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.

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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 Schweizer	Eric Schweizer Seeds, Switzerland	Conidia	G/WC	European cockchafer
	Betel	NPP, France	Conidia	G	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-

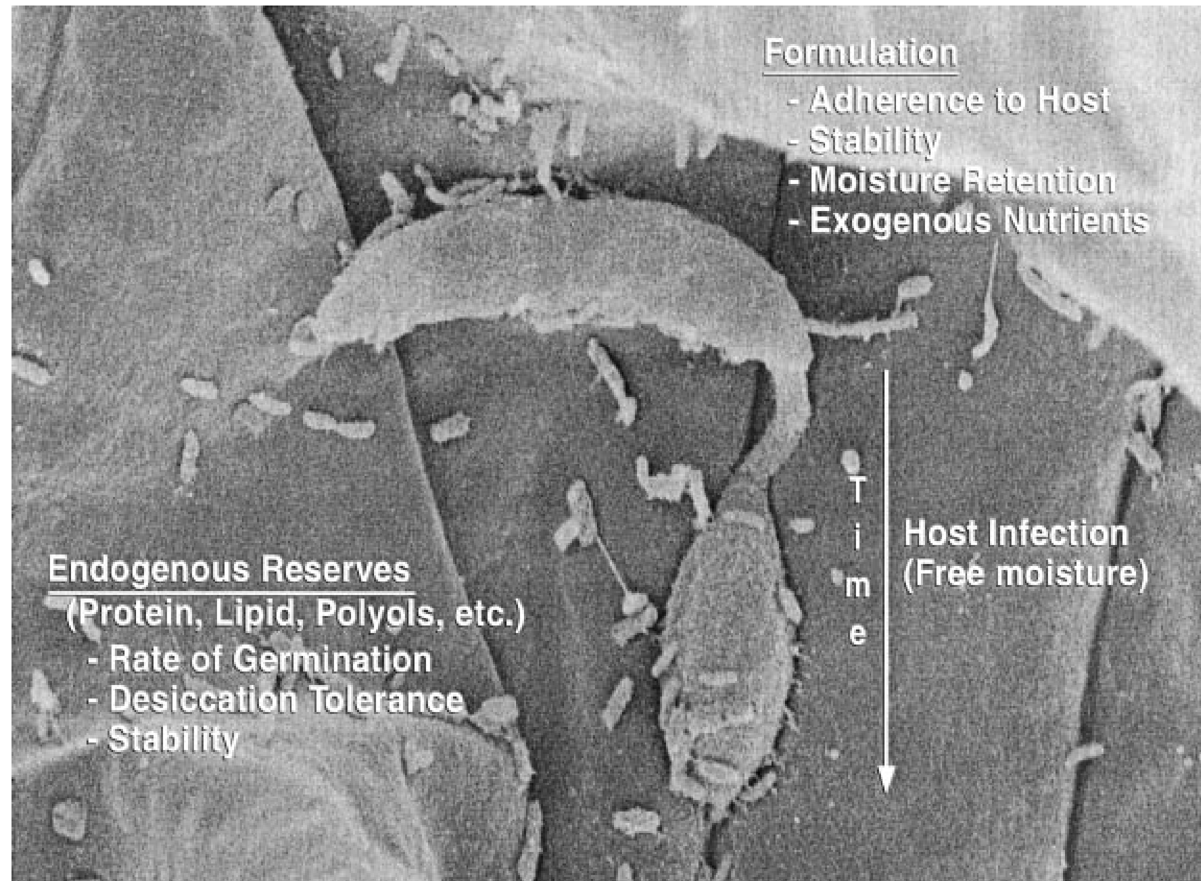

Fig. 10.1. Electron micrograph of a germinating conidium of the bioherbicide *Colletotrichum truncatum* on the leaf of the hemp sesbania (*Sesbania exaltata*). Consistent efficacy of fungal biocontrol agents under field conditions requires adequate free moisture for spore germination and penetration 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

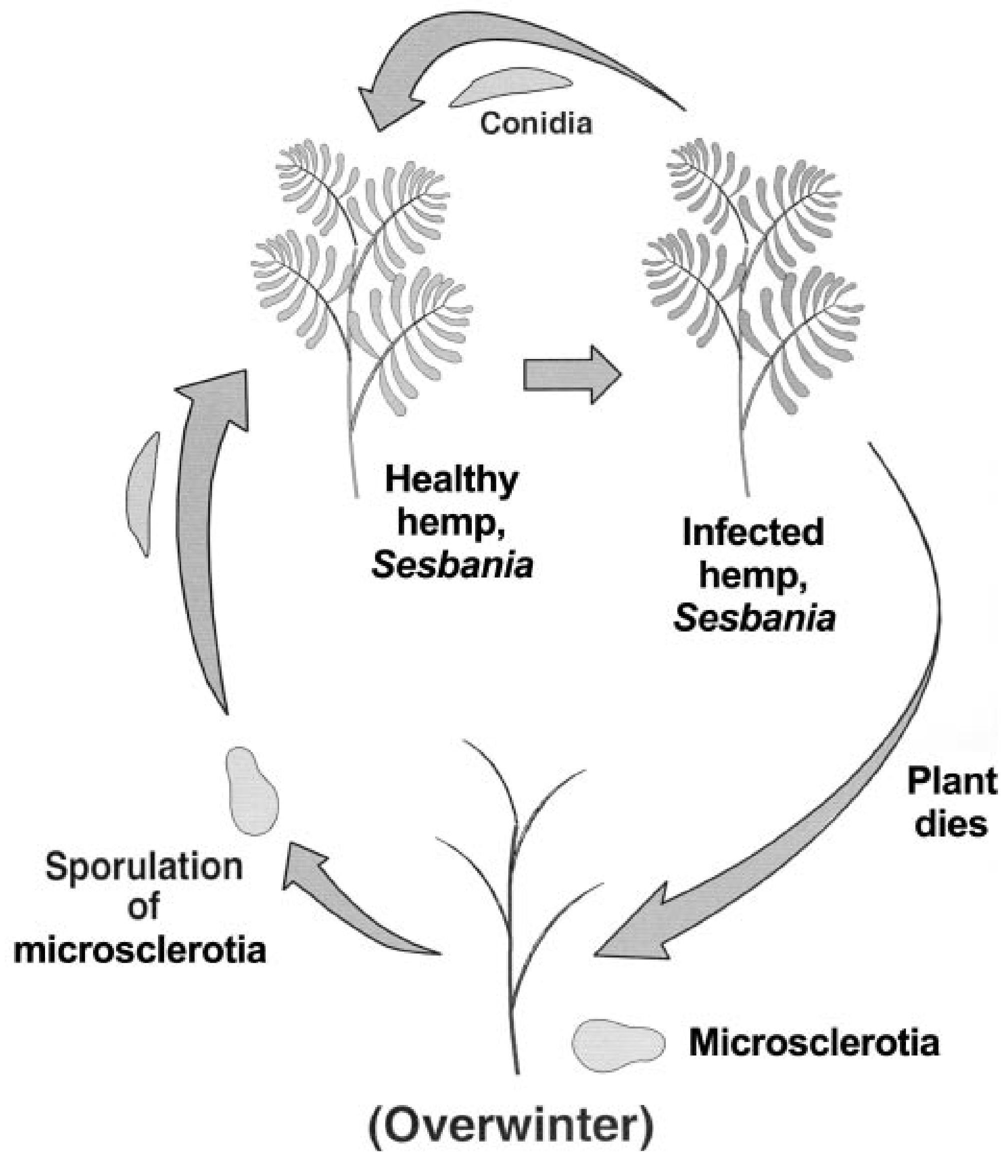

Fig. 10.2. The presumptive disease cycle for *Colletotrichum truncatum* on the weed hemp sesbania (*Sesbania exaltata*). Conidia of *C. truncatum* derived from microsclerotia or diseased plants infect healthy hemp sesbania plants. The pathogen overwinters as microsclerotia in dead plant material.

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.

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