R.S. and van Dyke, G.C. (1989) The pathogenicity, virulence, and biocontrol potential of two *Bipolaris* species on johnsongrass (*Sorghum halepense*). *Weed Science* 38, 84–89.

Wogan, H. (1992) Aflatoxins as risk factors for hepatocellular carcinoma in humans. *Canadian Research* 52, 2114–2118.

Wood, G.E. (1992) Mycotoxins in foods and feeds in the United States. *Journal of Animal Science* 70, 3941–3949.

Yeh, S.F., Pan, W., Ong, G.T., Chiou, A.J., Chuang, C.C., Chiou, S.H. and Wu, S.H. (1996) Study of the structure–activity correlation in destruxins, a class of cyclodepsipeptides pos-

sessing suppressive effect on the generation of hepatitis virus surface antigen in human hepatoma cells. *Biochemical and Biophysical Research Communications* 229, 65–72.

Yoder, O.C. and Turgeon, B.G. (1985) Molecular bases of fungal pathogenicity to plants. In: Bennet, J.W. and Lasure, L. (eds) *Gene Manipulations in Fungi.* Academic Press, New York, USA, pp. 417–441.

Yoshida, K., Okamoto, M., Shimazaki, N. and Hemmi, K. (1988) PAF inhibitors of microbial origin. Studies on diketopiperazine derivatives. *Progresses in Biochemistry and Pharmacology* 22, 66–80.

Zizka, J. and Weiser, J. (1993) Effect of beauvericin, a toxic metabolite of *Beauveria bassiana*, on the ultrastructure of *Culex pipiens autogenicus* larvae. *Cytobios* 75, 13–19.

13 Safety of Fungal Biocontrol Agents

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Introduction

Fungi contain a diverse array of taxa with a great diversity of properties. They are relatively common and are important in regulating pest populations. There has been considerable interest in the use of fungi as microbial control agents of pest insects, nematodes, weeds and plant pathogens and some have been developed as commercial biocontrol products. However, fungi also contain many species that are pests in themselves, causing untold losses to crops, forests, stored products and buildings and also affecting animal and human health. Consequently the development and use of fungi as biocontrol agents requires an assessment of unintended effects associated with their use.

In this chapter we provide an overview of the potential hazards and safety concerns associated with biocontrol fungi. We restrict our discussions to fungi with biocontrol potential for plants, plant diseases and arthropods. We provide examples of how fungi can be detrimental as well as how they have been used safely as biocontrol agents. Finally, we shall review how regulations and registration requirements attempt to address and mitigate potential safety issues. Previous reviews on the safety of biocontrol fungi are those of Austwick (1980), Goettel *et al.* (1990), Prior (1990), Goettel and Johnson (1992) and Evans (1998, 2000). Reviews on the safety of microbial control agents in general are those of Flexner *et al.* (1986), Laird *et al.* (1990), Cook *et al.* (1996) and Goettel and Jaronski (1997). Guidelines for testing the pathogenicity and infectivity of entomopathogens to mammals have been reviewed by Siegel (1997) and guidelines for evaluating effects of entomopathogens on invertebrate non-target organisms have been reviewed by Hajek and Goettel (2000). Several authors have also addressed the protocols and guidelines that should be followed in order to introduce exotic agents for weed biocontrol (Wapshere, 1974, 1975, 1989; Klingman and Coulson, 1982).

Some Examples of the Detrimental Effects of Fungi

In addressing the potential detrimental effects of fungi used for biological control, it is useful to reflect on the detrimental effects of fungi in general. This is especially so because, to date, the detrimental effects of fungi used specifically as biological control agents are either non-existent, have been minimal or have generally gone unnoticed. We have therefore included several examples of the detrimental effects of various fungal pathogens, both endemic and introduced, in order to illustrate the 'potential' detrimental effects of a biological control fungus in what we would consider a 'worst-case scenario'. We once again stress that these are examples of detrimental effects that have not arisen from attempts to use fungi as biocontrol agents.

Beauveriosis in the silkworm industry

Prior to the early 20th century, silk production throughout the world suffered because of the devastating effects of several diseases in the silkworm, *Bombyx mori* (Steinhaus, 1975). In France and Italy especially, annual losses due to 'muscardine' were tremendous. For instance, silk production in Italy suffered losses of approximately 5 million kg $year^{-1}$ until *c.* 1925 (Bell, 1974). However, initially, the role of pathogens in disease was not understood. Agostino Bassi (1835, as cited by Bell, 1974, and Steinhaus, 1975) first demonstrated the germ theory of disease using mycosis in the silkworm. He showed that the muscardine disease was caused by a 'vegetable parasite', which grows and develops within the silkworm, eventually killing its host. He demonstrated how the 'seeds' produced on the surface of the cadaver were responsible for disease in new individuals and how these seeds could be destroyed by chemical and physical means. He recommended disinfection practices using lye, wine and brandy, boiling water, burning and exposure to sunlight. Thus he was also one of the founders of disinfection. The muscardine in question was a fungus, which was later named *Beauveria bassiana*, in his honour.

With time, strict hygienic practices and legislation were adopted and muscardine disease in silkworm insectaries was brought under control. For instance, in China and Japan, the application of *B. bassiana* and other microbial control agents was restricted to areas where no silkworms are bred, to avoid contamination of the mulberry leaves used to feed the silkworms (Hussey and Tinsley, 1981; Goettel *et al.*, 1990). And, through careful strain selection, it may even be possible to use *B. bassiana* to control a pest in the immediate vicinity of a silkworm rearing facility; in a pilot test in a Chinese 110 ha pine plantation, good control of pine caterpillars (*Dendrolimus* spp.) was achieved through six applications of 2100 kg of *B. bassiana*, with no significant effect on the silkworms within a rearing shed built within the plantation (Anon., 1981a, as cited by Goettel *et al.*, 1990). The strains of *B. bassiana* used against the pine caterpillars were 100 times less virulent to silkworms than strains isolated from silkworms themselves.

This example illustrates the potential that entomopathogenic fungi have in devastating insect colonies in insectaries. It also illustrates the importance of differences in the specificity of different fungal strains or isolates. Through modern disinfection and hygienic practices, diseases in insectaries can, for the most part, be kept under control.

Chalkbrood in honey-bees

Reports of diseases in the honey-bee date as early as 700 BC (Steinhaus, 1975). The most important fungal disease of the honey-bee is chalkbrood, caused by the ascomycete *Ascosphaera apis* (Gilliam and Vandenberg, 1990). Although the disease is not generally considered serious, infections of certain colonies can be persistent and damaging. Chalkbrood is present on all continents where honey-bees are found (Bradbear, 1988; Anderson and Gibson, 1998). Although known in Europe since the early 20th century, the fungus has only been documented more recently in honey-bee colonies in North America and elsewhere.

The origins of the more recent findings remain a mystery and several possibilities exist: (i) the fungus was recently introduced into North America, possibly on imported pollen; (ii) the fungus has gone unnoticed until recently; or (iii) the fungus has occurred in feral and solitary bees and only recently invaded honey-bee colonies. Nevertheless, the fungus is spreading to honey-bee colonies throughout the world and is causing serious problems in some locations in some years. The disease is little understood and could be triggered by stress factors, such as chilling.

There is no chemotherapeutic agent registered for its control. However, Gilliam *et al.* (1988) demonstrated that genetically inherited hygienic behaviour is an important component of resistance of bee colonies to the disease. Colonies in which workers were able to detect and remove infected or dead bees were less susceptible to the disease.

Chalkbrood in honey-bees provides a rare example of a behavioural mechanism of disease resistance. It also demonstrates the difficulties of preventing the spread of a detrimental fungal pathogen. For instance, despite strict hygiene and importation restrictions of bees, bee equipment and bee products, chalkbrood has recently been detected in Australia and has rapidly spread over a wide geographical area within that country (Anderson and Gibson, 1998).

Plant pathogens

Plague or 'pestilence' affecting cultivated crops has been recorded since biblical times, and fungal plant pathogens have been implicated in most of these catastrophes (Large, 1940; Agrios, 1997). However, the first scientifically documented and certainly one of the most dramatic examples of the detrimental effects of plant pathogens, or indeed of any pest, concerns the potato late blight in Europe during the 19th century. The disease or 'murrain' was first reported in 1845, but was initially ascribed to low temperatures, and it was not until several years later, after disastrous crop losses throughout Europe, that it was identified as being caused by a fungus, *Phytophthora infestans*, by the leading mycologist of the time, M.J. Berkeley, who further speculated that the pathogen had arrived from the New World (Large, 1940). This biotic invasion has been described by the latter author as 'a historic determinant of human affairs', since, in Ireland alone, at least 1,000,000 people died between 1846 and 1860 as a consequence of the potato famine, and more than 1.5 million emigrated. It also had immediate political implications in the UK because it led to the repeal of the Corn Laws and the fall of the government (Ramsbottom, 1953). To quote Disraeli: 'The mysterious malady of a single tuber changed the political history of the world.' As correctly deduced by Berkeley, the fungus had co-evolved with potato and its relatives in the

neotropics – the specific locale is now considered to be Mexico (Fry *et al.*, 1992) – and had arrived in Europe almost certainly through the introduction of infected germplasm.

Unfortunately, the story does not end with this initial invasion. The disease had a major resurgence in Europe in the 1980s. Prior to this recent event, it was determined that the European pathogen populations comprised only a single mating type and thus they were relatively uniform, often dominated by a single genotype. However, a second mating type was identified and its source was eventually traced to a second invasion from Mexico, probably in the 1970s (Fry *et al.*, 1992). Indeed, the threat from invasive plant pathogens should not be underestimated, because many of the world's major food crops are genetically vulnerable (Kingsolver *et al.*, 1983). In addition, fungal pathogens also pose an actual and potential threat to natural ecosystems, as well as to amenity trees and forestry. The catastrophic impact of successive invasions of Dutch elm disease, caused by *Ceratocystis ulmi*, on the UK and North American landscapes has been well documented and needs little introduction (Brasier, 1990; Liebold *et al.*, 1995). However, other tree diseases of fungal origin are less well known but have had equally detrimental socio-economic and environmental effects. For example, the Asian chestnut blight fungus, *Cryphonectria parasitica*, after its arrival on infected nursery stock in New York at the beginning of the 20th century, spread in several decades over large areas of the eastern seaboard, destroying almost all native American chestnut trees (*Castanea dentata*) by the early 1950s (Hepting, 1974). Before this invasion, American chestnut had dominated many forest ecosystems, forming up to 25% of the tree cover and constituting not only a high-value timber but also a vital source of food for wildlife (Anagnostakis, 1987). Whilst these pathogens have shown host specificity at the generic level and therefore have had an impact only on segments of the forest community, the root pathogen, *Phytophthora cinnamomi*, has a much broader host range, attacking plants from at least 48 different families, and has caused up to 75% losses in the native eucalyptus forests of western Australia (Weste and Marks, 1987). Since its suspected arrival in the 1920s, this plant pathogen has transformed the sclerophyll forests with a species-rich understorey into open woodland dominated by sedges (Burdon, 1987).

An even more recent threat from an invasive plant pathogen involves anthracnose disease of native dogwood (*Cornus* spp.) in the USA, caused by the fungus *Discula destructiva*, thought to have been imported on contaminated ornamentals from Asia in the 1970s (Daughtrey and Hibben, 1994). The devastation by this pathogen is worsening, as demonstrated by an increase in tree mortality in some areas from 33% in 1984 to nearly 80% 4 years later. Clearly, the long-term detrimental effects from the elimination of an important component tree on native forest ecosystems in North America will be immense, following changes in species composition and community dynamics (Hiers and Evans, 1997).

The Potential Hazards and Safety Concerns

The safety of fungi being contemplated for use in biological control must be considered at many levels, with primary concern for direct effects on vertebrates, especially humans. In considering safety toward all organisms, vertebrates, invertebrates and plants not intentionally being affected by the biological control fungus are referred to as 'non-target organisms'. The North American Microbial Biocontrol Working Group

identifies the following potential safety issues: (i) competitive displacement of non-target organisms; (ii) allergenicity; (iii) toxigenicity to non-target organisms; and (iv) pathogenicity to non-target organisms (Cook *et al.*, 1996). In addition, indirect effects, such as those that could come about through the depletion of the target host itself, must be considered as a potential safety issue (Goettel and Hajek, 2000). The potential unintended effects will differ depending on the potential target and non-target organisms and the ecosystems they inhabit.

Allergenicity

Fungi are capable of producing spores that cause allergies or allergic reactions; however, actual or potential fungal microbial control agents are not among the species that are responsible for the production of common allergens (Latgé and Paris, 1991). Nevertheless, there are reports of allergic reactions to microbial control fungi, principally with those exposed during mass production and application (Austwick, 1980). For instance, a group of scientists working with *B. bassiana* reported moderate to severe allergic reactions, and consequently the US Environmental Protection Agency (EPA) lists this fungus as a dermal sensitizer (Saik *et al.*, 1990). Furthermore, it has been demonstrated that crude extracts of *Metarhizium anisopliae* contain one or more potent allergens (Ward *et al.*, 1998). Since all fungi are potentially allergenic, it is necessary to avoid exposing unprotected humans during production and application.

Toxicity

Fungi secrete a wide variety of compounds, many of which are toxic to plants, invertebrates or vertebrates. *M. anisopliae* produces destruxins and cytochalasins; *B. bassiana* oosporein, beauvericin, bassianolide and beauveriolide, *Hirsutella thompsonii* hirsutellin, and *Trichoderma harzianum* peptaibols, to name a few. The role that many of these toxins play in pathogenesis is little understood; however, some may contribute to the ability of the fungus to overcome its host (see Chapter 12). However, toxin production varies according to isolate and does not necessarily play an important role in virulence in every case.

The production of toxins by a candidate fungal biocontrol agent should not necessarily immediately preclude its use in biocontrol. Considerations must be made as to possible effects from the presence of the toxin in the formulated product (e.g. danger to applicator), fate of the toxin after application (i.e. rate of degradation) and possible bioaccumulation of the toxin within the host or environment (e.g. danger to scavengers feeding on cadavers). For instance, the fungal pathogen *Fusarium nygamai* has been considered as a promising mycoherbicide for control of witch-weed (*Striga hermonthica*), a serious constraint on grain production in Africa (Abbasher and Sauerborn, 1992). However, recent work has shown that this fungus produces mycotoxins, including several novel compounds (Capasso *et al.*, 1996). Due to the potential threat to vertebrates, particularly humans and their animals, interest in developing this fungus as a mycoherbicide has waned.

Pathogenicity

Pathogenicity towards the target host is usually the desired effect. However, pathogenicity towards non-target organisms could be an unintended effect. Fungi, including species intended for biological control, can infect a wide variety of hosts, which sometimes include mammals. For instance, there are reports of *B. bassiana* infecting captive American alligators and a giant tortoise that had been stressed by chilling (Heimpel, 1971; Saik *et al.*, 1990; Semalulu *et al.*, 1992). Results of laboratory assays demonstrate that a strain of this fungus can also be pathogenic to embryos of the inland silverside fish, *Menida beryllina,* and the grass shrimp *Palaemonetes pugio*; conidia attached and germinated and hyphae subsequently penetrated the chorionic membrane within 5 days (Genthner and Middaugh, 1992; Genthner *et al.*, 1997). Using a similar laboratory assay system, Genthner and Middaugh (1995) reported that, *M. anisopliae* conidia adversely affected both embryos and newly hatched larvae of the inland silverside fish. These effects included decreased cardiac output, chorionic rupture and teratogenic expressions in embryos and larvae.

More recently, several cases of human infection by *M. anisopliae* have been reported in both immunocompetent and immunoincompetent individuals, with one fatality in an immunoincompetent child (Burgner *et al.*, 1998; Revankar *et al.*, 1999) There are numerous case reports that the nematode-destroying fungus *Paecilomyces lilacinus* is a causative agent of human infections in both immunoincompetent and immunocompetent individuals (Itin *et al.*, 1998; Gutierrez-Rodero *et al.,* 1999). This species was listed in 1999 as an important emerging nosocomial fungal pathogen by the National Foundation for Infectious Diseases (www.nfid.org/publications/clinicalupdates/fungal/noso.html) and has been recovered from tortoises, lizards, snakes, crocodiles and alligators at the London Zoo (Austwick, 1983). Another entomopathogenic fungus, *Conidiobolus coronatus*, is commonly associated with lesions in both humans and horses (Saik *et al.*, 1990). Clearly, evaluation of potential fungal microbial control agents must include an evaluation of their virulence towards non-target organisms, especially vertebrates, with consideration given to potential human exposure scenarios.

For many pathogens of invertebrates and weeds, the ability to infect is usually constrained within a host group (e.g. many pathogens of weeds can only infect other plants and cannot infect animals). Among non-vertebrate non-target organisms, safety concerns first focus on safety towards invertebrates and crop amenity plants used by humans. In addition, plants and animals that constitute the flora and fauna of release areas but with little direct relation to humans could be affected and safety evaluations should consider direct effects at this level also.

Depletion of hosts

The goal of any biological control programme is to lower the population of a pest. However, this reduction in the pest population may in turn detrimentally affect other non-target organisms that in one way or another depend on this pest. The extent of harm to the non-target population will very much depend on the extent and speed of the depletion of the host and on the length of time that the host has been in its targeted location (e.g. is it introduced or endemic?).

Over the years, the biocontrol of weeds – and specifically classical biological control

– has consistently been plagued by conflicts of interest, centring on the misconception that there will be a sudden depletion of the target weed host (Harris, 1985). Any exotic plant that has been deliberately imported for economic or ecological benefit or has subsequently acquired local added value, but which later invades and dominates native ecosystems or agriculture, may still have its 'supporters'. Thus, any threat to that plant resulting from the introduction of a natural enemy, such as a fungal pathogen, can create controversy and seriously or irrevocably disrupt a weed biocontrol programme. As Harris (1985) concluded, classical biological control must be done as a matter of public interest and with enabling legislation since the long-term effects cannot be restricted to individual properties or release sites. Thus, even if a biocontrol programme is clearly in the interest of the general public and supported by it, this support must be unanimous and unambiguous because of the rule-of-law principle.

Although depletion of alien weed populations may well be popular with both farmers and conservationists alike, individuals or organizations who derive or perceive (more usually, misperceive) financial or ecological benefits from the presence of the 'weed' have the power to prevent the release of biocontrol agents. Because this process usually involves court cases, there are a number of examples in which lengthy and often costly battles have had to be fought (McFadyen, 1998). Such a conflict of interest – still one of the most contentious disputes in the history of biocontrol – occurred in Australia with the boraginaceous plant, *Echium plantagineum*. This plant is variously known as Paterson's curse by farmers and Salvation Jane by bee-keepers, who rely on it as a dependable source of pollen during dry years. The case was eventually settled out of court after a considerable delay and cost to the biocontrol programme. On the positive side, however, it did result in the Australian Biological Control Act, which, for the first time, provided a legal basis for the introduction of exotic biocontrol agents (Cullen and Delfosse, 1985). Ironically, of course, classical biocontrol aims not to eliminate an alien plant – indeed, this can never be achieved with a co-evolved natural enemy – but to gradually reduce its competitive ability and thus restore the natural balance (see Chapter 6). In all probability, there will always be sufficient flowers from the remaining weed populations to satisfy the needs of the bee-keepers.

An even more extreme example, in the USA, involves salt-cedars (*Tamarix* spp.); alien shrubs from Eurasia, which have become the most serious threat to riparian ecosystems in western states, often completely replacing the native flora. Such has been the habitat change that the endangered southwestern willow flycatcher now relies almost entirely on salt-cedars for nesting (DeLoach *et al.*, 1996). Consequently, ornithologists, a powerful lobby in the USA, have thus far successfully blocked any attempts to implement a classical biocontrol strategy, despite the fact that a suite of potentially useful natural enemies has been identified in the plant's native range (DeLoach *et al.*, 1996). Ironically, nine species of rare birds and at least five other species of vertebrates have been identified as being endangered as a direct result of the salt-cedar invasion. It has taken more than a decade of research to overturn these well-meaning but sadly misguided and even irresponsible objections. Further historical and ecological studies have now demonstrated that the flycatcher populations had actually declined since the arrival of salt-cedar and that experimental removal of the weed has resulted in increased growth and density of willows, the preferred vegetation type of this bird species. Biological control is expected gradually to reduce *Tamarix* populations, with a corresponding increase in native shrubs, but without the intervening loss (originally predicted by the ornithologists) of nesting habitats (DeLoach *et al.*, 1999).

This issue is even more contentious when the target host is indigenous. The

arguments centring around the recent introduction of an Australian pathotype 3 (= *Entomophaga praxibuli*) of the grasshopper-pathogenic fungus *Entomophaga grylli* into the USA illustrate this point (Carruthers and Onsager, 1993; Lockwood 1993a, b). Lockwood (1993a) speculated that suppression or even extinction of target as well as non-target acridids may result in the loss of biodiversity, proliferation of new weed species and otherwise innocuous acridid species, disruption of plant community structure, suppression of essential organisms vectored by grasshoppers and disruption of food-chains and other nutrient cycling processes. In rebuttal, Carruthers and Onsager (1993) pointed out that endemic *E. grylli* pathotypes 1 and 2 (= *Entomophaga calopteni* and *Entomophaga macleodii*) already periodically produce epizootics in grasshopper populations and reduce outbreak populations of grasshoppers. However, pathotype 3 from Australia, although biologically very similar to pathotype 1, differed in that it could produce conidia in *Melanoplus* spp., an attribute that was lacking in pathotype 1. This was seen as advantageous as it would allow infection of two major pest species by a single species within a single season.

Extensive evaluations of the *E. grylli* species complex in both the USA and Australia provided detailed information on the biology and epizootiology of the fungus, and models were used to predict the response to the introduction of the Australian pathotype into the USA (Carruthers and Onsager, 1993). A detailed proposal was submitted to the US regulatory agency and permits were granted for the field release of this pathotype into North Dakota and Alaska. Releases were made and the pathogen was monitored. Despite initial evidence that the fungus established and increased in prevalence (Carruthers and Onsager, 1993), current evidence suggests that the frequency of infection has declined to levels such that long-term survival of this pathotype in North America is questionable (Bidochka *et al.*, 1996). Only time will tell if the fungus will resurface in future years.

Competitive displacement

Fungi introduced or applied as biocontrol agents have the potential to competitively occupy a niche, thereby adversely affecting one or more native organisms within that niche. This may be the intended effect with some fungal biocontrol agents. For instance, the saprophytic fungus *Phlebia gigantea* applied to freshly cut pine stumps competitively displaces *Heterobasidion annosum*, the cause of root rot of pine (Rishbeth, 1975). Unintended effects would occur if this competitive displacement were to seriously affect a non-target organism, perhaps even leading to its extinction or in some other way detrimentally affecting a component of the ecosystem. For example, fungal pathogens and insect parasitoids may compete within the tissue of the host insects (Goettel *et al.*, 1990; Vinson, 1990). The pathogen usually out-competes younger parasitoids, while older parasitoids are often capable of completing their development within fungus-infected insects.

Lockwood (1993a) suggested the possibility that the introduction of an exotic pathotype of *E. grylli* from Australia could suppress native North American grasshoppers, seriously affecting the 'natural' control of those species currently being regulated by the native *E. grylli* species through competitive displacement of the native *E. grylli* pathotypes. However, Carruthers and Onsager (1993) point out that there should be little concern if the introduced pathotype became the dominant grasshopper pathogen in rangeland and crop environments. Although exotic natural enemies may displace

native species, there are usually habitats in which these native species are able to coexist (Bennett, 1993).

Specific Fungal Attributes and Use Patterns Related to Hazard

The potential hazards and the degree of difficulty in assessing hazards will very much depend not only on the pathogen in question, but also on its intended use. Fungal biocontrol agents can be used in augmentative, classical and conservation biological control. In augmentative approaches, the fungi are introduced either in low numbers (i.e. inoculative augmentation) or in very large numbers (i.e. inundative augmentation), essentially as pesticides. In the classical approach, fungi are introduced into geographical habitats where they have not previously occurred. The intent here is that they become established and provide self-perpetuating, long-term control. In the conservation approach, the habitat or management practices are manipulated in order to favour the naturally occurring fungi.

Host range

As a group, fungi exhibit a very wide range of host specificity. Some species are very host-specific, while others are generalists and are known from a very wide array of hosts. Many species within Hyphomycetes are facultative pathogens and consequently have broad host ranges. However, individual strains generally exhibit specificity for a limited number of hosts (Glare and Milner, 1991). In contrast, many obligate pathogens, such as *E. grylli*, are restricted to several closely related host species (i.e. several species within Acrididae) (Carruthers and Onsager, 1993).

The range of species that a fungus can infect often differs between that found in the laboratory (physiological host range) and that found in nature (ecological host range) (Hajek and Butler, 2000). The physiological host range is determined from laboratory infection assays and demonstrates which hosts could potentially be infected under field conditions. The ecological host range can only be determined from field studies. Differences between physiological and ecological host ranges are thought to be the result of the complex biotic and abiotic conditions that occur in the field and have an impact on both pathogen and host susceptibility. Such conditions are not normally replicated in laboratory host-range tests. Consequently, in order to make laboratory-acquired data as applicable to the field situation as possible, every effort must be made to mimic the field situation (Butt and Goettel, 2000; Hajek and Goettel, 2000).

Dose-related susceptibility

Host susceptibility to fungal pathogens is, for the most part, dose-dependent. A threshold for infection is presumed to exist whereby a minimum number of fungal propagules is required. Thereafter, increasing numbers of propagules increase the probability of a successful infection. This dose–mortality relationship provides a built-in safety factor in the inundative-augmentation use of many facultative fungal pathogens as microbial insecticides, because high doses are present for only a relatively brief time.

Even though infection may be dose-dependent, susceptibility and disease transmission are also very much dependent on many abiotic factors. For instance, most fungi depend on high relative humidity for sporulation. If humidity conditions during periods when large numbers of host cadavers are present are not conducive to sporulation, adequate numbers for infection of subsequent generations will not become available and the induced epizootic will subside.

Persistence and dispersal

Spore dispersal in most fungi is passive, relying on wind and water. In the Oomycetes, however, the spores are motile and are reliant on the presence of water (e.g. a film of water on a leaf surface). In the Entomophthorales, spores are forcibly discharged and can land centimetres from the host or be carried longer distances on air currents.

For the most part, most spore types are very sensitive to ultraviolet (UV) radiation and consequently spores exposed to sunlight are short-lived. The half-life of some spores exposed to direct sunlight can be a matter of minutes. Persistence is generally much increased in shaded habitats and in soil. In contrast, the thick-walled resting spores produced by species within the Oomycetes and Zygomycetes and some ascomycetous fungi are capable of persisting for many years under adverse abiotic conditions.

Genetically altered fungi

Some of the goals of genetic modification of fungi intended for biological control are to expand the host range, increase the speed of kill by incorporating more toxic modes of action and extend persistence (see Chapter 8). Through this direct genetic manipulation, and especially through the introduction of foreign genetic material from organisms within and outside the fungal kingdom, it may be possible, either directly or indirectly, to change many attributes of a given fungus, which could drastically change its safety. Genetically altered organisms are unique in that they have never existed in nature in their altered form and consequently their potential hazards can only be definitively evaluated once the pathogen has been released into the environment. Changes in the ecological attributes of a fungus which, for instance, favour its virulence against a target host may also inadvertently allow the fungus to persist in new niches and affect non-target hosts previously not encountered. Consequently, it would be prudent to engineer fungi in such a way as to minimize their ability to persist in nature. Nevertheless, there is still the ever-present concern that the genetically modified organism may pass its newly acquired traits to another microorganism.

Although there are several entomopathogenic fungi that have been or soon will be genetically altered, none to date have been released in the wild, and therefore any predictions as to their potential detrimental effects would be purely speculative. Permission has recently been granted to release a genetically altered strain of *M. anisopliae* (R. St Leger, personal communication), which overproduces the cuticle-degrading Pr1 protease required for pathogenesis. This strain causes more rapid death of its host and the subsequent melanization response of the host prevents the fungus from further colonizing it and subsequently sporulating on the cadaver (see Chapter 8).

Non-indigenous vs. indigenous organisms

Fungal pathogens have the ability to replicate, spread, persist and adapt to new environments. Regardless of whether pathogens are detrimental or beneficial within their area of endemicity, their population levels are more or less in 'ecological balance'. In other words, the pathogens themselves, as well as their hosts and other organisms dependent on the hosts, adapt to the ecosystem and coexist together. If inoculum levels of indigenous fungi are artificially raised, as in inundative augmentation, there can be a relatively short-term perturbation of the ecosystem, which is usually the desired effect on the target host. Inoculum levels eventually return to pre-augmentation levels, and the host–pathogen 'balance' is eventually restored. Consequently, Goettel (1995) argues that very little attention need be paid to the host range of an indigenous fungus that is to be used inundatively in its native area. Similar arguments are brought forth by Keller (1998) who suggests that the closer the use of a fungus is to the natural situation, the fewer the requirements that should be imposed on hazard identification.

However, when a pathogen is introduced into a new environment – that is, to an area where it was previously non-indigenous – three outcomes are possible. The pathogen can simply not adapt and die out. It may survive and reproduce, but with little consequence to the overall ecosystem. Or the microorganism can spread, unimpeded, sometimes with devastating consequences to its host. These devastating consequences are usually the desired effect in classical control if the effects are restricted to the target host. But, if the host range was not as predicted, or if the host is beneficial, the ecological or economic results can be devastating, as seen in the example of the accidental introduction of Dutch elm disease into North America (see examples in the section on plant pathogens).

Mode of use: inundative vs. inoculative release

These very different strategies differ significantly in concerns regarding non-target effects. For classical biological control, the establishment of natural enemies is generally considered permanent and irreversible, so predicting the host range in the area of release is critical before release. The intent of this strategy is that, after establishment, the fungus will increase in response to host increases; in particular, highly host-specific pathogens are sought for these programmes so that their life cycles are closely tied to host populations.

In inundative augmentation, it is assumed that the fungus is already resident in the release area and that organisms in that area will only experience higher levels for relatively short periods. Therefore, this strategy would only have a potentially temporary impact on the release area. Even if there were detrimental effects to non-target organisms, these effects would only be temporary and, in essence, no different from using a synthetic chemical insecticide, although fungal pathogens are invariably much more host-specific. The strategy for inundative or augmentative biocontrol typically involves the mass production and application of a formulated product (e.g. a mycoherbicide). This, of course, can pose significant hazards, especially as regards human exposure. Potential human health effects include allergenicity, pathogenicity and exposure to toxic metabolites. However, these hazards are minimal if the same precautions in the application of mycopesticides are used as those that have been adopted for the

application of chemical pesticides. Nevertheless, it is indisputable that basic knowledge on possible effects on humans needs to be evaluated prior to the registration of a product based on a fungal pathogen, as is the basic requirement for any microbial control agent today.

Addressing the Hazards – Examples of Safe Use

Despite the many hazards that fungi possess as a whole, numerous species are being safely mass-produced and used for inundative augmentation to control pestiferous arthropods, nematodes and weeds without any apparent detrimental environmental or safety effects. Fungal pathogens have also been used for classical biological control of insects and weeds, although use of this latter strategy has been much more common with introductions of arthropods as natural enemies. Effects on the environment are considered before releases, irrespective of use strategy. Effects could differ by release strategy (e.g. introductions for classical biological control are permanent and this strategy usually focuses on perennial or natural systems while inundative augmentation usually involves release of high doses of a native pathogen in annual systems (see above)). The effects of outbreak pest populations on non-targets if no control is undertaken versus use of alternative controls (e.g. synthetic chemical pesticides) must always be weighed against potential effects of biological control agents on non-target organisms.

Hazards must be assessed and addressed at every step of the development and use of a fungal biocontrol agent. Steps must be undertaken in the manufacturing process to minimize human exposure, especially as concerns potential allergenicity or toxicity to workers, as is required in any industrial process involving allergens or toxic chemicals. This concern can be resolved by monitoring workers combined with production procedures that minimize human contact with the fungal agent. Formulations must be developed that maximize pathogen targeting and minimize drift or exposure to the applicators. Labels must clearly define the environments and targeted hosts where application is suitable. Potential dangers to non-target organisms must be addressed.

For preliminary estimates of host specificity, laboratory studies are frequently conducted exposing a diversity of species to a fungal pathogen, especially including predators and parasitoids that are already important in providing partial control of the pest. As previously mentioned, such assays should mimic the field situation as much as possible. Another type of preliminary data on host range is that gathered by collecting infected organisms in the field and identifying the cause of death. Although not always possible, the most accurate method for determining non-target impact is data from actual trials when fungi were released. However, few field studies have been conducted. Below we discuss issues and the relevant data that were used to determine safety of fungi that are currently used for biological control.

Fungi against insects

Inundative

At present, there are over a dozen commercial products based on nine principal fungal species registered worldwide for inundative use against invertebrate pests (Shah and

Goettel, 1999; see also Chapter 3). No apparent detrimental effects have been reported due to their use. The most common insect-pathogenic fungus produced for inundative augmentation is the hyphomycete *B. bassiana* (see Chapter 3). *B. bassiana* has been recorded from over 700 species of arthropods, many of which are non-target or beneficial hosts (Li, 1988; Goettel *et al.* 1990). However, most isolates are much more host-specific. For instance, isolate GHA, currently registered in several countries against an array of pests (Shah and Goettel, 1999) has been demonstrated as being innocuous under field conditions to many species that are included in the host list of *B. bassiana* (Goettel and Jaronski, 1997).

B. bassiana has been extensively tested, both in the early 1960s, by Nutrilite Products, Inc., and more recently, by Mycotech Corporation and Troy Biosciences, Inc. (Goettel and Jaronski, 1997). Tests have included repeated subcutaneous, intravenous and intraperitoneal injection of rats, as well as feeding studies. A human volunteer even taped 0.2 g of conidia on one arm for 8 h day^{-1} every other day for 12 doses with no evidence of dermatitis. No incidents of human hypersensitivity reactions have occurred despite years of mass production of the fungus by Mycotech Corporation (Goettel and Jaronski, 1997). Clearance studies in mice reported that a low dose (2×10^5 spores) delivered subcutaneously cleared within 2 days (Saik *et al.*, 1990; Semalulu *et al.*, 1992). No mortality occurred in embryos and larvae of the fathead minnow, *Pemephales promelas* (Goettel and Jaronski, 1997). Although there is no evidence that *B. bassiana* is infectious in these laboratory studies, there are reports in the literature of *B. bassiana* infecting vertebrates, especially reptilians (see section on 'Pathogenicity' above). However, there has not been an upsurge in reptilian infections since *B. bassiana* was commercialized. In fact, Mycotech and Troy Biosciences obtained exemptions from the requirement of a tolerance for residues of their strains in or on all food commodities when applied or used as a ground spray and aerial foliar sprays on terrestrial crops (Anon., 1995, 1999).

Field studies conducted in rangeland treated with *B. bassiana* to control grasshoppers demonstrated no infection among 2500 non-target arthropods killed at the time of field collection and assessed for overt colonization by the fungus (Goettel *et al.*, 1996). In lucerne, 10 days after application with *B. bassiana*, *c.* 20% of the leaf-cutting bees killed at time of collection demonstrated overt colonization by *B. bassiana*. However, there was no evidence that the fungus affected leaf-cutting bee larvae, diapausing prepupae or emerging next-generation adults. In lucerne, prevalence of *B. bassiana* colony-forming units in coccinellids and phalangids increased 2 days after application, but these effects were later demonstrated to be the result of ingested conidia within the digestive tract, rather than blastospores within the haemocoel, which would have been indicative of infection (M.S. Goettel, unpublished). It was concluded that the application of *B. bassiana* during their studies in rangeland and lucerne caused only minimal effects on non-target organisms.

M. anisopliae is another entomopathogen that is currently registered against several hosts, including cockroaches, grasshoppers, weevils and scarabs (Shah and Goettel, 1999; see also Chapter 3). This fungus has undergone extensive mammalian safety testing including inhalation and intraperitoneal injection in mice and rats, intraocular injection in rabbits, oral dosing of frogs and long-term feeding studies in rats (Burges, 1981; Shadduck *et al.*, 1982; Saik *et al.*, 1990) and birds (Smits *et al.*, 1999). No adverse effects were reported and there was no evidence of infectivity. However, Mycotech observed extreme toxicity to mice by an isolate of *M. anisopliae* and one of *M. anisopliae* var. *acridum* (Goettel and Jaronski, 1997). In addition, another isolate

of *M. anisopliae* var. *acridum* was associated with severe dermal hyperallergenic response in humans by Mycotech (S. Jaronski, personal communication).

Interestingly, Genthner and Middaugh (1995) reported that, in their laboratory assay system, *M. anisopliae* conidia adversely affected both embryos and newly hatched larvae of the inland silverside fish (see section on 'Pathogenicity' above). It is difficult to judge the utility of the assay system of Genthner and Middaugh for predicting the hazard posed by a fungal control agent in the environment. Their system is highly sensitive and demonstrates the ability of certain fungi to penetrate the chorion, but rejecting a candidate agent based on their assay seems unwarranted. It is more useful to use their assay to label candidates that may need more extensive vertebrate safety testing.

Although several cases of human infection have been reported (see section on 'Pathogenicity' above), *M. anisopliae* has been registered and deemed safe when used according to the label instructions. If reports of human infections increase, it is inevitable that the fungus will need to be re-evaluated.

The hyphomycete *Beauveria brongniartii* has been developed for application against the scarab pest *Melolontha melolontha* in Switzerland (Baltensweiler and Cerutti, 1986). After aerial applications to the forest/pasture ecozone where adult beetles aggregate to mate, 10,165 insects and spiders were collected and reared to detect infection. Overall infection among non-targets was only 1.1%, with no infections in coccinellids and neuropterans but up to 9% infection among spiders.

Classical

There are relatively few examples where fungi have been used for classical control of invertebrate pests. The most recent has been the attempted establishment of *E. grylli* from Australia for control of native North American grasshoppers. Although many predictions of detrimental effects were made, indications are that the fungus failed to establish (see section on 'Depletion of hosts' above). Another example is the introduction of the entomophthoralean fungus *Zoophthora radicans* into Australia from Israel to control the introduced spotted alfalfa aphid. Non-target effects were never seen, especially with regard to the hymenopteran parasitoid *Trioxys complanatus*, which was also introduced as part of this control programme (Glare and Milner, 1991).

Releases of the Asian entomophthoralean pathogen *Entomophaga maimaiga* were made in 1910–1911. However, the pathogen was not found to be established in the target North American populations of *Lymantria dispar* until 1989 (Hajek, 1999). It is unknown whether this pathogen became established during releases in 1910–1911 (it was not detected from 1911 to 1989) or from a more recent accidental introduction. Initial studies demonstrated that this fungus would only infect lepidopteran larvae. Further evaluations of the host range demonstrated low levels of infection in a number of lepidopteran families (Hajek *et al.* 1995). These studies were followed by collecting and rearing non-target Lepidoptera from foliage during epizootics caused by *E. maimaiga* in *L. dispar* populations (Hajek *et al.*, 1996). Among the > 1500 insects collected and reared, only two individuals of two common species were found to be infected by *E. maimaiga*. Because later instars of *L. dispar* spend significant amounts of time in the leaf litter, where large titres of *E. maimaiga* spores occur, lepidopteran larvae in the litter were also sampled. Although high levels of infection occurred among *L. dispar* larvae, only two other litter-dwelling lepidopteran larvae, of two different species, were infected by *E. maimaiga* (Hajek *et al.*, 2000). It was concluded that this pathogen is highly specific with very little risk to non-target organisms, including non-target lepidopterans.

Nevertheless, an ecologist questioned whether fungal epizootics causing declines in *L. dispar* populations might have an adverse impact that was indirect (i.e. due to rapid host depletion (see section on 'Depletion of hosts' above). With huge declines in *L. dispar* populations, would the other natural enemies linked with *L. dispar* then decline due to lack of hosts and not be able to respond quickly enough in order to control this pest if the fungus were not active? In this hypothesized scenario, the resulting unstable system would be even more prone to occasional outbreak populations of *L. dispar* (Valenti, 1998). This theory was proposed based on 1992 epizootics that devastated *L. dispar* populations, but to date (2000) there is absolutely no evidence that *E. maimaiga* drives other natural enemies of *L. dispar* to such low levels that they cannot respond if *L. dispar* begins once again to increase (A.E. Hajek, unpublished data). In fact, semi-field (Malakar *et al.*, 1999) and empirical field (Hajek, 1997) studies of interactions between *E. maimaiga* and the *L. dispar* nuclear polyhedrosis virus suggest minimal to no negative interactions between these two virulent pathogens.

Fungi against nematodes

Many studies have been conducted investigating the use of fungi for control of plant-parasitic nematodes and, more recently, nematodes parasitizing livestock. In fact, nematode-attacking fungi are considered to be fairly common members of the soil community. Several fungal species attacking plant-parasitic nematodes are now mass-produced for control. Care must be taken so that fungi introduced to control plant-parasitic nematodes do not also affect insect-parasitic nematodes, beneficial fungi, such as mycorrhizae, or other beneficial members of the rhizosphere (see Chapter 5). To date, no detrimental effects on the rhizosphere microbial community have been documented, but few studies have been conducted to address non-target effects of nematophagous fungi. Due to the great diversity of soil inhabitants and the complexity of interactions within the soil, non-target studies are difficult to conduct. However, we can draw inferences from knowledge of the host specificity of nematophagous fungi. Nematode-attacking fungi utilize a diversity of types of associations with their hosts but these can generally be grouped as predatory or parasitic (Stirling, 1991). Predatory fungi have specialized structures, e.g. sticky rings and/or pegs for trapping nematodes. Among the predatory fungi, *Arthrobotrys* has received the most attention and methods for mass-producing and applying *Arthrobotrys irregularis* have been developed (Cayrol, 1983). Predatory fungi are not considered very specific to certain species of nematode prey (Barron, 1977; Kerry, 2000). Rosenzweig *et al.* (1985) found that, for nine nematodes tested, including free-living as well as plant and insect parasites, seven adhesive-producing nematode-trapping fungi were non-selective and were able to trap and consume all of the different nematodes. For a different fungus (*Monacrosporium ellipsosporum*), which traps nematodes using sticky knobs, ten of the 15 nematode species tested were trapped (Gaspard and Mankau, 1987).

More recently, emphasis has shifted to the use of parasitic fungi attacking sedentary stages of nematodes, e.g. saccate females and eggs. Among these parasitic fungi, much research has been directed toward two species: *P. lilacinus* and *Verticillium chlamydosporium* (Kerry, 2000). These fungi are opportunistic parasites, showing little host specificity, although isolates can differ in their ability to attack eggs of different nematode species. In addition, these fungi do not only attack nematodes; in particular, *V. chlamydosporium* is known to infect other organisms, including fungal spores and

eggs of snails and slugs, and it occurs in soil when root-knot and cyst nematodes are not present (Kerry and Crump, 1998; see also Chapter 5). Interestingly, although *V. chlamydosporium* populations do not depend only on populations of nematode hosts, a direct association has been found between the numbers of chlamydospores and the numbers of the plant-parasitic *Heterodera avenae* in the soil (Kerry and Crump, 1998).

P. lilacinus has been registered as a product for the control of nematodes. Mammalian safety tests included acute oral, dermal and pulmonary toxicity tests in rats and irritation studies in rabbits, and they indicated the relative safety of this microorganism (http://www.ticorp.com.au/safety.htm). However, there are numerous case reports that *P. lilacinus* is a causative agent of human infections (see section on 'Pathogenicity' above). This fungus is currently undergoing the tests necessary for registration in Australia. Given the numerous citations of its ability to infect humans, it would not be surprising if more extensive vertebrate testing is required for registration. It has been suggested that possibly the isolates of *P. lilacinus* collected from nematodes do not present a human health risk (see Chapter 5).

Perhaps the most unusual use of a fungus for nematode control is that of *Myrothecium verrucaria*. This soil-dwelling hyphomycete is mass-produced for the control of numerous species of endo- and ectoparasitic nematodes, but, after the fungus has been grown *in vitro*, it is killed before application (Warrior *et al.*, 1999). Anti-nematode effects are caused by multiple active ingredients that act synergistically to cause indirect effects; after application, the soil microcosm becomes an inhospitable habitat for nematodes, affecting motility, host/mate finding and egg development and increasing the parasitism of nematode eggs. Applications of this heat-killed fungus have been shown to enhance antagonism toward root-knot nematodes and are associated with structural and functional changes in the rhizosphere bacterial community (J.W. Kloepper, personal communication). Toxicological testing has demonstrated no effect of this product on aquatic invertebrates and tests have demonstrated no effects on an animal-parasitic nematode (*Nippostrongylus brasilense*) or free-living species of nematodes (*Caenorhabditis elegans* and *Panagrellus redivivus*) (Warrior *et al.,* 1999).

Fungi against weeds

Inundative

As discussed previously, the fungal pathogens currently being used or evaluated as mycoherbicides can either have narrow or wide host ranges and this will be dependent upon the crop ecosystem in which that particular product is being targeted and/or the dispersal capacity of the constituent pathogen. One of the first products, Collego™, developed for use against the leguminous weed, *Aeschynomene virginica*, in rice ecosystems in the southern USA, was based on a supposedly host-specific strain or *forma specialis* of *Colletotrichum gloeosporioides* (Templeton, 1982). Subsequently, however, it was shown to have an expanded host range within the Leguminosae, attacking several economically important plants, including several bean species (TeBeest, 1988). Nevertheless, this spectrum has been assessed as posing no danger to agriculture, since the product is used exclusively within rice-based cropping systems, far removed from potentially susceptible crops, and, in addition, the pathogen has poor dispersal ability.

A similar strategy has been adopted for the use of the high-profile crop pathogen *Phytophthora palmivora* and, in what was a pioneering venture, a product (DeVine™)

was developed for the control of strangle-vine (*Morrenia odorata*) in citrus orchards in Florida (Ridings, 1986). Although the strain used is also pathogenic to cucurbitaceous crops, the product can be safely applied as long as there are no susceptible crops within 200 m of the treated orchard: appropriate labelling to this effect is, of course, mandatory (Charudattan, 1991).

A more recent and potentially more hazardous venture has involved the development of a mycoherbicide based on the silver-leaf fungus, *Chondrostereum purpureum*, which was once a notifiable disease in Europe and subject to strict legislation. This was targeted specifically at the invasive North American black cherry (*Prunus serotina*), but is also used to control not only this woody weed but also other exotic hardwood species in the Netherlands. The product, Biochon™, is applied as a mycelial suspension to cut stumps to prevent re-sprouting. The risk analysis of *C. purpureum*, which is a well-documented pathogen of plantation and ornamental *Prunus* spp., was based almost entirely on epidemiological data (De Jong *et al.*, 1990).

Conceptual and simulation models, supplemented by inoculation experiments, were developed to predict the risks posed to non-targets by artificially increased populations of pathogens (De Jong *et al.*, 1991). It was demonstrated that, although the risk to susceptible crops is high up to 500 m from the treated area, this falls dramatically thereafter and is negligible at 5000 m. Thus, the product has been approved for safe use as long as there are no *Prunus* orchards within this range.

Classical

The inherent safety and stability of co-evolved natural enemies has been emphasized recently (Marohasy, 1996; McFadyen, 1998). After more than 100 years of experience of classical biocontrol of weeds, involving the release of over 600 exotic agents, there are only eight examples of damage to non-target plants. Five were anticipated, all were entirely predictable behavioural responses and not the result of host 'shift' and, most importantly, none had any significant economic or environmental impact. All these examples involved insect agents, whilst all of the 20 or more fungal pathogens so far used for classical weed biocontrol have proved to be extremely safe, with a high success rate (Evans, 2000). Nevertheless, there is still a general fear or mistrust of the concept of exploiting exotic plant pathogens by many countries, which is rarely expressed if the project involves insects for weed control. For various reasons, plant pathogens are adjudged to pose more of a risk, probably based on historical associations with invasive crop diseases (see section on 'Plant pathogens' above).

In contrast, Australia has shown some degree of plasticity or leniency in vetting or approving the release of exotic plant pathogens that have extended host ranges, attacking plant species other than the target weed, and these cases have been documented recently (Evans, 2000). An example is the introduction of a rust, *Uromyces heliotropii*, into Australia for control of the alien weed *Heliotropium europaeum*. Despite the fact that endemic *Heliotropium* spp. had proved to be susceptible in greenhouse tests, Hasan and Delfosse (1995) argued successfully that the Australian and alien *Heliotropium* spp. never overlapped in their ranges and that climatic conditions in the non-target areas were unsuitable anyway for rust establishment and development, and, therefore, that the risks were low. A similar, potential impasse was also faced and surmounted in the case of rubber-vine weed, *Cryptostegia grandiflora* (*Asclepiadaceae*), in Queensland. The co-evolved rust *Maravalia cryptostegiae*, from Madagascar, was shown to be pathogenic to a rare, endemic asclepiad (Evans and Tomley, 1994). However, it

was concluded that the risks involved were minimal and that the rust was safe to release, since the symptoms were not severe, and probably due to the severity of the testing protocol (Wapshere, 1989), and the only known habitat of the native species is itself endangered, as well as being both geographically distant and climatically distinct from the predicted range of the weed. Moreover, the threat posed by the weed to entire ecosystems far outweighed the potential loss of one species (Evans, 2000).

Even more recently, the microcyclic rust, *Puccinia melampodii,* from Mexico, has been approved for importation into Australia as part of an integrated strategy for management of the composite weed *Parthenium hysterophorus*, even though it has been found to sporulate on a related indigenous, albeit weedy, plant species, as well as on several sunflower and marigold cultivars (Evans, 2000). Once again, it was concluded by the Australian Quarantine and Plant Inspection Service that the actual and potential hazards involved in not attempting to control this allergenic weed, especially the threat to human health, were significantly greater than the perceived risks to non-target plants.

In summary, based on the protocols now in place and the experience gained, the classical introduction of fungal pathogens for the control of alien invasive plants is a safe, environmentally benign, economic and potentially sustainable strategy for long-term weed management.

Regulations and Registration

We have seen how fungi can be successfully and safely used as both classical and inundative biological control agents. However, we have also seen that fungi as a whole can possess properties that make them potentially hazardous both to the user and to the environment in general. Consequently, the development and use of fungi as biocontrol agents requires an assessment of their potential hazards. In most countries, regulations and registration requirements serve two major purposes: (i) to ensure the safety of the agent; and (ii) to ensure efficacy. The major challenge facing the regulatory community is to adequately address safety issues without at the same time unduly slowing research and impeding the development and implementation of microbial control.

In many cases, the intended use of the organism and its origin will determine the type of regulatory oversight that will be required. For instance, in most countries, the importation of a non-indigenous organism intended for classical biological control will be regulated differently (and in many cases fall under different legislation) from the regulation of microorganisms intended for inundative use, be they indigenous or not. Here we attempt to provide an overview of the regulations and registration requirements for microbial control in general, while emphasizing as much as possible the specific requirements or concerns related to fungal microbial control agents.

Regulation of fungi as classical biocontrol agents

Most countries regulate the importation and release of biological control agents, including pathogens, through legislation designed specifically for the exclusion of noxious agents. For example, the Animal and Plant Health Inspection Service (APHIS) of the US Department of Agriculture (USDA) until recently regulated the importation and release of microorganisms under the Federal Plant Pest Act and the Plant Quarantine

Act (OTA, 1995). Regulation of biological control agents through such plant pest statutes often posed difficulties, in that the acts addressed biological control agents in the context of noxious weeds or other concerns. For instance, in the USA, there were jurisdictional problems concerning the granting of permits for release of agents because the acts only covered the movement of agents and not their release (OTA, 1995). On 20 June 2000, a new Plant Protection Act was passed by the US Congress. This Act repealed ten statutes, including the Plant Quarantine Act and the Federal Plant Pest Act. For the first time, it recognizes biological control agents as beneficial tools, separating them from 'pests' in the context of the definitions (Henstridge, 2000; P. Henstridge, personal communication). It adds a new definition of biological control based on the definition under the International Plant Protection Convention. It also separates the definition entirely from the definition of plant pest. This changes the focus from negative to positive and places the correct emphasis on biocontrol as a beneficial control method. It contains language that emphasizes the need to avoid impeding commerce where there is no risk. For example, it includes provisions that clarify the Secretary's authority to allow for organisms or groups of organisms to be exempt from regulation once it is determined that they do not present a risk. In addition, there is a provision clarifying an individual's right to petition the Secretary to have an organism added or removed from regulation and contains language that emphasizes the need to facilitate commerce in beneficial organisms.

Australia is the only country that has enacted legislation – the Australian Biological Control Act – that deals specifically with biological control. Nevertheless, biological control agents are still regulated under other acts, such as the Quarantine Act and the Wildlife Protection Act; the Biological Control Act is invoked only as a last resort in controversial situations.

Recently, the Food and Agriculture Organization of the United Nations developed a code of conduct for the import and release of exotic biological control agents (FAO, 1996). The objectives of the code are to facilitate the regulation of exotic biological control agents through introducing internationally acceptable procedures and practices.

The most important part of the code as far as safety is concerned is paragraph 4.5. It states that the importer of biological agents must include:

> …an analysis of the risks posed to possible nontarget organisms and to the environment generally and should entail available emergency procedures should the biological control agent after release display unexpected adverse properties. The dossier should also contain a report detailing laboratory tests, and or field host range of the candidate agent. Testing should be based on recommended procedures and approved by the authority. These tests should relate to the candidate agent only and different procedures should apply to any additives used in formulations of products which contain biological control agents.

The difficulty lies in that the tests and information that are appropriate and are adequate to ensure that these conditions are met are not apparent and are certainly open to interpretation. Nevertheless, this code addresses the importance of conducting a risk assessment prior to the introduction of an exotic agent.

When the concept of employing plant pathogens for weed control was first put into practice in the 1970s, it was almost taken for granted that the fungal agents had to have an extremely narrow host range and, preferably, that they should be specific to the target weed. As was discussed (see section on 'Addressing the Hazards' above), this is no longer a necessary prerequisite. For inoculative or classical biological con-

trol, the main emphasis is, and must always be, placed on high specificity to the weed target or its near relatives. Thus, the most intensive, time-consuming and expensive part of the programme involves determining the host range of the candidate pathogen, for which the centrifugal, phylogenetic screening protocol or methodology has been developed and subsequently modified (Wapshere, 1975, 1989). The strategy that underpins the classical biological control approach is based on the theory that only those natural enemies that have co-evolved with the target weed in its centre of origin or diversity will have the necessary attributes of high virulence and specificity for successful classical agents. The initial biocontrol programmes concentrated on testing plants of economic value in the release area instead of the more relevant concept of genetic relatedness. The centrifugal, phylogenetic screen now offers a scientific and dependable method of host-range testing with a proven track record (McFadyen, 1998). In fact, the aforementioned Australian Biological Control Act does not demand that the exotic agent selected for importation be host-specific, but only that it should not cause any significant harm to any person or to the environment. The Act thereby allows a certain degree of latitude, both in interpreting the results of host-range screening and in the predicted host range of the candidate agent (see section on 'Addressing the Hazards' above).

One of the main difficulties that remain as far as entomopathogenic fungi are concerned is the lack of acceptable evaluation methods that would adequately address host range (Hajek and Goettel, 2000). Laboratory bioassays, which are at present almost exclusively relied upon to establish host range as far as most regulations for the importation of exotic entomopathogens are concerned, measure the 'physiological host range'. However, it has been established that the physiological host range does not adequately predict the 'ecological host range', the range manifested under field conditions (Hajek and Butler, 2000). But, by the careful design and use of laboratory and semi-field assays that incorporate pertinent parameters and through detailed knowledge of the ecology and epizootiology within the area of endemicity of the pathogen in question, information for pertinent risk assessment for non-target organisms as a result of the introduction of entomopathogenic fungi can be obtained (Hajek and Goettel 2000).

Because agents intended for classical control are expected to have minimal contact with humans, they are generally exempt from mammalian safety testing. Concerns would arise if a potential agent were hyperallergenic and were spread aerially in large numbers during epizootics, but such fungi are not being pursued for introductions.

Regulation of fungi as microbial pesticides

Mycopesticides are often based on an indigenous rather than an exotic fungal pathogen. Hence, the selected pathogen is already 'in the system' but, for one reason or another, is not effective in controlling its host. The emphasis here is no longer on host specificity but on ensuring, through epidemiological analysis, that the mycopesticide poses no danger to neighbouring useful plants or insects and especially that it should not establish in a new, non-target host (see section on 'Addressing the Hazards' above). It is highly unlikely that an indigenous pathogen would establish itself in a new host as a result of inundative use. If some non-target organisms were to be affected, it would be expected that such an effect would be only temporary and only occur within the immediate area of application, as is currently the case with most chemical insecticides

used at present. Unfortunately, most registration requirements for indigenous microbial control agents still require relatively extensive laboratory host-range testing; this is a requirement that is not generally demanded of most chemical pesticides because, *a priori*, a wide host range is expected.

Most countries require registration of fungi destined to be used inundatively as microbial pesticides. And, as with agents destined for the classical control approach, one of the difficulties in regulation of these agents is that, once again, they are regulated by legislation initially designed for chemical pesticides. For instance, in the USA, microbial pesticides are regulated and registered by the EPA under the Federal Insecticide, Fungicide and Rodenticide Act. Even though the EPA developed special data requirements for microbial pesticides in the early 1980s, many problems existed until the mid-1990s when the EPA adopted a separate review system for microbial pesticides from that for conventional pesticides (OTA, 1995). Some problems with the methodology still exist.

An Organization for Economic Cooperation and Development (OECD) survey of data requirements for the registration of biological pesticides in OECD member countries found that there were real differences in certain data requirements among the respondent countries, most notably in physical-chemical properties, ecotoxicology and environmental fate. This situation exists even though a similar approach was used to structure data requirements (Table 13.1) and emphasis was placed on the same test areas or study categories (OECD, 1996). These differential data requirements have been one of the most detrimental aspects of regulations of microbial control products. In many cases, a company wishing to register a product in several countries would have to provide different registration packages for each country, each with different data requirements and formatting, thereby significantly increasing the costs of registration of products, many of which are useful for niche markets at best.

A promising development in recent years has been the move towards harmonization of regulatory requirements among countries. For instance, under the auspices of a North American Free Trade Agreement Technical Working Group on Pesticides, the USA, Canada and Mexico have worked together to harmonize data requirements for registration of microbial pest control products between their countries (NAFTA, 1998), making joint reviews possible (NAFTA, 1999). Similar attempts are being made to develop common registration requirements for the European Union (Neale and Newton, 1999). An even more ambitious project is to standardize and harmonize core

Table 13.1. Data requirements for the registration of a microbial pathogen. (Adapted from OECD, 1996.)

Identity
Physical, chemical and biological properties
Function, mode of action and handling
Manufacturing, quality control and analytical methods
Residues
Efficacy[a]
Toxicology
Ecotoxicology
Fate and behaviour in the environment

[a]USEPA requires efficacy data only for pesticide products used to control pests that threaten public health.

data requirements for the registration of microbial pesticides within the 29 countries that are members of the OECD (OECD, 1999).

The goal of vertebrate safety testing is to assess the hazard posed by a candidate microbial pest control agent (MPCA) by means of carefully selected laboratory tests. The World Health Organization (WHO) was the first organization to propose a tiered testing strategy to evaluate the hazard posed by MPCAs to mammals (Anon., 1981b). Elements of this proposal are incorporated into the current regulatory guidelines for Canada, the USA, and the European Union. These tests replaced the long-term assays that are used in assessing chemical insecticides with short-term (1 month) exposures that utilize invasive routes, such as intravenous and/or intraperitoneal injection, as well as feeding studies. This battery of tests is referred to as Tier 1 (US Code of Federal Regulations 40 CFR 158.740c). If questions arise during Tier 1 testing, the candidate then goes through a second battery of tests that are more extensive. A third tier of tests is also available, but it is unlikely that an MPCA that does not clear Tier 2 will go to that level, because it would most probably no longer be considered as a viable control product at this stage. Unfortunately, many of these data are unavailable to the public because they are considered proprietary. However, the results of some of these acute tests have been published in peer-reviewed publications. Routes of exposure in these published studies include inhalation, subcutaneous injection, intraperitoneal injection, intravenous injection, dermal and ocular irritancy testing, dermal sensitization studies and feeding studies (Anon., 1981b; Burges, 1981; Shadduck *et al.*, 1982; Siegel and Shadduck, 1987; Mier *et al.*, 1989, 1994; Kerwin *et al.*, 1990; Saik *et al.*, 1990; Semalulu *et al.*, 1992; Goettel and Jaronski, 1997; Ward *et al.*, 1998; Smits *et al.*, 1999).

The MPCA does not have to be harmless in all tests and at all concentrations, but, rather, the circumstances under which it produces infection or mortality must be elucidated. Historically, these tests have emphasized assessing the infectivity and pathogenicity of a candidate organism, but recently concerns have been raised about assessing the allergenicity of MPCAs and these tests may be added to the protocols necessary for registration (Ward *et al.*, 1998). Aspects of the biology (such as maximum temperature tolerated) of the MPCA are considered when designing tests as well as possibly vulnerable organ systems, based on literature reviews. This latter point may be complicated by the taxonomic status of an MPCA. Medical reports may only identify a vertebrate isolate to genus or, in some cases, the vertebrate isolate may have been misidentified (Siegel *et al.*, 1997). These questions can be addressed during safety testing by choosing a particular route of exposure, such as inhalation or intravenous injection, and/or by extensively characterizing the isolate proposed as the MPCA. Characterization includes both taxonomy (this can include differentiating between entomopathogenic and non-entomopathogenic strains) and identification of toxins and/or toxic metabolites.

Regulatory agencies utilize the data on hazard to determine risk (hazard × exposure). It is unlikely that an MPCA that successfully passes these screens will cause problems when released in the field, but a possibility, perhaps remote, always exists that there may be susceptible species or a unique scenario in the field that may result in infection or mortality. The perspective necessary for evaluating both safety data and field reports was succinctly stated by Burges (1981):

> a no-risk situation does not exist, certainly not with chemical pesticides, and even with biological agents one cannot absolutely prove a negative. Registration of a chemical is essentially a statement of usage in which risks are acceptable and the same must be applied to biological agents.

Concluding statements

History has already demonstrated that fungi can be effectively and safely used in biological control. As we become more familiar with microbial control agents, and microbial ecology in general, regulations are being adopted to address the pertinent concerns. A key is that registration requirements for biocontrol fungi must remain flexible and address the hazards of the specific candidate in question and its proposed use on a case-by-case basis. The allowances for exemptions to many data requirements are of paramount importance. And hazards must also be weighed in consideration of the benefits of microbials, which, to date, almost always outweigh those of chemical pesticides.

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References

Abbasher, A.A. and Sauerborn, J. (1992) *Fusarium nygamai*, a potential bioherbicide for *Striga hermonthica* control in sorghum. *Biological Control* 2, 291–296.

Agrios, G.N. (1997) *Plant Pathology* (4th edn). Academic Press, San Diego.

Anagnostakis, S.L. (1987) Chestnut blight: the classical problem of an introduced pathogen. *Mycologia* 79, 23–37.

Anderson, D.L. and Gibson, N.L. (1998) New species and isolates of spore-cyst fungi (Plectomycetes: Ascosphaerales) from Australia. *Australian Systematic Botany* 11, 53–72.

Anon. (1981a) Studies on the relationship between the application of *Beauveria bassiana* against the pine caterpillar and the silkworm muscardine occurrence. In: *Proceedings on the Biocontrol of Forest Pests.* Forestry Press Cina, Beijing, p. 142.

Anon. (1981b) Mammalian safety testing of microbial control agents for vector control: a WHO memorandum. *Bulletin World Health Organization* 59, 857–863.

Anon. (1995) *Beauveria bassina* strain GHA: exemption from the requirement of a tolerance. *United States Federal Register* 60, 18547.

Anon. (1999) *Beauveria bassiana* (ATCC #74040): exemption from the requirement of a tolerance. *United States Federal Register* 64 (81), 22793–22796.

Austwick, P.K.C. (1980) The pathogenic aspects of the use of fungi: the need for risk analysis and registration of fungi. *Ecological Bulletin (Stockholm)* 31, 91–102.

Austwick, P.K.C. (1983) Some mycoses of reptiles. *Review of Medical and Veterinary Mycology* 18, 779.

Baltensweiler, W. and Cerutti, F. (1986) Bericht über die Nebenwirkungen einer Bekämpfung des Maikäfers (*Melolontha melolontha* L.) mit dem Pilz *Beauveria brongniartii* (Sacc.) Petch auf die Arthropodenfauna des Waldrandes. *Mitteilungen der Schweizerischen Entomologische Gesellschaft* 59, 267–274.

Barron, G.L. (1977) *The Nematode-destroying Fungi.* Canadian Biological Publications, Guelph, Ontario.

Bassi, A. (1835) Del mal del segno, calcinaccio o moscardino, malattia che affigge i bachi da seta e sul modo di laberarne le bigattaie anche le pi infestate. *Patre prima. Teoria*, Orcesi, Lodi, 67 pp. (Trans. by Ainsworth, A.C. and Yarrow, P.J. in *Phytopathological Classics*, 10, Monumental Printing, Baltimore, Maryland, 49 pp.)

Bell, J.V. (1974) Mycoses. In: Cantwell, G.E. (ed.) *Insect Diseases.* Marcel Dekker, New York, pp. 185–236.

Bennett, F. (1993) Do introduced parasitoids displace native ones? *Florida Entomologist* 76, 54–63.

Bidochka, M.J., Walsh, S.R.A., Ramos, M.E., St Leger, R.J., Silver, J.C. and Roberts, D.W. (1996) Fate of biological control introductions: monitoring an Australian fungal pathogen of grasshoppers in North America. *Proceedings of the National Academy of Sciences USA*, 93, 918–921.

Bradbear, N. (1988) World distribution of major honeybee diseases and pests. *Bee World* 69, 15–39.

Brasier, C.M. (1990) China and the origins of Dutch elm disease: an appraisal. *Plant Pathology* 39, 5–16.

Burdon, J.J. (1987) *Diseases and Plant Population Biology.* Cambridge University Press, Cambridge.

Burges, H.D. (1981) Safety, safety testing and quality control of microbial pesticides. In: Burges, H.D. (ed.) *Microbial Control of Pests and Plant Diseases 1970–1980.* Academic Press, London, pp. 738–767.

Burgner, D., Eagles, G., Burgess, M., Procopis, P., Rogers, M., Muir, D., Pritchard, R., Hocking, A. and Priest, M. (1998) Disseminated invasive infection due to *Metarhizium anisopliae* in an immunocompromised child. *Journal of Clinical Microbiology* 36, 1146–1150.

Butt, T.M. and Goettel, M.S. (2000) Bioassays of entomogenous fungi. In: Navon, A. and Ascher, K.R.S. (eds) *Bioassays of Entomopathogenic Microbes and Nematodes.* CAB International, Wallingford, UK, pp. 141–195.

Capasso, R., Evidente, A., Cutignano, A., Vurro, M., Zonno, M.C. and Bottalico, A. (1996) Fusaric and 9,10-dehydrofusaric acids and their methyl esters from *Fusarium nygamai. Phytochemistry* 41, 1035–1039.

Carruthers, R.J. and Onsager, J.A. (1993) Perspective on the use of exotic natural enemies for biological control of pest grasshoppers (Orthoptera: Acrididae). *Environmental Entomology* 22, 885–903.

Cayrol, J.-C. (1983) Lutte biologique contre les *Meloidogyne* au moyen d'*Arthrobotrys irregularis. Revue de Nematologie* 6, 265–273.

Charudattan, R. (1991) The mycoherbicide approach with plant pathogens. In: TeBeest, D.O. (ed.) *Microbial Control of Weeds.* Chapman & Hall, New York, pp. 24–57.

Cook, R.J., Bruckart, W.L., Coulson, J.R., Goettel, M.S., Humber, R.A., Lumsden, R.D., Maddox, J.V., McManus, M.L., Moore, L., Meyer, S.F., Quimby, P.C., Stack, J.P. and Vaughn, J.L. (1996) Safety of microorganisms intended for pest control and plant disease control: a framework for scientific evaluation. *Biological Control* 7, 333–351.

Cullen, J.M. and Delfosse, E.S. (1985) *Echium plantagineum*: catalyst for conflict and change in Australia. In: Delfosse, E.S. (ed.) *Proceedings of the Sixth International Symposium on Biological Control of Weeds.* Agriculture Canada, Ottawa, pp. 249–252.

Daughtrey, M.L. and Hibben, C.R. (1994) Dogwood anthracnose: a new disease threatens two native *Cornus* species. *Annual Review of Phytopathology* 32, 61–73.

De Jong, M.D., Scheepens, P.C. and Zadoks, J.C. (1990) Risk analysis for biological control: a Dutch case study in biocontrol of *Prunus serotina* by the fungus *Chondrostereum purpureum. Plant Disease* 74, 189–194.

De Jong, M.D., Wagenmakers, P.S. and Goudriaan, J. (1991) Modelling the escape of

Chondrostereum purpureum spores from a larch forest with biological control of *Prunus serotina. Netherlands Journal of Plant Pathology* 97, 55–61.

DeLoach, C.J., Gerling, D., Fornasari, L., Sobhain, R., Myartseva, S., Mityaev, I.D., Lu, Q.G., Tracy, J.L., Wang, R., Wang, J.F., Kirk, A., Pemberton, J.W., Chikatunov, V., Jashenko, R.V., Johnson, J.E., Zheng, H., Jiang, S.L., Liu, M.T., Liu, P.A. and Cisnero, J. (1996) Biological control programme against saltcedar (*Tamarix* spp.) in the United States of America: progress and problems. In: Moran, V.C. and Hoffmann, J.H. (eds) *Proceedings of the Ninth International Symposium on Biological Control of Weeds.* University of Cape Town, Cape Town, South Africa, pp. 253–260.

DeLoach, C.J., Carruthers, R. and Lovich, J. (1999) Ecological interactions in the biological control of saltcedar (*Tamarix* spp.) in the United States. *Abstracts of the Tenth International Symposium on Biological Control of Weeds.* University of Montana, Bozeman, p. 152.

Evans, H.C. (1998) The safe use of fungi for biological control of weeds. *Phytoprotection* 79, S67–S74.

Evans, H.C. (2000) Evaluating plant pathogens for biological control of weeds; an alternative view of pest risk assessment. *Australian Plant Pathology* 29, 1–14.

Evans, H.C. and Tomley, A.J. (1994) Studies on the rust, *Maravalia cryptostegiae*, a potential biological control agent of rubber-vine weed, *Cryptostegia grandiflora* (Asclepiadaceae: Periplocoideae), in Australia. III: Host range. *Mycopathologia* 26, 93–108.

FAO (1996) *Code of Conduct for the Import and Release of Exotic Biological Control Agents.* International Standards for Phytosanitary Measures, Secretariat of the International Plant Protection Convention, Food and Agriculture Organization of the United Nations, Publication No. 3, Rome, 21 pp.

Flexner, J.L., Lighthart, B. and Croft, B.A. (1986) The effects of microbial pesticides on non-target, beneficial arthropods. *Agriculture, Ecosystems and Environment* 16, 203–254.

Fry, W.E., Goodwin, S.B., Marusak, J.M., Spielman, L.J. and Milgroom, M.G. (1992) Population genetics and intercontinental migrations of *Phytophthora infestans. Annual Review of Phytopathology* 30, 107–129.

Gaspard, J.T. and Mankau, R. (1987) Density-dependence and host-specificity of the nematode-trapping fungus *Monacrosporium ellipsosporum. Reviews in Nematology* 10, 241–246.

Genthner, F. J. and Middaugh, D.P. (1992) Effects of *Beauveria bassiana* on embryos of the inland silverside fish (*Menidia beryllina*). *Applied Environmental Microbiology* 58, 2840–2845.

Genthner, F. J. and Middaugh, D.P. (1995) Nontarget testing of an insect control fungus: effects of *Metarhizium anisopliae* on developing embryos of the inland silverside fish *Menidia beryllina. Diseases of Aquatic Organisms* 22, 163–171.

Genthner, F.J., Foss, S.S. and Gals, P.S. (1997) Virulence of *Metarhizium anisopliae* to embryos of the grass shrimp *Palaemonetes pugio. Journal of Invertebrate Pathology* 69, 157–164.

Gilliam, M. and Vandenberg, J.D. (1990) Fungi. In: Morse, R.A. and Nowogrodzki, R. (eds) *Honey Bee Pests, Predators, and Diseases.* Comstock Publishing, Ithaca, New York, pp. 65–90.

Gilliam, M., Taber, S., III, Lorenz, B.J. and Prest, D.B. (1988) Factors affecting development of chalkbrood in colonies of honey bees, *Apis mellifera*, fed pollen contaminated with *Ascosphaera apis. Journal of Invertebrate Pathology* 52, 314–325.

Glare, T.R. and Milner, R.J. (1991) Ecology of entomopathogenic fungi. In: Arora, D.K., Ajello, L. and Mukerji, K.G. (eds) *Handbook of Applied Mycology*, Vol. 2. Marcel Dekker, New York, pp. 547–612.

Goettel, M.S. (1995) The utility of bioassays in the risk assessment of entomopathogenic fungi. In: *Biotechnology Risk Assessment: USEPA/USDA, Environment Canada, Agriculture and Agri-Food Canada Proceedings of the Biotechnology Risk Assessment Symposium, June 6–8, 1995, Pensacola, Florida.* University of Maryland Biotechnology Institute, College Park, Maryland, pp. 2–7.

Goettel, M.S. and Hajek, A.E. (2000) Evaluation of nontarget effects of pathogens used for management of arthropods. In: Wajnberg, E., Scott, J.K. and Quimby, P.C. (eds) *Evaluating*

Indirect Ecological Effects of Biological Control. CAB International, Wallingford, UK, pp. 81–97.

Goettel, M.S. and Jaronski, S.T. (1997) Safety and registration of microbial agents for control of grasshoppers and locusts. In: Goettel, M.S. and Johnson, D.L. (eds) *Microbial Control of Grasshoppers and Locusts. Memoirs of the Entomological Society of Canada* 171, 83–99.

Goettel, M.S. and Johnson, D.L. (1992) Environmental impact and safety of fungal biocontrol agents. In: Lomer, C. and Prior, C. (eds) *Biological Control of Locusts and Grasshoppers*. CAB International, Wallingford, UK, pp. 356–361.

Goettel, M.S., Poprawski, T.J., Vandenberg, J.D., Li, Z. and Roberts, W.D. (1990) Safety to nontarget invertebrates of fungal biocontrol agents. In: Laird, M., Lacey, L.A. and Davidson, E.W. (eds) *Safety of Microbial Insecticides.* CRC Press, Boca Raton, Florida, pp. 209–232.

Goettel, M.S., Inglis, G.D., Duke, G.M. and Lord, J.C. (1996) *Effect of* Beauveria bassiana (*Mycotech Strain GHA*) *on Invertebrates in Rangeland and Alfalfa Ecosystems.* Final Report for the United States Environmental Protection Agency, Washington, DC, pp. 81–97.

Gutierrez-Rodero, F., Moragon, M., Ortiz de la Tabla, V., Mayol, M.J. and Martin, C. (1999) Cutaneous hyalohyphomycosis caused by *Paecilomyces lilacinus* in an immunocompetent host successfully treated with itraconazole: case report and review. *European Journal of Clinical Microbiology and Infectious Diseases* 18, 814–818.

Hajek, A.E. (1997) Fungal and viral epizootics in gypsy moth (Lepidoptera: Lymantriidae) populations in central New York. *Biological Control* 10, 58–68.

Hajek, A.E. (1999) Pathology and epizootiology of the lepidoptera-specific mycopathogen *Entomophaga maimaiga. Microbiology and Molecular Biology Review* 63, 814–835.

Hajek, A.E. and Butler, L. (2000) Predicting the host range of entomopathogenic fungi. In: Follett, P.A. and Duan, J.J. (eds) *Nontarget Effects of Biological Control.* Kluwer Academic Publishers, Dordrecht, the Netherlands, pp. 263–276.

Hajek, A.E. and Goettel, M.S. (2000) Guidelines for evaluating effects of entomopathogens on nontarget organisms. In: Lacey, L.A. and Kaya, H.K. (eds) *Manual of Field Techniques in Insect Pathology.* Kluwer Academic Publishers, Dordrecht, the Netherlands, pp. 847–868.

Hajek, A.E., Butler, L. and Wheeler, M.M. (1995) Laboratory bioassays testing the host range of the gypsy moth fungal pathogen *Entomophaga maimaiga. Biological Control* 5, 530–544.

Hajek, A.E., Butler, L., Walsh, S.R.A., Silver, J.C., Hain, F.P., Hastings, F.L., O'Dell, T.M. and Smitley, D.R. (1996) Host range of the gypsy moth (Lepidoptera: Lymantriidae) pathogen *Entomophaga maimaiga* (Zygomycetes: Entomophthorales) in the field versus laboratory. *Environmental Entomology* 25, 709–721.

Hajek, A.E., Butler, L., Liebherr, J.K. and Wheeler, M.M. (2000) Risk of infection by the fungal pathogen *Entomophaga maimaiga* among Lepidoptera on the forest floor. *Environmental Entomology* 29, 645–650.

Harris, P. (1985) Biocontrol of weeds: bureaucrats, botanists, beekeepers and other bottlenecks. In: Delfosse, E.S. (ed.) *Proceedings of the Sixth International Symposium on Biological Control of Weeds.* Agriculture Canada, Ottawa, pp. 3–13.

Hasan, S. and Delfosse, E.S. (1995) Susceptibility of the Australian native *Heliotropium crispatum*, to the rust fungus *Uromyces heliotropii*, introduced to control common heliotrope, *Heliotropium europaeum. Biocontrol Science and Technology* 5, 165–174.

Heimpel, A.M. (1971) Safety of insect pathogens for man and vertebrates. In: Burges, H.D. and Hussey, N.W. (eds) *Microbial Control of Insects and Mites.* Academic Press, New York,. pp. 469–489.

Henstridge, P. (2000) New Plant Protection Act passed by Congress. *Inoculum* August 2000, p. 6. (Supplement to *Mycologia* 51, 6.)

Hepting, G.H. (1974) Death of the American chestnut. *Forest History* 18, 60–67.

Hiers, J.K. and Evans, J.P. (1997) Effects of anthracnose on dogwood mortality and forest composition of the Cumberland Plateau (USA). *Conservation Biology* 11, 1430–1435.

Hussey, N.W. and Tinsley, T.W. (1981) Impressions of insect pathology in the People's Republic

of China. In: Burges, H.D. (ed.) *Microbial Control of Pests and Plant Diseases 1970–1980*. Academic Press, London, pp. 785–795.

Itin, P.H., Frei, R., Lautenschlager, S., Buechner, S.A., Surber, C., Gratwohl, A. and Widmer, A.F. (1998) Cutaneous manifestation of *Paecilomyces lilacinus* infection induced by a contaminated skin lotion in patients who are severely immunosuppressed. *Journal of the American Academy of Dermatology* 39, 401–409.

Keller, S.K. (1998) Use of fungi for pest control in sustainable agriculture. In: *Sustainable Pest Management: Safe Use of New Organisms in Biological Control. Phytoprotection* 79 (suppl.), 56–60.

Kerry, B.R. (2000) Rhizosphere interactions and the exploitation of microbial agents for the biological control of plant-parasitic nematodes. *Annual Review of Phytopathology* 38, 323–441.

Kerry, B.R. and Crump, D.H. (1998) The dynamics of the decline of the cereal cyst nematode, *Heterodera avenae*, in four soils under intensive cereal production. *Fundamental and Applied Nematology* 21, 617–625.

Kerwin, J.L., Dritz, D.A. and Washino, R.K. (1990) Confirmation of the safety of *Lagenidium giganteum* (Oomycetes: Lagenidiales) to mammals. *Journal of Economic Entomology* 83, 374–376.

Kingsolver, C.H., Melching, J.S. and Bromfield, K.R. (1983) The threat of exotic plant pathogens to agriculture in the United States. *Plant Disease* 67, 595–600.

Klingman, D.L. and Coulson, J.R. (1982) Guidelines for introducing foreign organisms into the U.S. for biological control of weeds. *Plant Disease* 66, 1205–1209.

Laird, M., Lacey, L.A. and Davidson, E.W. (eds) (1990) *Safety of Microbial Insecticides.* CRC Press, Boca Raton, Florida.

Large, E.C. (1940) *The Advance of the Fungi.* Jonathon Cape, London.

Latgé, J.P. and Paris, S. (1991) The fungal spore: reservoir of allergens. In: Cole, G.T. and Hoch, H.C. (eds) *The Fungal Spore and Disease Initiation in Plants and Animals.* Plenum Press, New York, pp. 379–401.

Li, Z.Z. (1988) A list of insect hosts of *Beauveria bassiana.* In: Li, Y.W., Li, Z.Z., Liang, Z.Q., Wu, J.W., Wu, Z.K., and Xi, Q.F. (eds) *Study and Application of Entomogenous Fungi in China.* Academic Periodical Press, Beijing, PRC, pp. 10–14.

Liebold, A.M., MacDonald, W.L., Bergdahl, D. and Mastro, V.C. (1995) Invasion by exotic forest pests: a threat to forest ecosystems. *Forest Science* 41, 1–49.

Lockwood, J.A. (1993a) Environmental issues involved in biological control of rangeland grasshoppers (Orthoptera: Acrididae) with exotic agents. *Environmental Entomology* 22, 503–518.

Lockwood, J.A. (1993b) Benefits and costs of controlling rangeland grasshoppers (Orthoptera: Acrididae) with exotic organisms: search for a null hypothesis and regulatory compromise. *Environmental Entomology* 22, 909–914.

Malakar, R., Elkinton, J.S., Hajek, A.E. and Burand, J.P. (1999) Within-host interactions of *Lymantria dispar* L. (Lepidoptera: Lymantriidae) nucleopolyhedrosis virus (LdNPV) and *Entomophaga maimaiga* (Zygomycetes: Entomophthorales). *Journal of Invertebrate Pathology* 73, 91–100.

Marohasy, J. (1996) Host shifts in biological weed control: real problems, semantic difficulties or poor science? *International Journal of Pest Management* 42, 71–75.

McFadyen, R.E.C. (1998) Biological control of weeds. *Annual Review of Entomology* 43, 369–393.

Mier, T., Perez, J., Carrilli-Farga, J. and Toriello, C. (1989) Study on the innocuity of *Hirsutella thompsonii.* I. Infectivitiy in mice and guinea pigs. *Entomophaga* 34, 105–110.

Mier, T., Rivera, F., Rogriguez-Ponce, M.P., Carrillo-Farga, J. and Toriello, C. (1994) Infectivity of the entomopathogenic fungus *Verticillium lecanii* in mice and guinea pigs. *Reviews of Latin-American Microbiolology* 36, 107–111.

NAFTA (1998) *Harmonization of data requirements for microbials.* NAFTA Technical Working Group on Pesticides, Ottawa, Project Sheet, 16 June.

NAFTA (1999) *Joint Review of a Microbial Pesticide.* NAFTA Technical Working Group on Pesticides, Project Sheet R9904FE, February.

Neale, M. and Newton, P. (1999) Registration/regulatory requirements in Europe. In: Hall, F.R. and Menn, J.J. (eds) *Methods in Biotechnology*, Vol 5, *Biopesticides: Use and Delivery.* Humana Press, Totowa, New Jersey, pp. 453–471.

OECD (1996) *Data Requirements for Registration of Biopesticides in OECD Member Countries: Survey results.* OECD Environment Monograph No. 106, Organization for Economic Cooperation and Development, Paris, 121 pp.

OECD (1999) *OECD Pesticide Projects.* Organization for Economic Cooperation and Development, Report OECD99-02, Paris.

OTA (1995) *Biologically Based Technologies for Pest Control.* US Congress, Office of Technology Assessment, OTA-ENV-636, Government Printing Office, September, Washington, DC.

Prior, C. (1990) The biological basis for regulating the release of micro-organisms, with particular reference to the use of fungi for pest control. *Aspects of Applied Biology* 24, 231–238.

Ramsbottom, J. (1953) *Mushrooms and Toadstools: a Study of the Activities of Fungi.* Collins, London.

Revankar, S.G., Sutton, D.A., Sanche, S.E., Rao, J., Zervos, M., Dashti, F. and Rinald, M.G. (1999) *Metarhizium anisopliae* as a cause of sinusitis in immunocompetent hosts. *Journal of Clinical Microbiology* 37, 195–198.

Ridings, W.H. (1986) Biological control of stranglervine in citrus – a researcher's view. *Weed Science* 34, 31–32.

Rishbeth, J. (1975) Stump inoculation: a biological control of *Fomes annosus.* In: Bruehl, G.W. (ed.) *Biology and Control of Soil-borne Plant Pathogens.* American Phytopathological Society, St Paul, Minnesota, pp. 158–162.

Rosenzweig, W.D., Premachandran, D. and Pramer, D. (1985) Role of trap lectins in the specificity of nematode capture by fungi. *Canadian Journal of Microbiology* 31, 693–695.

Saik, J.E., Lacey, L.A. and Lacey, C.M. (1990) Safety of microbial insecticides to vertebrates-domestic animals and wildlife. In: Laird, M., Lacey, L.A. and Davidson, E.W. (eds) *Safety of Microbial Insecticides.* CRC Press, Boca Raton, Florida, pp. 115–134.

Semalulu, S.S., MacPherson, J.M., Schiefer, H.B. and Khachatourians, G.G. (1992) Pathogenicity of *Beauveria bassiana* in mice. *Journal of Veterinary Medicine* B39, 81–90.

Shadduck, J.A., Roberts, D.W. and Lause, S. (1982) Mammalian safety tests of *Metarhizium anisopliae*: preliminary results. *Environmental Entomology* 11, 189–192.

Shah, P.A. and Goettel, M.S. (1999) *Directory of Microbial Control Products and Services.* Society for Invertebrate Pathology, Gainesville, Florida, 31 pp.

Siegel, J.P. (1997) Testing the pathogenicity and infectivity of entomopathogens to mammals. In: Lacey, L.A. (ed.) *Manual of Techniques in Insect Pathology.* Academic Press, London, pp. 325–336.

Siegel, J.P. and Shadduck, J.A. (1987) Some observations on the safety of the entomopathogen to mammals. *Journal of Economic Entomology* 80, 994–997.

Siegel, J.P., Smith, A.R. and Novak, R.J. (1997) Cellular fatty acid analysis of a human isolate alleged to be *Bacillus sphaericus* and *Bacillus sphaericus* isolated from a mosquito larvicide. *Journal of Applied and Environmental Microbiology* 63, 1006–1010.

Smits, J.E., Johnson, D.L. and Lomer, C. (1999) Pathological and physiological responses of ring-necked pheasant chicks following dietary exposure to the fungus *Metarhizium flavoviride*, a biocontrol agent for locusts in Africa. *Journal of Wildlife Diseases* 35, 194–203.

Steinhaus, E.A. (1975) *Disease in a Minor Cord*. Ohio State University Press, Columbus, 488 pp.

Stirling, G.R. (1991) *Biological Control of Plant Parasitic Nematodes: Progress, Problems and Prospects.* CAB International, Wallingford, UK.

TeBeest, D.O. (1988) Additions to host range of *Colletotrichum gloeosporioides* f. sp. *aeschynomene. Plant Disease* 72, 16–18.

Templeton, G.E. (1982) Status of weed control with plant pathogens. In: Charudattan, R. and Walker, H.L. (eds) *Biological Control of Weeds with Plant Pathogens.* John Wiley and Sons, New York, pp. 29–44.

Valenti, M.A. (1998) *Entomophaga maimaiga*: salvation from the gypsy moth or fly in the ointment? *American Entomologist* 44, 20–22.

Vinson, S.B. (1990) Potential impact of microbial insecticides on beneficial arthropods in the terrestrial environment In: Laird, M., Lacey, L.A. and Davidson, E.W. (eds) *Safety of Microbial Insecticides.* CRC Press, Boca Raton, Florida, pp. 43–64.

Wapshere, A.J. (1974) A strategy for evaluating the safety of organisms for biological weed control. *Annals of Applied Biology* 77, 201–211.

Wapshere, A.J. (1975) A protocol for programmes for biological control of weeds. *PANS* 21, 295–303.

Wapshere, A.J. (1989) A testing sequence for reducing rejection of potential biological control agents for weeds. *Annals of Applied Biology* 114, 515–526.

Ward, M.D.W., Sailstad, D.M. and Selgrade, M.K. (1998). Allergic responses to the biopesticide *Metarhizium anisopliae* in Balb/c mice. *Toxicological Sciences* 45, 195–203.

Warrior, P., Rehburger, L.A., Beach, M., Grau, P.A., Kirfman, F.W. and Conley, J.M. (1999) Commercial develoment and introduction of DiTera™, a new nematocide. *Pesticide Science* 55, 376–379.

Weste, G. and Marks, G.C. (1987) The biology of *Phytophthora cinnamomi* in Australasian forests. *Annual Review of Phytopathology* 25, 207–229.

14 Fungal Biological Control Agents – Appraisal and Recommendations

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It is clear from an analysis and synthesis of the preceding chapters that considerable progress has been made in the development of fungal biological control agents (BCAs). This has been achieved in part through multidisciplinary approaches, often involving multinational research groups. Although niche markets exist for fungal BCAs, considerable progress still needs to be made in the following areas:

- Technical – production, formulation and application systems.
- Agronomic – integration of BCAs into cropping systems.
- Socio-economic – public perception, economic feasibility.
- Political – improved registration procedures, improved extension services, support for small–medium-size enterprises (SMEs) and organic growers.

In this chapter we summarize our analysis of the preceding chapters, briefly discuss the technical and agronomic issues, including the perception of SMEs, and, where appropriate, make recommendations. The socio-economic and political issues are outside the scope of this book. Some of these issues have recently been reviewed by Butt and Copping (2000). It should be stressed that all these issues have to be put in perspective: if there is no market, the issues become irrelevant. Numerous niche markets do exist for fungal BCAs. The estimated world market for pesticides in 1995 was *c.* $29 billion, with the biopesticide share being *c.* $380 million (Menn and Hall, 1999). The growth rate for biopesticides is expected to increase over the next 10 years, with fungal BCAs probably having a substantial share of this market (Moore and Prior, 1993).

Technical Issues

The following need to be dealt with for the development of more efficacious fungal BCAs.

Improved speed of action of the fungal BCA

One major criticism of fungal BCAs is that they act slowly and give limited protection to crops from pests and diseases. For effective pest control, more aggressive strains of fungal BCAs should be sought, i.e which work more quickly and require less inoculum. Virulence determinants should be identified and used in strain selection and quality control. Some progress has been made in this area; enzymes and metabolites have been identified which are important determinants of virulence or antagonism (e.g. Butt *et al.*, 1998; Amiri-Besheli *et al.*, 2000; Bandani *et al.*, 2000, see also Chapter 8). For disease control, priming of inocula, particularly of spores, needs to receive more attention, especially with regard to effective control in the rhizosphere and phyllosphere.

Greater ecological fitness

Many fungi that perform well in the laboratory are less effective in the field. The quality of fungal BCAs prior to use has received very little attention. For effective field performance, the formulations must consistently be able to tolerate a wide range of climatic (fluctuating temperatures, humidities, ultraviolet (UV) light), edaphic (soil types) and biotic (antagonists) factors. The ecological fitness of strains can be improved through physiological manipulation of growth conditions to channel useful endogenous reserves into the inoculum (Magan, Chapter 9). The physiologically modified inoculum will germinate at humidities slightly lower than unmodified inoculum. This, combined with improved formulations, could greatly improve the field efficacy of fungal BCAs.

Cost-effective production of fungal BCAs

Production costs need to be reduced to allow competitive pricing with conventional pesticides. The products must remain viable for up to 2 years under commercial storage conditions and maintain biocontrol efficacy. The end-product should be easy to handle and package. Cultural conditions must be identified, without increasing production costs, to overcome problems of loss of biocontrol. For pest control, particular attention needs to be paid to problems of attenuation of virulence. At present, little progress has been made in this area, partly because the underlying mechanisms of attenuation have not been elucidated.

Improved formulation of fungal BCAs

Good results are often obtained in trials carried out with fresh inoculum but when the inocula have been formulated control is often less effective. For any crop protection agent, efficient formulation is necessary to translate laboratory activity into adequate field performance (Burges, 1998; see also Chapter 10). In many cases the formulation of BCAs is very different from that of chemicals. This has not often been appreciated and has resulted in less progress being achieved in this area. For BCAs, different methods of preparation, from wet pastes to fluidized-bed drying, spray drying and freeze-drying, have not been investigated exhaustively. During this process,

additives can be incorporated to enable viability to be conserved. Some progress has been made, but it is evident that investment is needed to improve field efficacy of fungal BCAs and expand their niche (and ultimately market). While experience exists with pesticide formulations, less knowledge is available in relation to biological material. Furthermore, formulation with appropriate targeting and spraying systems could further improve the consistency and efficacy of BCAs (see Chapters 10 and 11). New, more effective formulation additives and appropriate stickers need to be investigated that are compatible with other BCAs (viruses, bacteria and entomophilic nematodes). The net result will be that the farmer can integrate BCA products in spray regimes in a single tank mix or at different times without one formulation/BCA affecting the other, and concomitantly reducing pesticide use.

Effective targeting of fungal BCAs

This is needed for the efficacious control of pests, weeds and diseases. Because control is dose-related, strategies need to be devised to ensure that sufficient amounts of the pathogen contact the target. Autodissemination/autoinoculation devices are being tested (Pell *et al.*, 1993; Vega *et al.*, 2000); insects are attracted to a device by specific olfactory and visual cues, they become contaminated with a BCA and, on leaving the device, they then act as vectors of the BCA, which could be for the control of pests, weeds or diseases. Recently, honey-bee-mediated dissemination of BCAs has been patented (Gross *et al.*, 1994); the bees can be more efficient than conventional sprayers in delivering the inoculum to the pest-infested flowers – trials with the insect pathogen *Metarhizium anisopliae* increased pollen beetle (*Meligethes* spp.) control in oil-seed rape (Butt *et al.*, 1998). To optimize the impact of the BCA, particularly for pest control, it could be used in the 'push–pull' pest control strategy. Briefly, this entails insect pests being driven ('pushed') out of the main crop with feeding deterrents and drawn ('pulled') into a trap crop, where they could be controlled by inundation with pathogens. To encourage pests into the trap crop, lures, such as favoured plant varieties that are more attractive than the crop and chemical attractants such as sex pheromones and gustatory stimulants, could be used. Feeding attractants incorporated into the formulation may be useful for encouraging insects to feed on the BCA. As yet, very few inexpensive, effective lures and deterrents have been developed for commercial use. Recently, Amiri *et al.* (1999) demonstrated that antifeedants can drive pests to the underside of leaves treated with *M. anisopliae*. The abaxial surface of leaves was a conducive environment for fungal infection of mustard-beetle larvae. Ideally, BCA products should be capable of application through the standard hydraulic sprayer or application equipment that is common in a particular market and should have as few unique requirements as possible. Growers are unlikely to invest in new spray equipment solely to apply a BCA, nor are they going to accept a very different spray regime or more frequent applications than is normal practice. Therefore, knowledge is required of how well formulations of BCAs survive such applications and how, using standard application techniques, targeting to prevent wastage can be included as part of the strategy for delivery of BCAs for effective control.

Improved packaging, shelf-life and sales

The formulation type and packaging materials must be similar to those with which the grower is already familiar. The grower will also want to purchase his/her BCAs through the same distribution chain as his/her agrochemicals. Distributors will want to handle BCA formulations in the same way as their normal chemical stock, and expect the product to be packaged in standard sizes and types of containers as used throughout the agrochemical industry. Storage stability must be such that product purchased at the start of one season is good for the whole of that season and the next, without any special storage requirements. If the shelf-life of a BCA formulation is very limited, a distributor may be prepared to buy only small quantities, thus limiting availability, or will only stock products on a consignment basis, which is a major inconvenience to the BCA supplier. Furthermore, some BCAs have a specific need for refrigeration for conserving viability; few distributors in Europe have such facilities and even fewer would be prepared to invest in them.

Understanding tritrophic interactions

There is growing evidence that the host plant can affect the efficacy of BCAs through dilution of the inoculum during growth, physical interference of the inoculum (trapping spores in epicuticular waxes) and via exudates and allelochemicals (Inyang *et al.*, 1998, 1999a, b). Some plants when ingested by insects can increase the susceptibility of insects to fungal infection (T.M. Butt, unpublished observations; see also Chapter 3). The exact mechanisms for this are unknown. This is an important area for future research.

Population dynamics

Although information has been generated on selected pests, weeds and diseases, comparatively little work has been done on the environmental fate of fungal BCAs and their impact on target organisms and plants. There is an urgent need to study the relationships between entomogenous fungi and their host(s) in the field and to identify vulnerable stages of the target. This could optimize the impact of BCAs. Studies on population dynamics could also provide invaluable information on fungal persistence in the environment, the fate of the inoculum and any genetic shift due to parasexual or sexual recombination (Leal-Bertioli *et al.*, 2000). Indeed, registration documentation requires information on the environmental fate of and potential side-effects in non-target fauna and flora. Besides models, there is a need to develop molecular markers to characterize fungal strains (Leal *et al.*, 1994; Driver *et al.*, 2000). The models could be useful in the study of epizootics/epidemics and lead to more effective deployment of fungal BCAs. When combined with safety studies (i.e. impact on non-target organisms), they could lead to timely applications of the pathogen to minimize any negative impact on beneficial non-target organisms, thus optimizing the impact of each component. Furthermore, these studies would reveal if pests could develop resistance to fungal BCAs. Evidence is growing that insects possess potent antifungal peptides, which could also be specificity determinants (Ekengren and Hultmark, 1999; T.M. Butt, unpublished observations).

Bioactive compounds

Bioactive compounds of BCAs, sometimes referred to as toxins, are a major concern, because some people believe they are a health risk (Strasser *et al.*, 2000). Very little is known about these compounds. The following topics need urgent study:

1. Development of the methods and tools to screen for bioactive compounds from fungal BCAs. This would facilitate rapid screening of toxins from BCAs and identification of strains that are efficacious BCAs but low toxin producers. The methodologies and tools developed could also help detect toxins in foodstuffs and the environment (target and non-target hosts, plant, soil, water).
2. Studies to determine the role of bioactive compounds. Are they pathogenicity determinants? Do they help in the survival of the BCA? Are they waste products?
3. Studies of the mode of action of bioactive compounds to see: (i) if they pose a risk to living systems; and (ii) if they have any commercial value as pharmaceutical drugs, agrochemicals or research tools.

Safety

Safety is a major concern of all parties developing fungal BCAs. More studies are needed to evaluate the risks involved in the use of fungal BCAs, and particular attention should focus on: (i) allergenic properties; (ii) risks of toxic metabolites; (iii) genetic recombination and displacement of natural strains; and (iv) effect on biodiversity (i.e. impact on non-target organisms). These data would be useful for registration purposes and could reduce development costs considerably.

Agronomic Issues

There are still many agronomic issues that need to be resolved, a few of which are discussed below.

Compatibility of fungal BCAs with other BCAs and agrochemicals

This needs investigation so that growers know which agents can be used in the same tank mix. For example, entomogenous fungi may be harmful to entomophilic nematodes, or fungicides used for disease control may kill entomogenous fungi. Industry should work closely with researchers and extension service workers in resolving these matters before the products reach the market.

Development of crop protection strategies

The problem faced by developers of fungal BCAs for the control of disease is complex and difficult. Crops are grown under a variety of climatic and environmental conditions, and temperature, rainfall, soil type, crop variety and pathogen can change from farm to farm or some even within one field. The producer of a crop protection product has to be able to give some assurance to the farmer that the product will be

robust in order for the product to be used. The availability of effective chemical controls for foliar pathogens has made it unlikely that a biological agent will compete effectively. It is therefore not surprising that the majority of effort in research has been concentrated on soil-borne or postharvest diseases. Even in these situations, the lack of robustness has limited the penetration of such products.

Natural plant resistance and transgenic plants

There is still considerable scope for exploiting plant natural resistance to pests and diseases and integrating this into an overall sustainable crop protection strategy. However, the widening opportunities offered by genetic modification have influenced research and development quite dramatically during the past decade. Large companies are more likely to support this area because of the intellectual property rights (IPR) afforded by producing unique products. Widespread use of transgenic plants is also seen as an opportunity and a potentially major constraint, through engineering for both herbicide tolerance and for inclusion of insecticidal activity. Both have potential, either indirectly, by removal of non-crop plants through widespread herbicide usage, or directly, by continuous expression of insecticidal genes, to induce resistance in target organisms. There may also be potential effects from reducing the effectiveness of more conventionally applied microbial agents. It is important that risk assessment of these new agents is improved, supported by ecological research to assess both the beneficial and potentially non-beneficial effects of the new technology.

Organic farming

Organic farming currently forms a minor part of European agriculture but is rapidly expanding. In some countries, it has gained acceptance on up to 8% of farms. Recent emphasis in policy towards more environmentally friendly farming practices and the importance of surplus reduction has led to more attention to organic farming and the establishment of specific policy provisions, e.g. European Community (EC) regulation 2078/92 (agricultural production methods compatible with the requirements of the protection of the environment and the maintenance of the countryside) and EC regulation 2092/91 (certification of organic food). The premium prices for organically grown produce offer some incentive to farmers to convert to organic farming. But there is an urgent need for more investment in this area, particularly in topics that would benefit both organic and conventional growers, e.g. funding of projects that integrate agronomic practices used by organic growers with natural agents for increased productivity. Another area for collaboration is in the development of tools and strategies to monitor pest influxes (e.g. traps or trap crops) and optimize the impact of artificially introduced BCAs and natural predators and parasitoids (e.g. application of BCAs at night, when conditions favour these organisms but minimize risk to parasitoids).

Perception of SMEs

Large commercial companies have relatively little interest in wild-type microbial agents, because these have more limited markets than chemical pesticides and, even where

markets have been established, they may not be able to protect their IPR sufficiently to make the initial investment. These conclusions point to opportunities for exploitation in markets where there are problems of resistance to conventional pesticides or in niche markets where there are no viable conventional alternatives.

There is a lack of international harmonization in the registration process for microbials, which drives up costs. Although the toxicological criteria for safety are similar in most countries (usually a tier testing system), there is little international harmony in the methodology, interpretation and presentation of the dossiers. This variation makes costs higher for SMEs, while having less effect on larger companies with an established presence in many countries (Lisansky, 1999). Clearly, this must be redressed before significant progress can be made.

A common misconception is the widespread tendency to classify fungal BCAs as one-to-one substitutes for chemical pesticides. This may be true for some, but more attention should be paid to the biological and ecological characteristics of BCAs. In other words, the chemical paradigm must be replaced by a biological paradigm, constructed on the innate characteristics of the microbial agents and not trying to circumvent them by making them chemical analogues. In essence, more account should be taken of the strengths of microbial agents, rather than forcing them into a standard of assessment that, within the chemical paradigm, emphasizes some particular weaknesses relative to chemical insecticides. Furthermore, it must be remembered that there are common hurdles that need to be overcome in the successful development of BCAs for fungal disease, pest and weed control.

Finally, if there were to be any key messages to convey to government bodies regarding fungal BCAs, these would be for governments to:

- strengthen extension services to accelerate technology transfer from research institutes and industry to the grower;
- streamline or refine policies and/or procedures to reduce product development time and/or costs, e.g. harmonize registration procedures;
- support interphase research that bridges theory and practice.

References

Amiri, B., Ibrahim, L. and Butt, T.M. (1999) Antifeedant properties of destruxins and their use with the entomogenous fungus *Metarhizium anisopliae* for improved control of crucifer pests. *Biocontrol Science and Technology* 9, 487–498.

Amiri-Besheli, B., Khambay, B., Cameron, S., Deadman, M.L. and Butt, T.M. (2000) Inter- and intra-specific variation in destruxin production by the insect pathogenic *Metarhizium*, and its significance to pathogenesis. *Mycological Research* 104, 447–452.

Burgess, H.D. (ed.) (1998) *Formulation of Microbial Biopesticides, Beneficial Microorganisms, Nematodes and Seed Treatments.* Kluwer Academic, Dordrecht, 412 pp.

Butt, T.M. and Copping, L. (2000) Fungal biological control agents. *Pesticide Outlook* 11, 186–191.

Butt, T.M., Carreck, N.L., Ibrahim, L. and Williams, I.H. (1998) Honey bee mediated infection of pollen beetle (*Meligethes* spp.) by the insect-pathogenic fungus, *Metarhizium anisopliae*. *Biocontrol Science and Technology* 8, 533–538.

Driver, F., Milner, R.J. and Trueman, J. (2000) A taxonomic revision of *Metarhizium* based on phylogenetic analysis of rDNA sequence data. *Mycological Research* 102, 134–150.

Ekengren, S. and Hultmark, D. (1999) *Drosophila* cecropin as an antifungal agent. *Insect Biochemistry and Molecular Biology* 29, 965–972.

Gross, H.R., Hamm, J. and Carpenter, J.E. (1994) Design and application of a hive-maintained device that uses honey bees (Hymenoptera: Apidae) to disseminate *Heliothis* nuclear polyhedrosis virus. *Environmental Entomology* 23, 492–501.

Inyang, E., Butt, T.M., Ibrahim, L., Clarke, S.J., Pye, B.J., Beckett, A. and Archer, S. (1998) The effect of plant growth and topography on the acquisition of conidia of the insect pathogen *Metarhizium anisopliae* by larvae of *Phaedon cochleariae. Mycological Research* 102, 1365–1374.

Inyang, E., Butt, T.M., Beckett, A. and Archer, S. (1999a). The effect of crucifer epicuticular waxes and leaf extracts on the germination and virulence of *Metarhizium anisopliae* conidia. *Mycological Research* 103, 419–426.

Inyang, E., Butt, T.M., Doughty, K.J., Todd, A.D. and Archer, S. (1999b) The effects of isothiocyanates on the growth of the entomopathogenic fungus *Metarhizium anisopliae* and its infection of the mustard beetle. *Mycological Research* 103, 974–980.

Leal, S.C.M., Bertioli, D.J., Ball, B. and Butt, T.M. (1994a) Presence of double stranded RNAs and virus-like particles in the entomopathogenic fungus *Metarhizium anisopliae. Biocontrol Science and Technology* 4, 89–94.

Leal, S.C., Bertioli, D., Butt, T.M. and Peberdy, J. (1994b) Characterization of isolates of the entomogenous fungus *Metarhizium anisopliae* by RAPD-PCR. *Mycological Research* 98, 1077–1081.

Leal-Bertioli, S.C.M., Peberdy, J.F., Bertioli, D.J. and Butt, T.M. (2000) Genetic exchange in *Metarhizium anisopliae* strains co-infecting *Phaedon cochleriae* as revealed by molecular markers. *Mycological Research* 104, 409–414.

Lisansky, S. (1999) *CPL Scientific Study on Biopesticides.* CPL Scientific Information Services, Newbury, UK.

Menn, J.J. and Hall, F.R. (1999) Biopesticides, present status and future prospects. In: Hall, F.R. and Menn, J.J. (eds) *Methods in Biotechnology*, Vol. 5: *Biopesticides: Use and Delivery.* Humana Press, Totowa, New Jersey, pp. 1–10.

Moore, D. and Prior, C. (1993) The potential of mycoinsecticides. *Biocontrol News and Information* 14, 31N–40N.

Pell, J.K., MaCauley, E.D.M. and Wilding N. (1993) A pheromone trap for dispersal of the pathogen *Zoophthora radicans* Brefeld. (Zygomycetes: Entomophthorales) amongst populations of the Diamondback Moth, *Plutella xylostella* L. (Lepidoptera: Yponomeutidae). *Biocontrol Science and Technology* 3, 315–320.

Strasser, H., Vey, A. and Butt, T.M. (2000) Are there any risks in using entomopathogenic fungi for pest control, with particular reference to the bioactive metabolites of *Metarhizium*, *Tolypocladium* and *Beauveria* species? *Biocontrol Science and Technology* 10, 717–735.

Vega, F.E., Dowd, P.F., Lacey, L.A., Pell, J.K., Michael Jackson D. and Klein, M. (2000) Trapping for autodissemination of entomopathogens. In: Lacey, L.A. and Kaya, H. (eds) *Field Manual of Techniques in Insect Pathology*. Kluwer Academic Publishers, The Netherlands, pp. 153–176.

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