Chapter 11

Mass Production of Entomopathogenic Fungi: State of the Art

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Mention of trade names or commercial products in this chapter is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the U.S. Department of Agriculture.

11.1. INTRODUCTION

The potential to control insects with fungi dates back to Augostino Bassi's 1835 demonstration that a fungus could cause a deliberately transmissible disease in silkworm (Steinhaus, 1956; Lord, 2007). In the late 1870s Metschnikoff observed a high proportion of *Metarhizium*-killed sugarbeet curculio *Cleonus punctiventris* Germar and proposed the concept of controlling this insect with conidia artificially produced on sterile brewer's mash (Metchnikoff, 1880; Steinhaus, 1975). His work was extended by Krassilstschik, who established a production facility using beer mash to produce a considerable amount conidia for distribution (Krassilstschik, 1888). In the United States, Lugger (1888) suggested the use of another fungus, now known as *Beauveria bassiana* (Balsamo) Vuillemin, to control the chinch bug (*Blissus leucopterus* (Say); Kansas, Nebraska, and neighboring states produced the fungus in vivo and attempted to augment natural populations with cadavers having sporulating fungus in the 1880s and 1890s (Billing and Glenn, 1911). During the early and middle twentieth century, citrus growers in Florida observed a series of fungi to be significant mortality factors for citrus pests. The Florida State Experiment Station produced and sold one fungus, *Aschersonia aleyrodis* Webber, to growers, while fungi attacking scale insects were often obtained and produced by the growers themselves (McCoy et al., 1988). With the advent of chemical insecticides, interest in all biological agents waned in the United States and Western Europe. It was not until the 1970s and 1980s that interest in microbial

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TABLE 11.1 Commercial Mycoinsecticide Products Available in 2007

Species	Number of Products	Percent
Beauveria bassiana	45	37.2%
B. brongniartii	5	4.1%
Metarhizium anisopliae s l.	44	36.4%
M. acridum	3	2.5%
Isaria fumosorosea	7	5.8%
I. farinosa	1	0.8%
Lecanicillium longisporium	2	1.7%
Lecanicillium muscarium	3	2.5%
Lecanicillium sp.	10	8.3%
Hirsutella thompsonii	1	0.8%
Total	121	

Source: Adapted from Faria and Wraight (2007).

agents resumed as the adverse environmental effects of chemical pesticides were better understood and alternatives began to be sought. Commercialization of fungi in the United States was limited, but efforts increased in a number of countries—most notably Brazil, Cuba, the former Czechoslovakia, the former U.S.S.R, and China—for economic as well as environmental reasons. Bartlett and Jaronski (1988) described many of these efforts.

Today, there are more than 100 commercial products based on entomopathogenic fungi (Table 11.1). De Faria and Wraight (2007) conducted a survey in 2006 and identified 129 active mycoinsecticide products; another 42 had been developed since the 1970s but were not commercially available at the time of the survey. In the United States, there are nine mycoinsecticides currently registered by U.S. Environmental Protection Agency; in the European Union (EU), 21 different fungi are registered in the Organisation for Economic Cooperation and Development (Agriculture and Agri-Food Canada, 2012).

A common characteristic of the entomopathogenic fungi is that, with the exception of the Microsporidia, they infect their hosts percutaneously, not perorally. With all but the Lagenidiales and Peronosporomycetes, the infectious stage is passively dispersed; the former two groups have motile zoospores that actively seek out their aquatic hosts. The life cycle, schematized in Fig. 11.1 and based on the Ascomycetes, begins when the spore contacts the arthropod cuticle, attaching initially by van der Waals forces, but then adheres more firmly and germinates within a few hours. A penetration hypha is produced, as well as

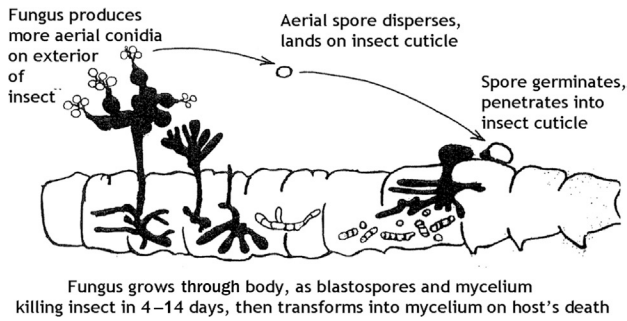

FIGURE 11.1 Schematic life cycle of the entomopathogenic fungi, exemplified by *Beauveria bassiana*.

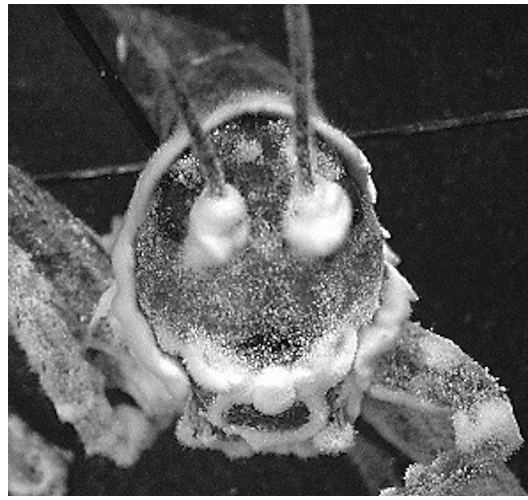

FIGURE 11.2 *Beauveria bassiana* emerging from intersegmental areas of its host a Mormon cricket (*Anabrus simplex* Haldeman), and conidiating on the surface of the cuticle.

an appressorium or anchoring structure in some species. The hypha penetrates the arthropod cuticle by means of several enzymes and mechanical pressure. Once in the hemocoel, the fungus proliferates by means of yeast-like bodies (hyphal bodies in Entomophthorales, blastospores and mycelium in Ascomycetes, mycelium in Oomycetes). As the host dies, the fungus rapidly transforms into mycelium and, under ideal conditions (particularly an extended period of high humidity), emerges to conidiate on the exterior of the insect (Fig. 11.2).

Mass production methods for the entomopathogenic fungi up to 1983 were reviewed in detail by Bartlett and Jaronski (1988); the reader is referred to that publication. Since then, there have been a few general review publications, namely Feng et al. (1994) and Jenkins and Goettel (1997), as well as many specific studies about some aspect of mycoinsecticide mass production. The

present chapter seeks to update the older literature, delineate important aspects, and identify areas of needed research.

11.2. PRODUCTION METHODS FOR THE IMPORTANT INSECT PATHOGENIC FUNGI

11.2.1. *Lagenidium giganteum* (Schenk)

Lagenidium giganteum (Oomycota: Lagenidiales) is the principal species studied for development, with larval mosquitoes as its principal target. A key aspect about this fungus is that the infectious stage is a motile zoospore that actively seeks out hosts and is produced by either mycelium or from germinated resting spores. Elution of nutrients from the mycelium and any carrier matrix (e.g. agar medium) is required for zoospore production from competent mycelium. Oospores—sexual resting bodies—can also produce zoospores upon rehydration, although the cues for relatively synchronous zoosporogenesis are unclear. Thus, efficient production of shelf-stable formulations is difficult. For a review of *L. giganteum* biology, see Kerwin (2007).

Exogenous sterols—specifically cholesterol, ergosterol, and campesterol—are essential nutrients for the production of both zoosporogenic mycelium (Domnas et al., 1977) and oospores (Kerwin and Washino, 1983). Attenuation of the zoosporogenic capability follows culture without these sterols (Lord and Roberts, 1986). Initially, production of zoosporogenic mycelium used agar-based media, primarily for experimental use (Jaronski et al., 1983). Subsequently, May and Vander Gheynst (2001) developed a solid substrate medium. This medium consisted of wheat bran supplemented with cholesterol, peptone, autolyzed yeast extract, glucose, corn oil, and lecithin, which was inoculated with a liquid preculture. Simpler media were subsequently developed by Maldonado-Blanco et al. (2011), who identified soybean meal and sunflower meal as suitable sources of the required sterols.

On a commercial scale, *L. giganteum* mycelium and oospores were produced in liquid fermentation using media consisting of crude carbon and nitrogen sources, with vegetable or fish oils providing the required sterols and unsaturated fatty acids (Kerwin and Washino, 1986); the unsaturated fatty acids, primarily triglycerides, were thought to help solubilize the sterols to optimize uptake and provide a higher percentage of fatty acids, thus increasing zoospore production (Kerwin and Washino, 1986). Another critical component is $CaCl_2 \cdot 2H_2O$. Fermentation output at pilot-scale level was 1–51 fermenter volume per hectare of mosquito habitat, with a production cycle of 3–4 days (Kerwin and Washino, 1987). Commercial production used this method, with harvesting of the fungus from medium and storage in refrigerated containers; effectiveness of such material lasted 1–3 weeks (Vander Gheynst et al., 2000). Kerwin (2007) still considered economical scale-up of liquid fermentation a serious challenge. Although mycelium and oospore formulations of the fungus

were registered and commercialized by Agraquest Inc. in the United States, the company abandoned the continued sale of the fungus. As an alternative, *L. giganteum* could be produced using wheat bran as a solid fermentation substrate (Vander Gheynst et al., 2000). The fungus retained its efficacy for 4 weeks. Glucose and wheat germ oil could increase the shelf life of the fungus and the whole culture could be efficacious against larval mosquitoes, at least in laboratory assays.

11.2.2. *Leptolegnia chapmani*

Leptolegnia chapmani Seymour (Straminipila: Peronosporomycetes) has been under study for a number of years as an alternative mosquito control agent (Zattau and McInnis, 1987). Much less work has been done regarding production of *L. chapmani*, primarily because the fungus remains in an experimental mode. Pelizza et al. (2011) evaluated a series of agar-based media for zoospore production. Although they observed that most media supported mycelial growth equally, zoospore production in an agar medium that contained 10% Fortisip (Nutricia, Wiltshire, UK), a complex human nutritional supplement, was 10-fold greater than any of the other media. The sterol requirement of *L. giganteum* does not seem to exist for *L. chapmani*.

11.2.3. *Coelomomyces* spp. Keilin

This group of more than 70 species, within the Phylum Chytridiomycota, Order Blastocladiales, are aquatic, obligatory pathogens of aquatic Diptera, including Culicidae, Psychodidae, Chironomidae, Simuliidae, and Tabanidae (Chapman, 1985; Whisler, 1985). These fungi are also notable for alternating sexual and asexual stages and involving copepods or ostracods as alternate hosts. Even though the *Coelomomyces* species have been observed to be very effective control agents of mosquitoes, the dependence on in vivo production has greatly limited their usefulness as inundative biocontrol agents (Scholte et al., 2004).

11.2.4. Entomophthorales

Previously classified within the Zygomycetes, Entomophthorales have been placed in a new subphylum, Entomophthoromycotina, representing a monophyletic lineage distinct from all the other fungi (Humber, 2012). Most species within this group are insect pathogens, with a few species also attacking nematodes and mites. A notable characteristic for most species is a degree of high host specificity. Many species have never been cultured in vitro because of their nutritional fastidiousness.

A major impediment in developing Entomophthorales—even for efficient inoculative biocontrol efforts, much less inundative applications—has been their biology. The conidia are short-lived after in vivo production and extremely

difficult to produce efficiently in vitro. In vivo, hyphal bodies or protoplasts are the basic vegetative stages of these fungi. Resting spores (azygospores, and zygospores in some species), however, have been deemed to be the more promising infectious propagule for biocontrol efforts. In *Zoophthora radicans* (Brefeld) Batko and other species, the azygospores are formed in vivo by the proliferative hyphal bodies in the insect's hemolymph. These azygospores are generally dormant after production and are typically in the overwintering stage in temperate climates. Under specific environmental conditions, the resting spores germinate to produce primary conidia, which then give rise to infectious conidia, which then continue the fungus life cycle by infecting insect hosts. In limited field introductions, azygospores have been typically produced in vivo, being collected from laboratory-infected cadavers or from a soil matrix in which the infected insects have died. Neither process is efficient nor amenable for large-scale introduction (Hajek, 1997). Laboratory methods for isolating and culturing these fungi have been thoroughly discussed by Hajek et al. (2012).

Bartlett and Jaronski (1988) reviewed the status of entomophthoralean production up to that time. Some limited research into mass production of Entomophthorales had been conducted prior to 1984, namely efforts by Latge and collegues (Latge, 1975, 1981; Latge et al., 1978). Since then, there has been some effort beyond laboratory-level media to better study a particular species, but only a few studies attempted to mass produce an entomophthoralean fungus. A review of the literature reveals that only the species listed in Table 11.2 have been mass produced in vitro.

Culturing entomophthoralean fungi in vitro varies widely in difficulty, depending on the species and even the isolate. In general, *Neozygites* Witlaczil species are among the more difficult species to grow, and *Conidiobolus* (Costantin) Batko are the easiest. The earliest advances in the in vitro production of the Entomophthorales were the use of Grace's insect cell culture medium supplemented with fetal bovine serum (5% v/v), which simulated the insect hemolymph (Dunphy et al., 1978). This approach is still being used; for example, Kogan and Hajek (2000) produced azygospores of *Entomophaga miamaiga* Humber, Shimazu, and Soper with this medium. Alternatively, some species could be grown on Sabouraud dextrose agar supplemented with egg yolk and milk or coagulated egg–yolk–milk medium (Hajek et al., 2012). However, neither approach is practical—much less economical—for any level of mass production. Beauvais and Latge (1988) advanced the art with a medium of glucose, yeast extract, lactalbumin hydrolysate, NaCl, and 10% fetal bovine serum (GLEN medium). GLEN and modified Grace's medium have also been used to grow *Entomophthora thripidum* Samson, Ramakers, and Oswald in the laboratory, but not on a large scale (Freimoser et al., 2003).

Nolan (1988) initially devised a defined medium, which allowed growth in both stationary and shaken cultures in the absence of fetal calf serum; it was developed for protoplasts of the fungus *Entomophaga aulicae* (Reichardt)

TABLE 11.2 Entomophthorales Species that Have Been Mass Produced In Vitro

Species	Reference
Pandora nouryi	Zhou and Feng, 2010
Pandora (Erynia) neoaphidis	Hua and Feng, 2003; Uziel and Kenneth, 1998; Shah et al., 2007
Zoophthora radicans	Senthikumar et al., 2011; Pell and Wilding, 1992
Pandora delphasis	Uziel and Kenneth, 1998
Entomophaga aulicae	Nolan, 1988
Entomophthora thripidum	Freimoser et al., 2003
Entomophthora maimaiga	Hajek and Plymale, 2010
Batkoa sp.	Leite et al., 2005
Furia sp.	Leite et al., 2005
Neozygites floridana	Leite et al. 2000, 2005; Delalibera et al., 2003
Entomophthora (Conidiobolus) coronata	Wolf, 1951
Entomphthora obscura	Latge, 1981
Neozygites tanajoae	Delalibera et al., 2003
Entomophaga grylli pathotype 1	Sanchez Pena, 2005
Neozygites parvispora	Grundschober et al., 2001

Humber. The protein "requirement" was obviated and growth was enhanced by the addition of hematin (0.5 pg/ml) and oleic acid (1.0 pg/ml). Nolan (1993) developed a low-cost liquid medium for production of *E. aulicae* hyphal bodies competent to form conidia. The medium consisted of a basal medium of a series of salts, amino acids, sucrose, glucose, and buffer, plus 0.8% tryptic soy broth and 0.4% calcium caseinate; it supported the growth of several isolates. Inoculant protoplasts were grown on "traditional" Grace's tissue culture medium supplemented with 5% fetal bovine serum.

Hyphal bodies of *Neozygites floridana* (Weiser and Muma) Remaudier and Keller were produced in vitro using Grace's cell culture medium plus 0.33% lactalbumen hydrolysate and 0.33% yeastolate (Leite et al., 2000). Yields were over 10^6/ml. These hyphal bodies would produce primary conidia from which the infectious capilloconidia could be generated. Leite et al. (2005) refined the medium for *N. floridana* further, using glucose, skim milk, yeast extract,

peptone, and trace salts, but the complexity of preparing this medium may preclude its use in large volume.

In seeking a mass production medium for *Z. radicans* azygospores, Senthikumar et al. (2011) tested a series of concentrations and ratios of sunflower oil or dextrose as a carbon source and a yeast extract or peptone as nitrogen source in liquid medium; they discovered the optimal ratio was 4:8 yeast extract to sunflower oil medium. Alternatively, *Z. radicans* could be produced as mycelium in a liquid culture medium of yeast extract, dextrose, and sunflower oil; mycelium was then harvested by filtration, washed, and prepared as a thin slurry. The slurry was treated with 10% maltose, matured at 4 °C, then air dried (McCabe and Soper, 1985; Wraight et al., 2003). This dry, marcescent mycelium was then prepared as a granular formulation, which when rehydrated and exposed to the appropriate environmental conditions would generate large numbers of the infectious conidia. The dry mycelium formulation produced equivalent or greater numbers of conidia than fungus on leafhopper cadavers. However, Li et al. (1993) discovered that dried mycelium preparations of *Z. radicans* and *Erynia (Pandora) neoaphidis* Remaudier and Hennebert did not survive milling or freezing.

The production process is complex and imposes serious constraints for mass production, especially for inundative releases of this fungus. The process could be feasible for inoculative release to establish epizootics, but it has not yet been capitalized upon. One impediment—at least in the United States, Canada, and the EU—is that any use of a microbial to control an insect is subject to registration with the respective regulatory authorities, and inoculative release be an insufficient commercial incentive.

Latge et al. (2004) described the best media for *Entomphthora virulenta* Hall and Dunn zygospores, consisting of dextrose and corn syrup as carbon sources and yeast extract, soybean flour, or cottonseed flour as the best nitrogen sources. The resulting zygospores had a 70% germination rate. A liquid culture medium for *E. neopahidis* was determined by Gray and Markham (1997) to consist of glucose, yeast extract, mycological peptone, KH_2PO_4, Na_2HPO_4, and 0.01% oleic acid in 1.5-l fermentation volumes. They obtained considerable mycelial biomass in batch but not continuous fermentations. A disadvantage was that large inoculum volumes were necessary to detoxify an essential nutrient, oleic acid.

Media for *Batkoa* (Keller) and *Furia* (Humber) species were devised by Leite et al. (2005). For *Furia*, the best medium consisted of 0.33% each of yeast extract, beef extract, and skim milk in a basal medium of 2.66% glucose and trace salts. Although several other combinations of nitrogen sources gave good yields, skim milk was associated with the best media. For *Batkoa*, in contrast, yeast extract was the best nitrogen source, whereas skim milk and peptone did not seem to be important nitrogen sources for this fungus. Both fungi produced mycelium in these media, which presumably could be processed and preserved using some modification of the McCabe and Soper (1985) process.

The Leite et al. (2005) study illustrates the differences in optimal media that can exist among species of Entomophthorales, especially differences in response to different concentrations of complex nitrogen sources, as well as to the sources themselves.

The most successful simple in vitro mass production of an entomophthoralean fungi using a simpler medium has been with *Pandora neoaphidis* (Remaud and Hennebert) Humber, *Pandora nouryi* (Remaud and Hennebert) Humber, and *Z. radicans* on broom corn millet (Hua and Feng, 2003, 2005; Zhou and Feng, 2010). Autoclaved broomcorn millet (with 36% moisture content) supplemented with Sabouraud dextrose broth and inoculated with the fungus evidently colonized the grains, which then served as surrogate aphid cadavers, allowing subsequent sporulation that was 2–3 times more abundant and with a greater duration than in aphid cadavers.

There have been several efforts of in vivo production. Mullens (1986) devised a method to infect large numbers of houseflies with *Entomophthora muscae* (Cohn) Fresenius. By this method, several hundred adult flies were exposed to conidial showers from infected insects in a small container, ensuring good dose transfer. Serial repetitions of these exposures could greatly increase the number of infected insects, which could then be released alive. Carruthers et al. (1997) described using *Entomophaga grylli* (Fresenius) Batko, produced in vivo, in inoculative releases against grasshoppers. The insects were infected by injecting protoplast cultures and were then released live into natural grasshopper populations.

The potential for using dried infected hosts (*Plutella xylostella* L. with *Z. radicans* resting spores) was presented by Pell and Wilding (1992). Second instar larvae were exposed to sporulating plate cultures, reared until death but before fungal sporulation occurred, and then dried at ambient temperature and 40% relative humidity. Steinkraus and Boys (2005) harvested large numbers of cotton aphids (*Aphis gossypii* Glover) infected with *Neozygites frezenii* (Nowak) Remaud and Keller from natural epizootics and preserved them by drying the aphid-infested leaves over silica gel. The dried cadavers had a very high level of sporulation when rehydrated, even 6 months later. In this manner, they were able to collect 25,572 infected aphids from 189 leaves. The key for success with such a method, however, is to find an appropriate epizootic, which may not always be possible.

11.2.5. Microsporidia

Microsporidia have been historically associated with the Protozoa, but in the last decade have been reclassified within the kingdom Fungi, as the Phylum Microsporidia (Keeling, 2009). Nevertheless, their biology and morphology are unique and distinctly different from the other fungi. A spore is the infectious agent, and the infection process most commonly involves the host ingesting the spores, explosive spore germination in the host gut, with insertion of the nucleus

and associated cytoplasm into a gut epithelial cell through a thin, hollow, polar filament rapidly everted from the spore. The microsporidian then develops within the host cell. Vertical transmission via transovarial and transovum routes is also common among the microsporidia, as are intermediate hosts. Hosts of microsporidia span a wide range, from Protista to warm-blooded vertebrates, but in general each species is somewhat host specific. See Solter et al. (2012b) for a review of microsporidian biology. One species, *Paranosema (Nosema) locustae* (Canning), has been registered as a microbial pesticide and commercialized in the United States for the control of grasshoppers (USEPA, 2000). In general, however, biocontrol efforts with microsporidians have focused more on inoculative or augmentative approaches, for which smaller amounts of infective spores are needed than in inundative use.

A salient feature of microsporidia is their obligate intracellular development. Thus, these organisms can be mass produced only in vivo, in their hosts (Henry, 1985) or in invertebrate cell culture (Visvervara et al., 1999). For example, *P. locustae* is produced by perorally infecting grasshoppers with a dose that is optimal for spore production, allowing the pathogens to multiply within the host, fragmenting the cadavers in a mill and suspending the macerate in water. After the insect parts are removed by filtration, the resulting spore suspension is further purified and formulated with wheat bran carrier (Henry, 1985). Spores of *P. locustae* can be stored in refrigerated sterile water, lyophilized, or vacuum dried before formulation. Infected cadavers can also be dried and stored until further processing. The reader is referred to Solter et al. (2012a) and Solter et al. (2012b) for further information about mass production methods for microsporidia.

11.2.6. Ascomycete Hypocreales

Most commercial development of entomopathogenic fungi has been directed towards this group of fungi. These particular species, once placed in the Deuteromycete (Imperfect) fungi, have now been assigned to the family Clavicipitaceae within the Ascomycete order of Hypocreales, based on their molecular association with teleomorph stages. These fungi include the genera *Beauveria*, *Metarhizium*, *Isaria* (formerly *Paecilomyces*, *Lecanicillium* (formerly *Verticillium)*, and the species *Hirsutella thompsonii* Fisher, *Nomuraea rileyi* (Farlow) Samson, *Aschersonia aleyrodis* Webber, *Culicinomyces clavosporus* Romney and Rao, and *Tolypocladium cylindrosporum* (Gams). For recent reviews about the three most important genera, see Zimmermann (2007a) for *Beauveria*, Zimmermann (2007b) for *Metarhizium*, and Zimmermann (2008) for *Isaria*. Each of these references contains considerable information about the biology of the respective genera.

In general, these fungi have four major propagule types that can be used. In nature, the aerial conidium is the primary infectious propagule. Conidia are the spores that are produced on the exterior of fungus-killed insects. Blastospores

are the proliferative stages within the insect for many of these fungi and can also be produced in liquid fermentation. (This ability is not surprising if one considers an insect as merely a six-legged flask of culture medium.) Under certain liquid fermentation conditions, mainly substitution of inorganic for organic nitrogen, *Beauveria* and *Metarhizium* can produce "microcycle" conidia (Thomas et al., 1987; Bosch and Yantoro, 1999; Zhang et al., 2009). These conidia are not true conidia and are produced on the ends of hyphal strands. Lastly, mycelium, the major form of fungal vegetative proliferation, or mycelial derivatives such as the microsclerotia, can be used. But in this last case, the mycelium or its derivatives are true producers of conidia—a way to deliver conidia to the insect.

Because of the significance of *Beauveria* and *Metarhizium* among the entomopathogenic Ascomycetes, emphasis on developments for their mass production follows. Specific production methods developed for *Isaria*, *Hirsutella*, *Aschersonia*, *Nomuraea*, *Lecanicillium,* and *Culicinomyces* are subsequently reviewed.

11.2.6.1. Solid Substrate Fermentation

The fundamentals of solid substrate fermentation to produce entomopathogenic fungi were detailed by Bartlett and Jaronski (1988); the reader is referred to that publication. Here, I will attempt to address process development since then. An overview of solid substrate fermentation systems has been presented by Krishna (2005). A description of practical small scale methods, yielding decagrams, even hectograms of conidia per kilogram of substrate may be found in Jaronski and Jackson (2012). Other descriptions of solid substrate production systems are presented in Aquino et al. (1977); Cherry et al. (1999); Perez-Guerra et al. (2003); and Bateman (2007).

11.2.6.1.1. The End Products of Solid Substrate Fermentation

Solid substrate fermentation, mimicking the natural conidiation processes, yields aerial conidia as the final product (Figs 11.3 and 11.4). Aerial conidia

FIGURE 11.3 Solid substrate (flaked barley) with well-sporulated *Beauveria bassiana*. Note the abundant conidia in the form of white powder on the substrate.

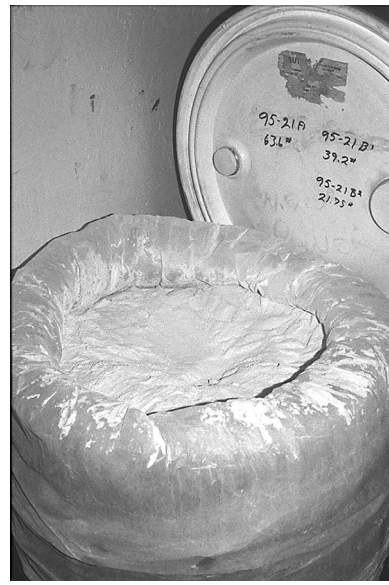

FIGURE 11.4 Commercial harvest of *Beauveria bassiana* conidia produced in a 1995 Mycotech Corporation pilot-scale, solid-substrate, fermentation system. This batch of conidia totaled 56 kg of conidial powder having a titer of 1.6×10^{11} conidia/g.

are the natural infectious bodies for these Ascomycetes. In a few isolated cases, the fermentation is terminated after the fungus has penetrated the nutritive substrate but before conidiation has begun (Kirchmair et al., 2007; Skinner et al., 2012). This process yields dried grain kernels colonized by *Beauveria* or, in particular, *Metarhizium anisopliae* (Metschnikoff) Sorokin, which remain competent for regrowth and sporulation upon rehydration. This rehydration occurs, for example, after such granules are applied into soil by a sowing machine in combination with a rotary harrow (Kirchmair et al., 2007) or mixed into horticultural soil (Skinner et al., 2012), creating focii of conidia within the habitat of the target insect.

11.2.6.1.2. Substrates and Media

A wide variety of organic materials have been evaluated as substrates for the Ascomycetes. Although rice and barley seem to be the major substrates used in the tropics and the Northern Hemisphere, respectively, there has been considerable effort to identify low-cost agricultural materials, especially byproducts and waste products, as suitable substrates. Such efforts have been particularly frequent in India, Pakistan, and China. Table 11.3 lists some of these alternative substrates. Comparisons, especially among different reports, are very difficult because of different fungus species and strains, different fermentation conditions, and different supplementations. A few studies have quantitatively examined certain basic nutritional aspects, such as C:N ratio (Ortiz-Urquiza

TABLE 11.3 Solid Substrates Evaluated for Production of the Principal Entomopathogenic Ascomycetes

Substrate	Fungus
Agricultural byproducts	*Metarhizium*
Bagasse ± 2% dextrose	*Beauveria*
Barley	*Beauveria*
Beetroot	*Beauveria*
Broken rice	*Metarhizium*
Broken rice + $CaCl_2$	*Beauveria, Metarhizium*
Carrot tubers	*Beauveria*
Cassava chips	*Metarhizium*
Chickpea	*Metarhizium*
Coconut cake	*Beauveria*
Cottonseed cake	*Beauveria*
Finger millet	*Beauveria*
Grade 3 unpolished rice	*Metarhizium*
Groundnut cake	*Beauveria*
Kodo millet	*Metarhizium*
Maize	*Beauveria, Metarhizium*
Maize bran ± 2% dextrose	*Beauveria*
Mijo grains + organic nitrogen	*Nomuraea*
Millet	*Beauveria, Metarhizium*
Neem cake	*Beauveria*
Pearl millet	*Beauveria*
Potato tubers	*Beauveria*
Prawn waste	*Beauveria*
Press mud ± 2% dextrose	*Beauveria*
Rice	*Metarhizium*
Rice + saccharomyces	*Beauveria, Metarhizium*
Rice bran ± 2% dextrose	*Beauveria, Metarhizium*
Rice flour	*Metarhizium*

Continued

TABLE 11.3 Solid Substrates Evaluated for Production of the Principal Entomopathogenic Ascomycetes—cont'd

Substrate	Fungus
Rice hulls, sawdust:rice bran ± 2% dextrose	*Beauveria*
Rice husk ± 2% dextrose	*Beauveria*
Sesame cake	*Beauveria*
Sorghum	*Beauveria, Metarhizium*
Soyabean	*Beauveria, Metarhizium*
Sugarcane bagasse ± yeast, molasses	*Beauveria, Metarhizium*
Sugarcane press mud	*Beauveria*
Tapioca rind	*Beauveria*
Tapioca tubers	*Beauveria*
Wheat	*Beauveria, Metarhizium*
Wheat bran + $Al(SO_4)_3$	*Beauveria*
Wheat bran + organic nitrogen	*Nomuraea*
Wheat bran ± 2% dextrose	*Beauveria*

et al., 2010) or controlled levels of carbohydrates (Domenech et al., 1998). Nevertheless, rice remains the substrate of choice where it is readily and cheaply available. In North America and Europe, barley—particularly dry, flaked barley—seems to be preferred because of its superior handling characteristics and cost relative to rice (Jaronski and Jackson, 2012).

Several inorganic substrates have been identified in recent years. These include granules of calcined diatomaceous earth (diatomite); (Jaronski and Jackson, 2012; Crangle, 2011; Wikipedia Contributors, 2012), and open-pored clay granules (e.g. Seramis®; Seramis GmbH, 2012). Use of clay granules to produce *B. bassiana* has been described in detail by Desgranges et al. (1993) and Guillon (1997). Diatomite can be obtained in a range of sizes from several millimeter in diameter to a coarse powder; it has a high surface to volume ratio and can absorb aqueous liquids up to 110–140% of its weight. Seramis is a processed, particulate clay composed of kaolinite, illite, and quartz, produced in the Westerwald region of Germany; it is used primarily in hydroponics, plant bedding, and interior landscaping industries in Europe. It also is open-pored and highly absorbent (Seramis, 2012).

Unlike cereal grains or other organic materials, mineral carriers have the advantages of allowing flexible control of nutrients tailored for each fungus

species and strain, as well as the ability to be recycled after washing and sterilization. This ability for recycling avoids disposal issues, which can be considerable, as is faced by one North American company having 7000-kg production runs. In the 1980s, I and colleagues developed a pilot-scale (100-kg batch size) process using Celetom granules with a liquid medium to produce $5–7 \times 10^{12}$ *B. bassiana* ARSEF252 conidia per kilogram of substrate (Jaronski, unpublished data). If harvesting was performed aseptically, it was possible to obtain a second round of conidiation after the first was removed and the substrate reincubated. A third flush became contaminated, however, with *Aspergillus*, and in general such practice is not recommended for a quality product.

Niedermayr et al. (2012) noted that an unidentified, open-pored clay granule produced only 10% of the spore yield of *Metarhizium brunneum* BIPESCO5 (also known as Met52) with several grains. Their comparison was flawed, however, because they used a packed bed column fermenter for the clay granules and plastic bags for the grains. Further, they did not optimize the liquid medium for the clay granules. The grain in bags yielded more typical yields for this fungus. Hemp-based animal litter (Hemparade; HempFlax, The Netherlands) has also been used as an inert carrier, impregnated with a nutrient medium (Breukelen et al., 2011). The hemp evidently afforded more than adequate porosity for good gas and heat transfer in a packed-bed column fermenter. Conidia could be removed from crushed, dried whole culture by simple mechanical classification. Although yields were excellent, comparable to those on barley in vented mushroom spawn bags (Jaronski, unpublished data), the cost of the substrate and nutrients was not revealed. In addition, the hemp does not seem to be recyclable. Another novel substrate is Amberlite IRA-900 ion exchange resin impregnated with nutrient medium, which has been used for *Aspergillus niger* van Tieghem but has not yet been assessed for any of the entomopathogenic Ascomycetes (Auria et al., 1990).

11.2.6.1.3. Equipment for Fermentation

By and large, most academic and industrial systems have used traditional solid substrate fermentation, either manually intensive or highly mechanized, for producing these Ascomycetes, with a few systems employing submerged liquid fermentation for specific species. In situations where labor costs are low, allowing labor-intensive approaches, polypropylene bags are used. These bags include specialized, vented mushroom spawn bags (e.g. Unicorn spawn bags; Unicorn Industries, Plano, TX, USA), $SacO_2$® microsacs (Combiness, Belgium), zipper-lock bags, and simple shopping bags (Jaronski and Jackson, 2012). A unifying characteristic is that the different plastic bags are autoclavable; unused, unopened, zipper lock bags are often sterile inside. Certain production operations in Africa use plastic bags for the initial mycelial colonization and growth, then transfer the cultures to open, nonsterile, plastic laundry hampers or tubs for the sporulation phase within a controlled environment (Cherry et al., 1999; Bateman, 2007). *Metarhizium* production in Brazil since the 1970s has

FIGURE 11.5 Typical plastic bag-based mass production of *Beauveria bassiana* as is practiced in many countries. *Photograph courtesy Itaforte Bioprodutos, Koppert and Miguel Rinçon Najera.*

involved solid substrate in plastic bags (Aquino et al., 1975; Mendonca, 1991) as has *Metarhizium* production in Nicaragua (Grimm, 2001). Figure 11.5 illustrates *B. bassiana* mass production in Latin America. Open trays of inoculated substrate have also been employed (Alves and Pereira, 1989; Claro, 2006).

Another direction of research pertains to the use of trays within controlled environment chambers. The technology underlying such systems is the Koji tray fermentation, whereby the inoculated substrate is a shallow bed in a tray with perforated bottom and open or mesh cover. Ye et al. (2006) described an upright incubation chamber containing 25 mesh-bottom, open trays, each with capacity of 2 kg of rice. The chamber had a substrate volume of 0.72 m^3 and occupied a surface area of 0.36 m^2. Although temperature could not be regulated, humidity could be controlled during the fermentation. With such a chamber, fully loaded, they obtained 2.4×10^{12} *B. bassiana* conidia per kilogram of substrate.

Several groups have further explored packed bed fermentation technology based on early research. Since the review by Bartlett and Jaronski (1988), there has been considerable development in such systems. Comprehensive reviews of these systems have been presented by Durand (1998, 2003), Durand et al. (1996), Raimbault (1998), and Krishna (2005), and will not be discussed further here. The most recent equipment development is the Prophyta packed bed fermenter (Luth and Eiben, 2003; Eiben and Luth, 2006; Prophyta, 2012). Problems with packed, aerated beds include uneven air flow through the substrate creating pockets of overheated, anaerobic conditions and pockets of substrate with moisture that are suboptimal for fungal growth, even with water-saturated air supply. With many of the Ascomycetes, the solid substrate culture should be broken up for optimal conidiation. This is not readily possible with most packed bed systems, but it is with plastic bags.

A major advance in large-scale solid substrate fermentation has been in the development of mushroom spawn production, using a steam-sterilized,

double-cone blender holding several hundred kilograms of substrate (Maul et al., 1980). An example of this blender is illustrated in Bateman (2006). The device allows in situ steam sterilization of substrate, controlled cooling, and subsequent thorough mixing of liquid inoculum with the substrate. A chute at the pointed end of the cone allows for aseptic filling of spawn bags of other containers in an efficient, rapid manner. This system is in use not only by a large North American mushroom spawn producer but also by several other entities to produce atoxigenic *Aspergillus flavus* Heinrich and Link and mycoinsecticides. It overcomes a major impediment to very large-scale production—efficient preparation and sterilization of a large amount of substrate.

11.2.6.1.4. Fermentation Parameters: Moisture, Temperature, Gas Environment, and pH

Strictly speaking, solid substrate fermentation is really biphasic, with an initial step being a liquid fermentation to produce inoculum, the most common practice, although some researchers use an aqueous suspension of conidia to initiate the solid substrate phase. There are obvious advantages to liquid fermentation in producing inoculum for the solid phase. Conidia require 24 h to fully germinate and begin colonization of the substrate, while a liquid fermentation (blastospores and mycelia) begins immediately. Further, a liquid fermentation phase greatly multiplies the inoculum potential compared to conidia, because an inoculum should contain 10^7–10^8 propagules per milliliter with 60–100 ml inoculum per kilogram of grain substrate. Inoculum concentration affects the duration of the solid substrate phase to reach maximum conidial yield. Nuñez-Gaona et al. (2010) developed a model for *B. bassiana* grown on wheat bran substrate amended with sugarcane bagasse to predict the effect of inoculum concentration. The time to produce 1×10^{10} conidia per gram of substrate could be halved to 148 h by increasing the conidial concentration from 1×10^6 to 5×10^7 conidia per gram of dry substrate.

The ideal liquid fermentation should produce mostly, or entirely, blastospores and short hyphae for optimal dispersion of inoculum through the substrate. Abundant mycelial production, especially in balls or clusters, prevents good dispersion and even colonization of the solid substrate. A wide range of media have been used for the liquid fermentation phase. The simplest recipe is dextrose/sucrose as the carbon source and yeast extract as source of nitrogenous compounds and vitamins; trace salts are not necessary (Cherry et al. 1999; Bateman, 2007; Jaronski and Jackson, 2012). A medium that works very well with a wide range of *Beauveria* and *Metarhizium* isolates consists of 20–30 g/l glucose or sucrose and 15 g/l yeast extract, supplemented with 17 g/l liquid corn steep or 8 g/l corn steep liquor powder (e.g. Solulys®, Roquette Chemical and Bioindustries, Lestrem, France; Jaronski and Jackson, 2012). Corn steep evidently stimulates vigorous blastospore production of both fungi, with minimal mycelial formation. The liquid fermentation requires vigorous aeration, which can be achieved using 200–250 rpm for flask production or an air-lift bubbler

fermentation vessel for larger quantities. A typical liquid fermentation cycle takes 72 h with conidia as inoculant or 48 h if a blastospore preculture is used.

In all cases, the solid substrate, whether organic or inert, must be hydrated and sterilized. The role of water in solid substrate fermentation of fungi was discussed in detail by Gervais and Molin (2003). In situations of potential contamination, the substrate can be hydrated with 0.04% H_2SO_4 and 0.097% KH_2PO_4 to inhibit bacterial growth (Jaronski, unpublished data). In some cases, published protocols using rice substrate have used nutritional additives in hydrating the substrate to increase conidial production: 2% dextrose (Mazumder et al., 1995), cane molasses and torula yeast (Calderon et al., 1995), urea, $(NH_4)_2SO_4$, and yeast extract (Domenech et al., 1998), yeast and molasses (Calderon et al., 1990), and sugar molasses (Sene et al., 2010). No supplements are needed for barley, oats, or wheat, presumably because these have more nitrogen and micronutrients than polished rice (Jaronski, unpublished data).

The typical duration of the solid substrate phase is 7–14 days. Depending on the nature of the inoculum (conidia or blastospores/mycelia), the substrate is colonized by fungus within the first 24–48 h, after which there is active mycelial proliferation through the substrate. Then, the culture needs to be dried in most cases. During the initial drying process, as water activity (a_w) begins to decrease through 0.99, there can be a burst of additional conidiation.

Fermentation parameters—particularly in packed bed approaches—have been dealt with in detail elsewhere (Raimbault, 1998; Krishna, 2005) and the reader is referred to these works. An extremely good discussion of the role of moisture in solid substrate fermentation may be found in Gervais and Molin (2003). A critical moisture level is needed for optimal fungal growth and sporulation. In terms of water activity, the critical level for *Beauveria* and *Metarhizium* is a_w 0.97–0.98 (Humphreys et al., 1989; Hallsworth and Magan, 1999; Nunez-Gaona et al., 2010). Significant interspecific differences in tolerances to a_w exist (Hallsworth and Magan, 1999). They reported optimal a_w ranging between 0.99 and 0.97 for *M. anisopliae* and *Isaria farinosa* (Holmskiold; formally *Paecilomyces farinosus* Holmskiold) and 0.998 for *B. bassiana*. Tarocco et al. (2005) reported that the greatest conidia yield of a *B. bassiana* on rice was obtained at an initial a_w of 0.99.

The amount of water to achieve this level will vary based on the nature of the substrate. For flaked barley substrate, optimal moisture is 52–56% (w/w) before autoclaving (Jaronski and Jackson, 2012). For rice and sorghum, 22–30% and ~75–76% moisture, respectively, seem to be optimum (Prakash et al., 2008). A researcher investigating production of a new isolate of either fungus should experiment to determine optimum moisture levels because there are differences among isolates of any species (Jaronski, unpublished data).

Because the solid substrate phase is an active fermentation, not only does oxygen need to be readily available to all parts of the substrate, but carbon dioxide and heat must be drawn off. In vented mushroom spawn bags O_2 falls to ~10% and CO_2 rises to 11% in the headspace of the bag within 24 h of

inoculation (Jaronski, unpublished data). In bag fermentation, the substrate mass must be less than 7–10 cm thick to allow proper heat dissipation and gas exchange. Additives to create more headspace within the grain substrate have included aluminum silicate (Saroha and Mohan, 2006), sugarcane bagasse (Nuñez-Gaona et al., 2010), rice bran/husk (Dorta et al., 1996), and wheat bran husk (Arcas et al., 1999). Forced-air packed bed fermentation, although potentially overcoming heat dissipation and gas exchange problems of still fermentations, often results in zones of poor growth and sporulation, especially if larger masses are employed. This aspect was investigated thoroughly by Underkofler et al. (1947). However, as Arzumanov et al. (2005) observed, forced aeration of packed beds may not be absolutely necessary, at least with certain Ascomycetes, as long as the bed geometry allows sufficient gas exchange and heat dissipation.

A recent advance in the empirical identification of environmental variables optimal for spore production with economy of effort has been the use of response surface methodology (Prakash et al., 2008; Dhar and Kaur, 2011; Deng et al., 2011; Qiu et al., 2013). Response surface methodology, which has been used in industry but rarely in academic studies, combines mathematical and statistical techniques to design experiments and identify optimal conditions using a reduced number of experiments; this approach eliminates the limitations and avoids the laboriousness of single-factor optimization in fermentation.

Conidial yields can vary among strains of each fungus species. For example, Arcas et al. (1999) determined that one strain of *B. bassiana* produced three times as many spores as a second under identical fermentation conditions. Conidial production of 15 *B. bassiana* isolates ranged from 1.11×10^{11} to 2.25×10^{13} conidia per gram of initial dry substrate when grown under identical solid substrate fermentation conditions (Jaronski, unpublished data). It has been my observation that, for the most part, conidial yield is genetically determined in both *M. anisopliae* and *B. bassiana* and can be inversely proportional to virulence for insects.

Kuźniar (2011) and Zhang et al. (2009) observed that exposure to light enhanced *B. bassiana* growth and conidiation; however, this is contradicted by the very high conidial yields obtained by Bradley et al. (1992) and subsequent commercial production of several *B. bassiana* strains in a completely dark fermentation environment. Similarly, although Onofre et al. (2001) stated that continuous illumination gave 2.5–5 times increase in spore production of *Metarhizium flavoviride*, this has not been my experience. Thus, a light requirement may be strain specific rather than a general phenomenon. On a practical level, a light requirement may be a considerable challenge in even shallow-packed bed fermentations. In bag fermentation, where transparent plastic is used to enclose the fermentation, light is more easily supplied but the substrate mass must be thin enough to allow light penetration through the entire mass.

11.2.6.1.5. Downstream Processing

For all purposes except immediate use, the conidia produced on solid substrate must be dried down to a moisture content <9% w/w or $a_w \leq 0.3$ (Bateman, 2007; Jaronski and Jackson, 2012). This low moisture is necessary for optimal shelf life regardless of whether conidia are formulated or not. Moore et al. (1996) observed that even trace amounts of moisture in the conidia result in foreshortened shelf life. A relatively short shelf life of a few weeks to a few months, depending on the fungus, can be obtained by refrigerating whole sporulated solid substrate within their original fermentation bags.

There are a number of published drying methods: simple opening of plastic fermentation bags; transfer of sporulated substrate to open trays (Bateman, 2007; Claro, 2006) or table tops (D.W. Roberts, personal communication; transfer to Kraft paper sacks (Jaronski and Jackson, 2012); or use of air-lift devices. In general, *Beauveria* conidia can be dried relatively quickly (within 2–3 days) without loss in viability, whereas *Metarhizium* conidia require slower drying (5–9 days; Hong et al., 2000; Jaronski and Jackson, 2012). There is only one study carefully examining the effect of drying temperature and duration of an entomopathogenic Ascomycete (*B. bassiana*) (Li et al., 2008). In that study, the viability of conidia was affected by different drying temperatures and speed of drying; 5 h at 35 °C had no effect on conidial viability. However, the effects of drying temperature and speed on the shelf life of formulated and unformulated conidia, an important commercial aspect, are not known.

Moisture endpoint is best measured using a water activity meter (see Jaronski and Jackson, 2012), but gravimetric moisture analysis is satisfactory. Water activity (a_w) is a measure of the biologically relevant moisture content of an object. The relationship between water activity and moisture content was graphically and algebraically presented for both absorption and desorption isotherms by Faria et al. (2009); the interested reader should refer to this report for more details.

Extreme desiccation of conidia ($a_w < 0.1$), however, can lead to two problems: one affects proper quality assurance testing and the second is a significant problem in operational use of such dry conidia. This situation stems from imbibitional damage of the plasma membranes within the conidia during improper dehydration. Problems with conidial rehydration were first observed by Moore et al. (1996) and subsequently studied in more detail by Faria et al. (2010). In a series of experiments, Faria et al. (2009) discovered that extreme desiccation of *Metarhizium* ($a_w < 0.3$) conidia requires careful rehydration before suspension in an aqueous medium. Use of cold water (≤15 °C) with dry conidia resulted in very low germination. Conidia of *B. bassiana* were more resistant to imbibitional damage, with conidia as dry as a_w of 0.02 having germination rates at normal levels, except when water was 0 °C. Proper rehydration of dry conidia can be by exposure of the conidia to moisture-saturated atmosphere for a minimum of 30 min, as described by Moore et al. (1996), or by use of warm (33–34 °C) water as described by Faria et al. (2009). Use of warm water has to be carefully

controlled, however; as Xavier-Santos et al. (2011) observed, different isolates of *Metarhizium* responded differently to immersion at 31 °C and all isolates tested were adversely affected by either 45 °C for 60 min or prolonged exposure.

In operational conditions involving more than a few decagrams of conidia, the need for careful rehydration of very dry conidia can pose logistical challenges, especially with *Metarhizium*. Of course, one can carefully observe the drying process and terminate it when the conidia have reached a water activity of 0.3. However, emulsifiable oil formulations seem to confer protection from imbibitional damage (Xavier-Santos et al., 2011).

Mechanical separation of conidia from the dried substrate is the predominate method in commercial use. There are two ways to do this: sieving (Fig. 11.6) and mechanical agitation of the substrate with air collection using a cyclone dust collector (Fig. 11.7) (Jaronski and Jackson, 2012; Bateman, 2007). Graded sieves, ideally on a vibratory rather than rotary shaker, can separate out the conidia from substrate with 60–80% efficiency (Jaronski, unpublished data). The second method can be mechanically simple with spore collection using an appropriate vacuum cleaner system (Jaronski and Jackson, 2012) or sophisticated cyclone dust collector. One device using the latter technology has been specifically developed for harvesting fungal conidia (Bateman, 2012). In both cases, the conidia are physically dislodged from the substrate in a rotating drum agitator.

Washing the conidia off in dry or semidry substrate has not been pursued to any great extent, at least in the published literature. The *Beauveria* production

FIGURE 11.6 Harvesting conidia of *Metarhizium anisopliae* by mechanical classification using sieves on a vibratory shaker.

FIGURE 11.7 A cyclone dust collector used to concentrate conidia mechanically dislodged from solid substrate. The conidia are collected in the barrel beneath the conical collector. There is a high-efficiency particulate air (HEPA) filter on the exhaust to prevent escape of conidia into the environment.

system that I developed in the 1980s employed washing conidia from moist substrate using cold 0.1 M NaCl, removing nonconidial material with self-cleaning filters, adding a diatomaceous earth filter aid, and then concentrating the conidia with a continuous flow centrifuge or, alternatively, a ribbon filter. The resulting semidry paste was then rapidly air-dried as a thin layer or lyophilized (Jaronski, unpublished data). Cold saline solution was necessary to keep the conidia from germinating, and the filter aid allowed creation of a fine friable powder at the end of the process.

11.2.6.1.6. Major Technical Problems/Solutions in Solid Substrate Fermentation

The greatest problem in solid substrate fermentation is the scale-up to large capacity at commercial levels. For a mycoinsecticide to succeed commercially, a very large number of conidia must be produced as cheaply and efficiently as possible to compete with chemical insecticides. There are two directions in the production of entomopathogenic fungi by solid substrate fermentation for inundative applications, whether it be uniphasic or biphasic. The first is a low-input, manually intensive, simple technology (e.g. in plastic bags). In practice, especially in emerging rural economies, this system is appropriate and often targets limited, local use. The other is a high-technology, high-input, industrial approach, as can be seen in companies in North America and the EU.

The industrial approach is mandated by the considerable costs of development and registration and expensive wage rates. The typical bag fermentation is not amenable to such large-scale needs, particularly in the developed world where salaries are considerable. The high-technology alternative is capital intensive, often requiring specialized equipment (Ravensberger, 2011), although sparing manpower costs.

Applied to large-scale solid substrate fermentation, the need for large conidial numbers mandates efficient sterilization of very large amounts of solid substrate. For example, for one U.S. company, the fermentation batch size is 10,000 kg. Traditionally, moist heat (steam) is the method used. The V-cone blender sterilizer mentioned in Section 11.2.6.1.3 is one solution. There are alternative methods (e.g. radiation sterilization) already in commercial use in other industries that may be adaptable to fungal mass production. Of course, with plastic bags, the unit volumes are small (0.1–2 kg) and so it is relatively easy to steam sterilize. Use of recyclable solid substrate is highly desirable, yet the current state of the art largely relies on a grain, causing considerable waste disposal issues. In countries with emerging economies, many different alternative materials have been identified, but few seem to have been adopted by commercial enterprises.

Jenkins (1995), in reviewing yields of conidia for 13 fungi produced on solid substrate at that time, found only one instance where yields reached 1×10^{13} conidia per kilogram of dry substrate. The product, based on *B. bassiana*, which is sold by Laverlam International (previously known as Mycotech and Emerald BioAgriculture), was reported to have an operational yield of 2.6×10^{13} conidia per kilogram of substrate (Bradley et al., 1992).

There is also the demand for sufficient, yet efficient, fermentation space, although the mushroom and soy sauce industries have dealt with such problems and offer potentially useful technology. Harvesting problems are largely solved with the advent of cyclone dust collector technology, as discussed in Section 11.2.6.1.5.

11.2.6.2. Submerged Fermentation

Submerged liquid fermentation to produce mycoinsecticides has been a goal of fermentation microbiologists for many years. The technology lends itself to massive scale-up, using existing commercial equipment, and allows closer control of environmental variables and shorter process times (i.e., hours rather than days as for solid substrate fermentation). Submerged fermentation is currently used to produce commercial mycoinsecticides of *Isaria (Paecilomyces) fumosorosea* (Wize) and *Lecanicillium* spp.

11.2.6.2.1. The End Products of Submerged Fermentation

Submerged fermentation generally yields different propagules than solid substrate fermentation: blastospores, submerged, “microcycle” conidia, stabilized

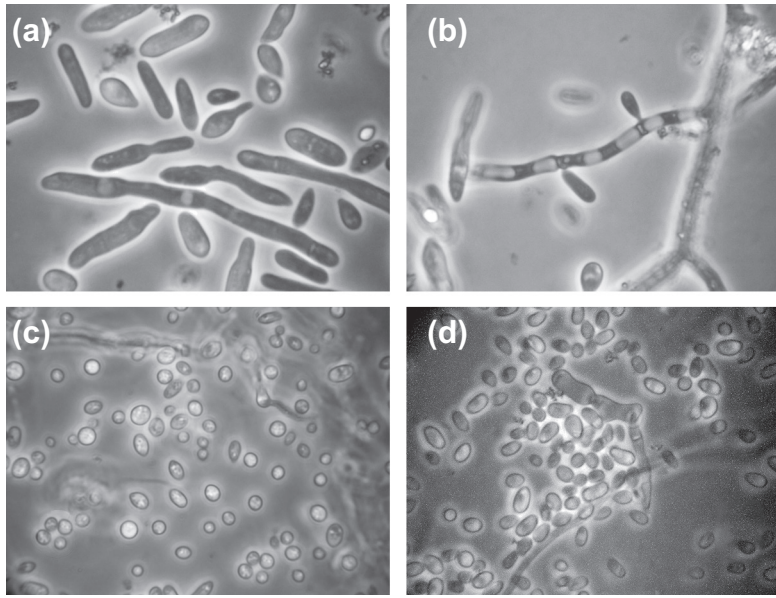

FIGURE 11.8 Ascomycete propagules obtained in submerged liquid fermentation: (a) blastospores of *Metarhizium anisopliae*; (b) hyphae of *Metarhizium robertsii* DWR346 giving rise to microcycle conidia; (c) blastospores of *Beauveria bassiana*; (d) microcycle conidia of *Metarhizium acridum*.

mycelial products, and microsclerotia. Blastospores (Fig 11.8(a), (c)) are vegetative cells by which the Ascomycetes proliferate through the body of an infected insect (Vestergaard et al., 1999), which could be considered a six-legged liquid fermentation vessel. Their growth is yeast-like, but it is not the true budding of yeasts. *Beauveria*, *Metarhizium*, *Nomuraea* and *Isaria* will grow as blastospores under the appropriate liquid fermentation conditions. The blastospore is a more environmentally fragile propagule than the aerial conidium, and special approaches are necessary to make it desiccation tolerant.

Submerged (or microcycle) conidia are produced by *Beauveria* and *Metarhizium* (Fig 11.8(b), (d)) (Thomas et al., 1987; Zhang, 2001). Microcycle conidiation has been defined as the production of conidia directly by a spore without the intervention of hyphal growth (Anderson and Smith, 1971). These conidia are morphologically and ultrastructurally different from true, aerial conidia; they lack one layer in the spore wall and have some different physical properties (Hegedus et al., 1990). They also germinate at a rate intermediate between blastospores and aerial conidia. The microcycle conidia of *Metarhizium acridum* (Driver and Milner) can evidently also be produced on solid medium (Zhang et al., 2009).

Submerged fermentation can be used to produce mycelial masses, which are then dried with preservatives to make granular formulations, such as was described in Section 11.2.4 for the Entomophthorales (Rombach et al., 1988).

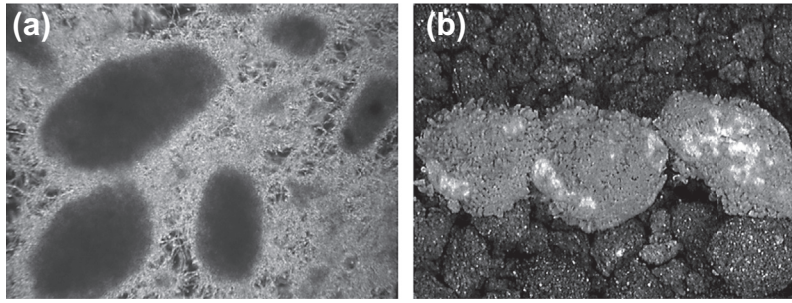

FIGURE 11.9 (a) Microsclerotia of *Metarhizium brunneum*; (b) granules formed from microsclerotia that have conidiated upon rehydration on moist soil.

When rehydrated, the mycelium generates large numbers of conidia. For a time in the 1990s, a *Metarhizium*-based, dried mycelial pellet, which would conidiate when rehydrated upon application to soil, was commercialized by Bayer AG as BIO1020. These pellets were produced in submerged fermentation using specific media and additives, which caused the mycelium to aggregate as large pellets. The pellets could then be harvested and dried to form 0.5–1 mm diameter granules (Andersch et al., 1993). This product and process were subsequently abandoned.

Fungi within the genus *Metarhizium*, except for *M. acridum*, can also produce compact melanized bodies, 50–200 μ in size, termed "microsclerotia" because of their similarity to such forms in plant pathogenic fungi (Fig. 11.8(d)). These microsclerotia are generated under certain nutrient and aeration conditions (Jackson and Jaronski, 2009a). So far, all strains within the nonacridum species of *Metarhizium* produce microsclerotia, although the numbers seem to vary by strain (Jackson and Jaronski, unpublished data). Experiments with multiple strains of *B. bassiana* and *Nomuraea rileyi* have failed to yield microsclerotia (Jackson and Jaronski, unpublished data). Microsclerotial fermentations can be simply filtered and air dried; the process has been scaled up to 100-l fermenters (Jackson and Jaronski, 2012). When microsclerotia are rehydrated, they rapidly conidiate and can thus be made into conidiogenic granules for use (Fig. 11.9). A noted feature is that microsclerotia can be simply air dried and have considerable shelf-life stability; in addition, the resulting granules can be sized as needed for particular applications. There are a number of considerations when choosing the most appropriate propagule for deployment against an insect. The reader is referred to Jackson et al. (2010) for an exploration of this topic; the discussion is not restricted to *Beauveria* and *Metarhizium*, but also includes relevant data about *Isaria fumosorosea*, which is otherwise discussed in Section 11.2.6.4.1.

11.2.6.2.2. Media

A variety of standard fermentation media can be and have been used. Carbon sources are typically glucose, while nitrogen sources can vary. Yeast extract,

peptone, and inorganic nitrogen compounds have been used in laboratory experiments, while commercial scale fermentations use cottonseed flour (Pharmamedia®, Archer Daniels Midland Company, Decatur, IL, USA), or casamino acids (e.g. Solulys, mentioned earlier).

The nature of the nitrogen source affects the predominant propagule obtained. Microcycle conidium production by *B. bassiana* requires inorganic nitrogen and very high carbohydrate levels (Thomas et al., 1987), whereas blastospore production is favored by organic nitrogen, especially corn steep liquor or corn steep solids. Thomas et al. (1987) first described production of microcycle conidia by *B. bassiana*. Their medium consisted of 5% glucose and 1% KNO_3. This medium preferentially produced high numbers of submerged conidia, whereas other carbon sources strongly favored blastospore production. In work refining the production of *B. bassiana* blastospores and microcycle conidia, Chong-Rodríguez et al. (2011) discovered that a medium of glucose plus casamino acids or one containing glucose, peptone, and KNO_3 produced high numbers of blastospores, whereas a glucose-KNO_3-corn steep liquor medium produced mostly microcycle conidia. Slight variations in the ratio of carbon and nitrogen, or the nature of the carbon source, will also affect blastospore yields. For example, with 5% sucrose as the carbon source, *B. bassiana* produced 4.6×10^8 blastospores and microcycle conidia per milliliter with a 9:1 ratio of the two. When the media was supplemented with 5% sugarbeet molasses, *B. bassiana* generated 2×10^9 spores per milliliter, but only 64% were microcycle conidia with the remainder being blastospores (Jakobs-Schönwandt et al., 2011). Strain and species differences increase the complexity in defining an optimal medium for either blastospores or microcycle conidia (Vega et al., 2003).

Organic nitrogen, preferably as brewer's yeast, and high levels of sucrose seem to be essential for submerged conidia production by *M. acridum* (Jenkins and Prior, 1993; Leland et al., 2005a, 2005b), which will produce blastospores in a medium of glucose and C:N ratio > 30:1, using casamino acids as the nitrogen source (Jaronski, unpublished data). Issaly et al. (2005) further refined medium and fermentation parameters, affirming the use of sucrose and yeast extract and identifying an optimal C:N ratio of 1:6 for blastospores. Zhang et al. (2009) described production of microcycle conidia by one strain of *M. acridum* on an agar medium consisting of 3% sucrose, 0.5% yeast extract, 0.3% $NaNO_3$, and trace salts. The same fungus conidiated normally on quarter-strength Sabouraud dextrose agar (1% dextrose, 0.25% neopeptone, 0.025% yeast extract). Whether other *M. acridum* strains do the same remains to be determined. A high-osmolarity medium (using polyethylene glycol) seems to have allowed formation of a spore intermediate between blastospores and microcycle conidia in morphology and physical-chemical properties. These spores germinated more quickly than submerged conidia at a rate similar to blastospores and were more pathogenic, than either submerged conidia or aerial conidia (Leland et al., 2005b). Fargues et al. (2002) added 0.4% polyoxyethylene sorbitan monooleate. Microsclerotia of *Metarhizium* spp. can be produced using glucose-rich

media having a C:N ratio > 30:1 (Jackson and Jaronski, 2009). The preferred nitrogen source can be casamino acids, such as HyCase M® (Kerry Group Services, United Kingdom) or Pharmamedia® (Archer Daniels Midland Company; Jaronski and Jackson, 2009b).

For production of marcescent mycelium, similar methods as for the Entomophthorales have been employed (Rombach et al., 1988). Magalhaes et al. (1994) identified 4% sucrose and 1% yeast extract as the best medium of several assessed for an isolate of *M. anisopliae*, yielding 9 g biomass per liter in 72 h and 1.6×10^{10} conidia per gram of dry mycelium after rehydration. This process has had very little implementation, however, and is best suited for small-scale applications. The Bayer mycelial pellet mentioned in Section 11.2.6.2.1 was produced in a glucose-yeast autolysate-K_2HPO_4 medium with $MgCl_2$ and trace salts. The medium was seeded at a very low rate (10^6 conidia/ml for small volumes; if a preculture was used, then it was added at 3% of final volume) to induce pellet formation (Andersch et al., 1993).

11.2.6.2.3. Fermentation Parameters: Air, Agitation, pH, Batch and Fed-batch Culture

A high degree of aeration for submerged fermentation is essential. The difficulty lies in the method of aeration. On a small, flask-scale level, rotary agitation at 300–400 rpm, ideally with baffled flasks, should be used. For *I. fumosorosea*, 20% dissolved oxygen is necessary for good blastospore production (Jackson, 2012). Machado et al. (1994) stated that the best air flow rate was 1.5 volumes of air per volume of medium per minute for mycelium production by *M. anisopliae*. De la Torre and Cardenas-Cota (1996) used air flow and agitation to achieve 20% saturation for optimal production of *I. fumosorosea*. The traditional stirring paddle method of agitation in larger volume fermenters can lead to mycelium and blastospore damage through the shear forces present at required speeds. Sparging fermenters overcome this problem but may not provide sufficient oxygen efficiently in larger volumes.

Recommended temperatures are those at which the particular fungus grows best, typically 24–28 °C, although there is one report that heat induction of *I. fumosorosea* inoculant for submerged fermentation strongly increased microcycle conidiation (De La Torre and Cardenas-Cota, 1996). In their study, they exposed the inoculum, a conidial suspension, to an initial 24-h incubation at 37 °C followed by 96 h at 30 °C. The germinating conidia immediately went into blastospore rather than mycelial growth and quickly gave rise to microcycle conidia. Anderson et al. (1978) saw a parallel heat induction of microcycle conidiation in *Paecilomyces varioti* Samson. Whether this phenomenon extends to *Beauveria* spp. or *Metarhizium* spp. remains to be determined.

11.2.6.2.4. Equipment for Submerged Fermentation

Discussion of equipment used in submerged fermentation—especially in large-scale, industrial production—is beyond the scope of this review. Typically, the

same fermentation equipment as is used for other microorganisms is used for the entomopathogenic Ascomycetes. The literature about submerged liquid fermentation to produce microorganisms is vast and the reader is referred elsewhere for more information (e.g. Stansbury and Whitaker, 1993; Rao, 2010).

11.2.6.2.5. Downstream Processing

The product of liquid culture is typically harvested by filtration, then processed further to stabilize the fungal material. Industrial-scale filtration uses a ribbon filter or continuous flow centrifugation. Often, diatomaceous earth is added to aid in filtration because fungal culture will often clog filters.

If the fungus material (blastospores, microcycle conidia) is to be used within a short time after harvest, it can be preserved by refrigeration. Otherwise, proper preservation of the propagules is necessary for acceptable shelf life and optimal handling characteristics in formulations. Conversion of the fungal propagules into a dry powder can be accomplished by simple air drying, freeze drying, or spray drying, but preservation of the viability of the propagules, especially blastospores, has been the greatest challenge. Most of the research regarding blastospore preservation has been directed towards *I. fumosorosea* rather than *Beauveria* or *Metarhizium*, but it is discussed here because of its relevance to the latter fungi. Typically, blastospores lose considerable viability upon drying (Inch et al., 1986; Fargues et al., 1994). Microcycle conidia are less prone to this phenomenon but do suffer viability loss.

In simple air drying, relative humidity of the drying air significantly affects desiccation tolerance of *I. fumosorosea* blastospores (Jackson and Payne, 2007). A humidity level >40% allowed significantly higher rates of initial blastospore survival after drying versus drying with lower humidity air; air with a relative humidity (RH) >50% improved the shelf-life of the air-dried blastospore preparations. Fargues et al. (1979) discovered good blastospore preservation by lyophilization using powdered milk supplemented with glycerol as cryoprotectants. Lyophilization, however, is not economically practical on a large scale.

Spray drying is a common technique used in many microbial fermentations. Stephan and Zimmermann (1998) obtained 90% blastospore/microcycle conidium viability after spray drying *B. bassiana*, *M. anisopliae*, *M. acridum* and *I. fumosorosea* when the blastospores were suspended in 20% skimmed milk powder and 2.5% sugar-beet syrup before spray drying; unprotected submerged spores were killed. Yeast extract, soluble starch, hydroxyethyl-starch and bentonite clay failed to protect the propagules. Spray dryer inlet and outlet temperature, as well as the flow rate, are also important to minimize damage to blastospores. Stephan and Zimmermann (1998) documented that 64 °C and 48 °C were the respective critical temperatures for inlet and outlet temperatures.

Of relevance to this discussion is the work of Horaczek and Viernstein (2004), who reported a detailed comparison of lyophilization, spray-drying, and fluid-bed drying for processing of aerial conidia of *Beauveria brongniartii* (Saccardo) and *M. anisopliae*, harvested with water from agar substrate. They

found that *M. anisopliae* was slightly more heat resistant than *B. brongniartii* (2 min at 50 °C). In addition, conidia of both fungi suffered considerable mortality (~65%) after spray drying, even with a 60 °C/40 °C inlet/outlet temperatures. *B. brongniartii* was best preserved with lyophilization, whereas the drying phase caused considerable mortality to *Metarhizium*. *M. anisopliae* was better preserved with either of two possible preservatives, skim milk and polyvinylpyrrolidone. Finally, fluid bed drying killed both fungi, even when the inlet temperature was lowered to 60 °C.

Chen et al. (2002), in examining several drying methods for *I. fumosorosea* conidia, observed that 20- to 24-h low-vacuum (0.1 MPa), low-heat (30 °C) drying was the best, yielding only a slight loss in conidial viability. High-vacuum freeze drying, high-vacuum room temperature drying, and heating-drying at 35 °C were all deleterious for conidial viability. Thus, desiccation and temperature intolerance are the most important constraints in processing conidia, and likely even more so for blastospores and microcycle conidia.

Effort has also been concentrated on media manipulations to produce more desiccation-tolerant blastospores. For example, Cliquet and Jackson (1999) evaluated the impact of amino acids, carbohydrates, trace metals, and vitamins on the freeze-drying tolerance of *I. fumosorosea* blastospores as well as hyphal growth and sporulation. Sodium citrate or galactose as the sole carbohydrate produced more desiccation-tolerant spores but yielded lower blastospore concentrations. Media containing glucose concentrations greater than 20 g/l and 13.2–40 g casamino acid per liter supported maximal production of desiccation-tolerant blastospores of *I. fumosorosea* using air drying but not necessarily the greatest yields per se (Jackson et al., 1997). Thus, there seems to be tradeoff between blastospore yield and desiccation tolerance. In the case of the BIO1020 mycelial pellets, they were simply harvested using a 0.1-mm pore sieve plate, washed, and dried in a fluidized bed granulator (Andersch et al., 1993) or freeze dried.

11.2.6.2.6. Major Technical Problems/Solutions in Submerged Fermentation

There are several major problems with blastospore or microcycle conidia production using submerged fermentation. Rarely are the desired propagules produced in high purity. Blastospores are "contaminated" with mycelium, whereas blastospores and mycelium are copresent with microcycle conidia. For example, a mixture of cell types dilutes out the desired type and necessitates larger fermentation volumes for a specified number of the desired spore. Such mixtures make harvesting and purification difficult or render the physical nature of the harvested and stabilized product incompatible with formulation. The hydrophilic nature of blastospores and microcycle conidia requires different liquid formulations than do the hydrophobic aerial conidia. Also, with few exceptions, the shelf life of dry blastospore or microcycle preparations is shorter than that of dry aerial conidia and often commercially unacceptable without

special measures, such as vacuum packaging, low-temperature storage, or some sort of encapsulation. All of these measures can add considerable cost to a commercial mycoinsecticide.

Microsclerotia production with *Metarhizium*, on the other hand, offers a viable alternative, in which sporulating granules (in soil, under a plant canopy, or in other humid, protected microhabitats) are practical. Microsclerotia seem to be considerably stable as a granular formulation and produce abundant conidia when rehydrated. Unfortunately, microsclerotia production seems to be restricted to the genus *Metarhizium*. A number of isolates of *B. bassiana* failed to produce microsclerotia under any conditions tested. (Jaronski and Jackson, unpublished data). Microsclerotia production by the genera *Isaria, Verticillium*, or *Nomuraea* has not yet been investigated.

11.2.6.3. Other Novel Methods

In addition to several atypical methods for fungus production described by Bartlett and Jaronski (1988), a novel technology using media-impregnated nonwoven fabric bands was developed and commercialized in Japan in the mid-1990s as Biolisakamakiri, using *B. brongniartii* (Higuchi et al., 1997). A U.S. patent subsequently described the methodology and specifics for mechanization and scale-up (Higuchi et al., 1996).

Wood pulp fabric (and in later modifications rayon-polypropylene or rayon polyester), typically 0.5–2 mm in thickness and sometimes laminated, is first impregnated with a liquid culture medium, then is heat dried, with the drying process sterilizing and stabilizing the media. This fabric can be stored dry. The patent describes a machine that can produce the dry, medium-impregnated fabric in long rolls. A hydrophilic polymer added to the fabric improves medium absorption and fungal conidiation. To cultivate a fungus for conidial production, the fungus is first grown in a liquid medium, which is then applied to the dry fabric. With suitable humid incubation (>80% RH, ~25 °C.) for 3–14 days, the fungus abundantly colonizes and conidiates on the surface and even within the fabric, which is then partially or completely dried and refrigerated until use. Levels of $1–2 \times 10^8$ conidia/cm^2 were obtained in this manner. The method was developed for deployment of *B. brongniartii* against tree-dwelling Chrysomelidae beetles, who have a habit of moving up and down tree trunks. A conidiated band is wrapped around the tree trunk, and the insects pass beneath or over it in their movements, coming into contact with the conidia. If the semidry band is rewetted, for instance by rain, there is another flush of conidiation. This fabric carrier was also used to obtain *Lecanicillium* conidia at similar levels (Higuchi et al., 1996). U.S. workers have adapted the fabric methodology for *Metarhizium* in an effort to evaluate its potential to combat the Asian longhorned beetle (*Anoplophora glabripennis* [Motschulsky]; Dubois et al., 2004; Hajek et al., 2006; Shanley et al., 2009). The approach was also evaluated and showed promise versus the pecan weevil (Shapiro-Ilan et al., 2009).

In parallel to the above development, Jenkins and Lomer (1994) developed a similar method for mass production of *M. acridum* conidia on cellulose cloths. In their case, however, the strategy was to remove the conidia off the cloths by washing or by mechanical separation and SIR classification. The method was abandoned in favor of grain-based solid substrate fermentation.

Bringing the liquid fermentation facility to the side of the field or in the form of a self-contained, portable fermenter was another novel concept developed in the 2000s (Jackson et al., 2004). By the late 1990s, production of beneficial bacteria on site for use as soil amendments in managed turf had been demonstrated, and that technology was adapted to entomopathogenic fungi. Jackson and his associates developed and validated a self-contained system for producing *I. fumosorosea* blastospores on site for immediate application. Prepared media concentrates were diluted in a chemically disinfected (but not completely sterilized), portable, 100-l fermenter that could be placed beside the field or glasshouse intended for application. A rehydrated, dry blastospore preparation was used as inoculum. Fermentation variables were monitored and controlled by a computer attached to the unit. Yields of 8×10^{11}/l blastospores were obtained after 48 h of fermentation starting with an inoculum level of 5×10^{9} blastospores per liter of medium. Bacterial contamination was a periodic problem, however, despite use of low pH (4). The system was designed to accommodate limited user expertise and the greatest self-containment possible. This technology is readily adaptable to *Beauveria*, *Metarhizium,* and the other Ascomycetes. Commercial development ensued, initially targeting high-value turf situations, but was eventually ended due to cost.

11.2.6.4. Other Ascomycetes

11.2.6.4.1. *Isaria* (*Paecilomyces*)

The Ascomycete *I. fumosorosea* has generated considerable interest and commercial exploitation for the control of several glasshouse pests in recent years. De Faria and Wraight (2007) identified seven commercial products containing *I. fumosorosea* at that time; in the United States and EU, there is currently one registered strain, Apopka 97, and a second undergoing registration review, FE9901 (U.S. Environmental Protection Agency, 2010, 2011). A second species, *I. farinosa* (Holmsk.) Fr., has been occasionally identified as an insect pathogen of potential use, but there has been little effort directed towards its mass production.

Although *I. fumosorosea* can be produced using solid substrate fermentation with substrates and methods developed for *Beauveria* and *Metarhizium*, many strains have a far blue-near ultraviolet (UV) light requirement for good conidiation (Sakamoto et al., 1985; Sanchez-Murillo et al., 2004; Kuźniar, 2011). In continuous darkness, conidiation seems to be reduced to continued, robust, vegetative growth. This situation may be the cause of the relatively low yields in at least one assessment of different grains for producing *I. fumosorosea* conidia

(Kuźniar and Krysa, 2011). A need for light:dark cycles may also be present in some *I. fumosorosea* strains (De la Torre and Cardenas-Cota, 1996). However, not all strains of *Isaria fumosorosea* display this behavior. In screening many isolates of this species for potential commercialization, Mycotech Corporation identified four to five isolates that conidiated abundantly on agar or on solid substrate in continuous darkness (Jaronski, unpublished data).

Current commercial production seems to be based on liquid fermentation. Eyal et al. (1994) described the liquid fermentation production of Apopka 97 *I. fumosorosea* using molasses, cottonseed flour, and corn steep liquor to produce blastospores and mycelium that are then encapsulated in an alginate matrix to yield prill having the potential of producing conidia upon rehydration. This system formed the basis of commercial production of PFR97®, at least in initial years of commercialization. Current processes are proprietary. FE9901 is produced by its company in submerged fermentation and consists of blastospores. Considerations in producing *Isaria* blastospores by submerged fermentation are discussed in Section 11.2.6.2.

11.2.6.4.2. *Lecanicillium*

Lecanicillium muscarium Zare and Gams and *L. longisporum* Zare and Gams (both formerly classified as *Verticillium lecanii* [Zimmerman]) have attracted some attention as biocontrol agents of Homoptera and spider mites. In their 2007 survey, De Faria and Wraight (2007) noted 16 commercial products in existence. Two have been in commercial use in Europe since the 1980s. This genus is notable in that conidia are borne in slime balls and rarely in dry chains, unlike the other Hyprocreales fungi, which produce hydrophobic conidia.

Both submerged liquid and solid substrate fermentation have been used to produce these two fungi, using methods similar to those for the genera *Beauveria* and *Metarhizium*. Both aerial conidia and submerged conidia are produced in the respective fermentation systems. There are differences in morphology, germination, and growth patterns between the two types, but there is no clear indication in the literature about their relative desiccation tolerance.

Derakhshan et al. (2008) reported that molasses-yeast broth was the best liquid medium while rice yielded the highest conidial production. A wheat bran-sugarbeet pulp mixture (9:1 w/w) has also been touted as an excellent solid substrate by Grajek (1994). Feng et al. (2000) identified rice bran to be the best medium, better than cooked rice; a bran:husk ratio of 1:1 was almost as good. Of note is that spore production in liquid media, at least shake flask culture, decreased significantly after 9 days, indicating a dynamic situation in that system. Several agricultural waste products have also been examined with a view of lowering production costs (Lopez-Llorca and Carbonell, 1998; Lopez-Llorca et al., 1999; Shi et al., 2009). Optimal temperatures for growth and sporulation in both systems are typically 20–25 °C. There is virtually no public information about harvesting and subsequent processing of *Lecanicillium*. Presumably, the aerial conidia can be washed off solid substrate because they are not

hydrophobic; submerged conidia can be harvested by methods described for other Hypocreales.

11.2.6.4.3. *Hirsutella thompsonii*

Hirsutella thompsonii Fisher has undergone several commercialization efforts, primarily in the United States in the 1970s and Cuba in the 1980s. Only one product made from this fungus was identified by De Faria and Wraight (2007) in their survey. Initial data (Van Winklehof and McCoy, 1984) indicated that microcycle conidia could be produced in submerged fermentation, but by only 1 of 15 strains evaluated, and conidial germination was poor. The earliest program used liquid fermentation to produce mycelial mats, which were refrigerated then formulated on the day of application by creating an aqueous slurry for spraying (McCoy et al., 1975). The medium consisted of dextrose, yeast extract, peptone, and essential mineral salts. Subsequently, solid substrate fermentation using wheat bran was substituted by the company commercializing the fungus (Jaronski, unpublished data). The fungus was marketed for the control of eriophyid mites in citrus, but sales were terminated in the 1980s for a number of technical reasons. In 1988, Latge et al. (1988) were also able to obtain microcycle conidia from submerged fermentation, but from a strain unique in this regard.

A low level of interest has continued, primarily in South Asia, but there do not seem to be any commercial products at present. Ground maize coated with molasses was the best solid medium for conidial production evaluated by Maimala et al. (1999). Sreerama Kumar et al. (2005) focused on submerged liquid fermentation and observed that low concentrations of polyethylene glycol in dextrose peptone medium allowed production of mycelial pellets that were competent to produce conidia. A powder formulation containing both mycelia and conidia was developed in India during the 2000s, followed by two liquid formulations, but efforts to commercialize were discontinued (Sreerama Kumar, 2010).

11.2.6.4.4. Nomuraea

Nomuraea rileyi (Farl.) Samson, a pathogen of certain Lepidoptera, has undergone relatively little development and commercialization. De Faria and Wraight (2007) identified only one product, but it is no longer being marketed. The conidia were produced on erect conidiophores much like in *Beauveria*, *Isaria*, and *Metarhizium*.

Both solid substrate and submerged liquid fermentation have been explored for mass production of this fungus, primarily in Latin America, South Asia, and China. Rice has been identified as an excellent substrate, although the best conidial yields require precooking in boiling water versus simple autoclaving (Mendez et al., 2010); the boiling presumably gelatinizes the starch in the rice, making it more available to the fungus. The liquid phase of this biphasic system

used molasses and yeast extract. In India, Vimala Devi (2000), Kulkarni and Lingappa (2002), and Lalitha et al. (2008) identified sorghum and rice grains as the solid substrates of choice. The second group also supplemented their grain with 1% yeast extract for better conidial production. Vimala Devi et al. (2000) determined that 2% barley extract and 1% soybean extract provided cheap carbon and nitrogen sources for the liquid production phase, whereas sorghum seeds with 0.5% yeast extract served as the best solid substrate. They also noted that good aeration of the solid substrate was essential and closed plastic bags prevented conidiation.

In evaluating a variety of agricultural products and byproducts, Tincilley et al. (2004) identified sugarcane spent wash liquid medium (still culture), rice, finger millet, and groundnut cake as suitable solid substrates. Thakre et al. (2011) continued examination of alternative substrates; rice, sorghum, and refuse raw bananas yielded the greatest conidial numbers. A liquid medium for submerged fermentation, consisting of molasses and yeast extract, was developed either for still culture or to produce inoculum for rice gruel semisolid substrate (Ramegowda et al., 2007). In Colombia, a mass production system was devised using plastic bags with autoclaved mijo grains, inoculated with 8-day-old fragments of agar grown with the sporulated fungus (Villamizar et al., 2004). A recent Chinese study indicated that illumination was essential for conidiation in at least one strain of *N. rileyi* (JianWen et al., 2009), yet Bell (1975) stated that light had no effect on growth or sporulation. This discrepancy may be due to strain differences.

11.2.6.4.5. *Aschersonia* spp.

Aschersonia spp. are specific to Aleyrodidae (whiteflies) and Coccoidea (scale insects). Because of the growing impact of the former insect, the genus—particularly the species *A. aleyrodis*—has received periodic attention by researchers (Ramakers and Samson, 1984; Fransen and van Lenteren 1993). It frequently causes severe epizootics in these insects in the tropics and subtropics. It also was one of the first fungi used in inoculative biocontrol; in the early 1900s, U.S. citrus growers introduced infected insects into their orchards. One European company briefly considered its commercialization but did not do so. An overview of the biology of *A. aleyrodis* is given by Fransen (1990). This fungus differs from the other Ascomycetes because the aerial conidia are produced within pyncnidia rather than on exposed structures.

Very little work on mass production of this fungus has been published, and most research has focused on *A. aleyrodis* rather than the other species in the genus. Ibrahim et al. (1993) observed that semisolid rather than liquid media were better for growth and sporulation, with macerated pumpkin being the best of the media they tested. They also observed that the fungus sporulated well on the surface of still liquid culture, a process developed by Czech scientists for *Beauveria* (Bartlett and Jaronski, 1988). Zhu et al. (2008) studied the nutritional requirements of one strain of *A. aleyrodis* in liquid culture (for mycelial

biomass) and on agar media (for conidial production). They thereby identified a semisynthetic liquid medium of soluble starch, tryptone, Ca^{2+}, and folacin, and a solid medium of lactose, tryptone, Fe^{2+}, and Vitamin B_1. Use of an orthogonal matrix method allowed Zhu and coworkers to define the concentrations of each medium component. Very recently, optimal solid substrate medium for *Achersonia placenta* Berk. was identified by response surface method analysis to contain millet, KH_2PO_4 $MgSO_4$, albeit in an agar medium (Qiu et al., 2013).

11.2.6.4.6. Culicinomyces

Culicinomyces clavisporus Couch was investigated beginning in the 1980s as a biocontrol agent for control of larval mosquitoes (Sweeney, 1985). Since then, however, interest in this fungus seem to have waned, probably because of the commercial success of *Bacillus thuringiensis israelensis* Berliner (Bti) and *B. sphaericus* Meyer and Neide for mosquito control. One exception was a small resurgence regarding its potential to control biting midges (*Culicoides* Latreille), reported by Unkles et al. (2004), but there is little published literature about the topic. Conidia have been produced on wheat-bran solid substrate or in liquid media (corn meal extract, corn steep liquor, or standard nutrient broths), but yields were very low in comparison to efficacious field rates. The fungus has been experimentally grown in 750–1000 l fermenters in Australia and harvested by filtration or centrifugation (A. Sweeney, personal communication), but no details about the process are known to the author.

Additional experimental production using the marcescent process as described earlier for Entomophthorales was evaluated by Roberts et al. (1987) and Goettel et al. (1984). Mycelia were produced in liquid peptone-yeast extract-glucose medium, harvested by filtration treated with 10% sucrose, air dried to 13% moisture, then granulated. Although freshly dried and granulated marcescent mycelium produced abundant conidia that could be stored at room temperature or 4 °C, the product lost viability within 2 weeks. Mycelium stored at −20 °C did retain viability at least for 63 days. Given the biology and ecology of *Culicinomyces,* this fungus has potential as a persistent biological control agent for mosquitoes and perhaps *Culicoides* spp., but considerable technical advances in mass production are still necessary to achieve success.

11.3. PROCESS AND QUALITY CONTROL IN MASS PRODUCTION

A quality mycoinsecticide or fungal biocontrol agent is critical for successful use. A quality mycoinsecticide demands a thorough quality assurance component to any production effort. Despite what ostensibly seems to be fine control of fermentation variables, the production process (be it liquid or solid substrate) is only semicontrolled, especially when on a large commercial scale (>1000-l liquid fermenters, 10,000 kg solid substrate fermentation batches). For example, during the initial commercial production of *B. bassiana* GHA by Mycotech in

the mid-1990s, meaningful differences were observed in the shelf life of conidial powders from 16 full-scale production runs, with the time to loss of 50% of the original conidial viability (LT_{50}s) at 25 °C ranging from 180 to 700 days. By 1999, after a new production facility had been established, the half lives of conidial powders ranged from 280 to 450 days (25 °C). These powders were produced under closely controlled environmental conditions and dried to 5–7% moisture. To ensure that a quality product will be produced over the long term, it behooves the mycoinsecticide enterprise to constantly monitor agent viability, physical specifications (e.g. moisture), contamination levels, virulence, and shelf life. Vigilance is paramount. See Bateman (2007) and Jenkins et al. (1998) for discussion of specific quality control parameters and methods.

There is another problem in mass production efforts—genetic changes (degeneration) in the fungus. Butt et al. (2006) expressed the situation succinctly, "Entomogenous fungi will degenerate when continuously cultured on nutrient-rich media." Genetic changes can encompass virulence determinants, changes in colony color and gross morphology, or decline in conidial or metabolite production. Some of the fungi can give rise to morphologically different sectors in radial colonies. I have found this particularly true of *Metarhizium* spp., less so with *B. bassiana*. Butt et al. (2006) presented a detailed discussion of this topic and the interested reader should refer to this work.

Changes in virulence with repeated subculture vary widely, depending on the fungus species and strain. Butt et al. (2006) summarized all the reports up to 2006. Since then, additional studies have been performed by Hussain et al. (2010), Shah et al. (2007), Ansari and Butt (2011), Rajanikanth et al. (2011), and Safavi (2011, 2012). Under commercial conditions, bioassays of 35 standard production runs of *B. bassiana* GHA, spanning 2 years, did not reveal any significant changes in virulence (LD_{50}) for the migratory grasshopper, *Melanoplus sanguinipes* (F.), nor did eight production batches for nymphal *Bemisia tabaci* (Gennadius) (Jaronski, unpublished data).

It is possible to restore, at least partially, lost attributes by in vivo passage through an insect (Shah et al., 2005). Other examples are given in Butt et al. (2006). In the course of selecting for improved heat tolerance of a *M. anisopliae* strain via continuous growth under selective conditions, the ability to sporulate was lost in two heat-tolerant clones but regained after one passage through a grasshopper (de Crecy et al., 2009); virulence was only partially restored.

Nevertheless, care must be taken to minimize the number of in vitro conidiation cycles. Typically in an industrial situation, multiple "mother" cultures are prepared from the second or third in vitro passage from an insect and preserved by freezing with cryoprotectants to −80 °C or lyophilized. From this mother culture, enough subcultures are prepared for 6 months or a year of production cycles, and they are frozen until used. When these are exhausted, another mother culture is thawed and the cycle repeated.

There is another aspect to potential genetic changes—the number of mitotic divisions and propagules generated during a typical production cycle. Consider

that in one commercial production process, an initial inoculum of 1×10^9 conidia results in 4.5×10^{16} conidia at the end of a biphasic production cycle, a 4.5×10^7-fold multiplication. Yet, no changes in the fungal restriction fragment length polymorphism pattern were noted, indicating considerable genetic stability (Jaronski, unpublished data).

11.4. CURRENT KNOWLEDGE ABOUT EFFECT OF CULTURAL CONDITIONS ON PROPAGULE ATTRIBUTES

Fungal spores (blastospores and conidia) have a proscribed environmental range that affects their infectivity (efficacy). Spores require high humidity for germination, although this requirement can be obviated by the microhabitat of the insect cuticle or phylloplane boundary layer or be modified with formulations. In addition, spores can be subjected to desiccation in the target habitat following application in an aqueous spray. Conidial germination, as well as vegetative growth within the insect, is limited in both speed and extent by high (>32 °C) and low (<15 °C) temperatures. The speed of conidial germination, even at optimal temperatures, can be critical with frequently molting insects such as aphids. UV-A (315–406 nm) and UV-B (280–315 nm) are lethal to conidia, greatly shortening their persistence in the foliar habitat and limiting efficacy when the target insects are more likely to acquire spores from treated habitat rather than direct spray. See Jaronski (2010) for a full discussion of ecological considerations in the inundative use of entomopathogenic fungi.

The different fungus species and isolates within each species vary in their tolerances to these environmental factors (Devi et al., 2005), as well as in virulence and mass production potential. Typical development programs therefore seek to identify the best fungus for a particular use and target, and formulations are devised to accommodate fungal deficiencies to a great or lesser degree of success. In recent years, there have been attempts to improve efficacy through genetic modifications. A developmental program can therefore expend considerable effort in screening candidate fungi; emphasizing virulence, spore production, and shelf life; and optimizing fermentation variables to maximize spore production.

However, there may be another approach, at least to improve an already acceptable fungal agent: manipulation of fermentation conditions to improve environmental stress tolerance. Magan (2001) posed four key questions on this subject: "(1) can one manipulate the physiology of non-xerophilic/tolerant fungi to accumulate useful endogenous reserves into inocula for improved environmental stress tolerance?; (2) would this result in improved germination/growth under environmental stress?; (3) can this improve the establishment of inocula and conserved biocontrol potential in the field? and (4) does ecophysiological manipulation have a role in improving the production and quality of inocula?" Magan (2001) pointed out that there are xerotolerant/xerophilic fungi able to tolerate a range of water availability that inhibits the entomopathogenic

species, which are inhibited by water activities below 0.95–0.93 a_w. The ability to tolerate water stress conditions in these fungi is associated with compatible solutes within the spores. The potential for such manipulations was identified by Matewele et al. (1994), who observed that mutants of *M. anisopliae* and *I. farinosa*, which were able to germinate and grow at lower water activities than parental strains and which were subsequently grown on low water activity medium (a_w = 0·969), were more virulent against green leafhopper than the parental strains. Also, *B. bassiana* conidia, having an increased glycerol and erythritol content, germinated in low a_w (0.935) media but conidia having low glycerol and erythritol failed to germinate below 0.951 a_w (Hallsworth and Magan, 1995). In addition, Magan (2001) noted that *M. anisopliae* conidia from an insect had significantly greater polyol content and different sugar/polyol ratios than conidia produced on an agar medium. These data strongly suggest that endogenous solutes can be manipulated. Can manipulation affect stress tolerance and improve efficacy? Although there is not an abundance of data on this subject, what does exist poses intriguing possibilities.

11.4.1. Age of Conidia

Hall et al. (1994) observed that young conidia produced by all the isolates of *M. anisopliae* and *Lecanicillium* tested germinated faster than older ones, whereas *B. bassiana* conidia germinated at the same rate, regardless of culture age. The impact of culture age on conidial germination appeared to be species- and strain-dependent. More recently, Smith and Edgington (2011) showed that the capacity of conidia to withstand water stress developed by low water activity was related to the age of fungal cultures, implying that prolonged production cycle (conidial ageing) may also improve the fitness of conidia.

"Old" spores (collected after 14 days of fungal growth) of *B. bassiana* and *M. anisopliae* produced on agars composed of whey permeate or millet were more thermotolerant than conidia from quarter-strength Sabouraud dextrose yeast agar, although the differences were inconsistent among the two isolates of each species (Kim et al., 2010c). A mixed message was described by Rajanikanth et al. (2011), whereby conidia of six strains of *B. bassiana* from 14-day-old cultures had greater virulence for larval *Spodoptera litura* F. than conidia from 7-, 21-, and 28-day-old cultures.

A clue to what may be going on with age-related differences in conidia is offered by Kim et al. (2010c), who observed that two isolates each of *B. bassiana* and *M. anisopliae* had conidia of two types with different degrees of hydrophobicity, termed young (7-day-old) versus old (mature, 14-day-old) conidia. Thermotolerance seems to have been directly associated with hydrophobicity (age) of the conidia, although there were differences among the isolates and media used to generate the conidia. Quarter-strength Sabouraud dextrose yeast agar had the most pronounced difference between the two spore ages.

11.4.2. Conidia Produced under Certain Nutrient Conditions or under Osmotic Stress

Conidia of *M. anisopliae* and *I. farinosa*, produced on agar media adjusted to an a_w of 0.96, were significantly more infective for *Galleria mellonella* (L.) larvae at 86% and 78% (Hallsworth and Magan, 1994b). The series of studies by Hallsworth and Magan (1994a, 1994b, 1994c, 1995, 1996) were significant in that they lay the foundation for manipulation of fermentation media to change solute content of conidia and thereby improve desiccation tolerance. There are inter- and intraspecies differences in the response to particular growth conditions (Hallsworth and Magan, 1999). Overall, however, lowering a_w to less than 0.98 reduces mycelial growth and conidial yield, so there is a fine line between obtaining xerotolerance and not seriously affecting spore yield. Also, it should be kept in mind that these studies were conducted with agar media; it may be difficult to control a_w within fine tolerances on a solid substrate, especially when in large quantities under commercial conditions.

Lane et al. (1991a, 1991b) examined the effect of C:N ratios on blastospore production by *B. bassiana*. They observed that inorganic nitrogen-limited blastospores had a longer shelf-life than blastospores from carbon-limited media, which was accompanied by differences in morphology and endogenous reserves. In addition, although there were no differences in the LC_{50}s of carbon- and nitrogen-limited blastospores for a leafhopper, the virulence (LT_{50}) of nitrogen-limited blastospores was greater than their carbon-limited counterparts and the former adhered more firmly to insect cuticle than the latter.

Virulence of *M. anisopliae* conidia seems to be slightly affected by the C:N ratio of the (agar) medium, with the most virulent conidia having an endogenous C:N ratio <5.2:1, per the claims of Shah et al. (2005). The conidial C:N ratios were affected by the agar medium used, with a glucose:peptone medium having a C:N ratio of >35:1, or an osmotically stressful medium, via KCl addition, causing the greatest endogenous C:N ratio. However, virulence for *Tenebrio molitor* L. was really unaffected from a biological perspective, varying from 3.5 to 4.1 days for one strain and 3.5–3.9 days for a second strain. A problem with interpreting these data is that virulence, as time to 50% mortality, is an expression of mycelial development rather than conidial fitness per se.

In a related study with *B. bassiana*, Safavi et al. (2007) did not observe the effect of endogenous C:N ratio as did Shah et al. (2005) with *M. anisopliae*. Although the endogenous C:N ratio was affected by the different media, Safavi and his associates could not see a clear relationship among C:N ratio, germination rate, conidial PR1 activity, and virulence in their *B. bassiana* isolates. Osmotic stress caused the lowest conidial production, however.

Rangel et al. (2004) noted that conidia of two isolates of *M. anisopliae* obtained from insect cadavers were more sensitive to UV-B irradiation than those produced on a rich artificial medium, and they also germinated more slowly. The artificial medium also had an effect with conidia from rice substrate

or two agar media having more UV-B tolerance than conidia on potato dextrose yeast agar. Rangel et al. (2006) subsequently observed that UV-B tolerance of *Metarhizium robertsii* (= *anisopliae*) conidia was increased at least twofold when the fungus was grown on agar medium containing nonpreferred carbon sources, such as fructose, galactose, or lactose versus a preferred carbon source, such as dextrose. With lactose, endogenous trehalose and mannitol accumulated to a significantly greater level. However, conidial yields were reduced, which could be a considerable disadvantage.

In follow-up work, Rangel et al. (2008a, 2008b) examined the effect of growing *M. robertsii* under different stress conditions. Conidia produced on a minimal agar medium and minimal medium supplemented with lactose had the faster germination and greater bioassay virulence for *T. molitor* than conidia from a rich medium (potato dextrose agar with yeast extract). These researchers also observed that conidia produced under conditions of carbon or nitrogen starvation possessed significantly greater heat and UV-B tolerance than conidia produced on nutrient-rich potato dextrose agar plus yeast extract (PDAY). The greater tolerances were associated with the greatest accumulation of trehalose and mannitol within the conidia. Similar results were seen with an osmotically stressful agar medium (0.8 M KCl or NaCl, a_w unknown). Conidial yield, however, could be severely affected by stressful conditions during mycelial growth.

Thermotolerance (conidial viability after exposure to 48 °C for 30 min) seems also to have been significantly increased when glucose (4% optimal) or starch (1%) were the carbon sources in agar media (Ying and Feng, 2006). When ≤50 μg/ml of Mn^{+2} was added, thermotolerance was extremely good and was greatly increased with sucrose and Fe^{+3} (Ying and Feng, 2006). The pH had some effect; thermotolerance was greatest when the fungus was grown at pH 5–6; osmotic stress, in the form of KCl, had an adverse effect. Amendments, such as Mn^{+2} or carbon supplementation with a grain-based solid substrate, are feasible, but again, whether this phenomenon extends to other strains or species remains to be elucidated.

An insight into the mechanisms of thermotolerance may be obtained from Leng et al. (2011). Using RNAi, they demonstrated that trehalose levels in *M. acridum* conidia were closely associated with tolerance to heat stress. There also seems to be a relationship between thermotolerance and formic-acid-extractable proteins in aerial conidia of *B. bassiana* and *I. fumosorosea* (Ying and Feng, 2004). The formic-acid-extractable proteins are affected by the nature of the carbon source, with glucose and sucrose causing significantly better thermotolerance than starch (in an agar medium).

Investigations about a_w effects can also be extended to solid substrate media. The a_w of rice substrate (modified by use of different normalities and amounts of HCl) affected the polyol content of *B. bassiana* conidia and the relative proportions of mannitol, arabitol, glycerol, and erythritol (Tarocco et al., 2005). The last two are the most biologically important: high intraconidial concentrations of glycerol and erythritol were associated with ability of conidia to

germinate at much lower a_w than otherwise (Hallsworth and Magan, 1995). The optimal a_w for glycerol/erythritol accumulation was 0.980 +/− 0.005. Conidial production, however, was severely reduced by 95–99.8% (optimal a_w for conidial production was 0.999). A compromise a_w yielding acceptable polyol levels still resulted in a 72% reduction in yield.

Kim et al. (2011) discovered that millet grain as a solid substrate produced *B. bassiana* and *M. anisopliae* conidia that were more thermotolerant (to 43–47 °C) than conidia from agar-based media. Their data indicate that certain substances in the millet contributed to the observed thermotolerance. Use of millet supplemented with plant-derived oils, especially corn oil, further induced greater heat tolerance of *B. bassiana* conidia (45 °C for 90 min) (Kim et al., 2010a).

In a subsequent paper, Kim et al. (2010b) demonstrated that, for *I. fumosorosea*, ground corn solid substrate produced conidia with the most heat tolerance, compared to rice, soybean of red kidney bean substrates, and corn oil supplementation of the corn meal increased that heat tolerance (Kim et al., 2010c). They hypothesized that unsaturated fatty acids, such as linoleic acid and oleic acid, in the corn oil possibly explained the improved thermotolerance.

The addition of 1–4 mM salicylic acid, a plant cell-signaling metabolite, to an agar medium yielded conidia of one *M. robertsii* isolate with a doubling in heat tolerance, but not UV-B tolerance (Rangel et al., 2012). It should be noted that the amendment did reduce conidial yield somewhat and a defined agar base was used in the study. Whether the same phenomenon would result in either solid substrate or submerged liquid fermentation—or with other *Metarhizium* strains, species, and *B. bassiana*—remains to be determined. Many of the fermentation manipulations caused a significant decrease in spore production, to the extent that the manipulations were self-defeating. Nevertheless, further research may find a satisfactory compromise between stress tolerance and spore production.

11.4.3. Conidia Produced after Photoirradiation

Conidia produced from mycelium of *M. robertsii*, irradiated with UV-A while growing on PDAY, had somewhat elevated virulence levels similar to that of conidia produced on nutritionally minimal medium, but their germination rate was not increased, nor were UV-B resistance and thermotolerance improved (Rangel et al., 2008a, 2008b). This approach needs further investigation to determine the applicability of the observations to other strains and other species. Nevertheless, extension of this approach to solid substrate fermentation seems limited, although it may be possible when plastic bags are used.

11.5. THE CHALLENGE IN MASS PRODUCTION OF ENTOMOPATHOGENIC FUNGI

The fundamental consideration in potential commercialization of any of these fungi is whether an efficacious product can be developed and produced cheaply

enough to compete with existing controls, especially chemicals. To return an acceptable profit on the research and development investment, low-cost mass production of an entomogenous fungus is only one of a number of technical constraints. Yet, mass production is basic to commercialization.

Bartlett and Jaronski (1988) examined the capacities of the different technologies at the time and concluded that high-efficiency biphasic solid substrate, such as that now practiced by at least one company in the United States, was the only commercially feasible system for very large mycoinsecticide production needs. For example, let us look at a potential U.S. market, European corn borer, *Ostrinia nubilaris* (Hübner), in maize, ignoring for the moment the dominance of Bt-maize. In 2012, there were 26 million hectares of maize subject to attack by European corn borer and corn earworm, *Helicoverpa zea* (Boddie) (U.S. Department of Agriculture, Economic Research Service, 2012; U.S. Department of Agriculture, 2012). Assuming 5% of the corn market is open to mycoinsecticide use, that is 1.3 million hectares. Much of the published literature on efficacy indicates that a rate of $1–2.5 \times 10^{13}$ conidia per hectare is needed for fungal products (De Faria and Wraight, 2007); however, *M. acridum* has a use rate of 5×10^{12} per hectare for locust control. If the use rate of mycoinsecticide is the more optimistic 5×10^{12} conidia per hectare, then a company would need to produce 6.5×10^{18} conidia for one growing season. With the public Mycotech/Laverlam yields of 2.6×10^{13} conidia/kg, that need would require 250,000 kg of substrate (25 production runs at Laverlam). Lower yields, such as those reported in the literature, or higher efficacious rates of fungus would require much larger amounts (Table 11.4).

A comparison of the economics and efficiencies of the two processes for producing *L. muscarium* was presented by Ravensberger (2011). For the manufacturer, liquid submerged fermentation yielded 2.1×10^{13} spores/l within 7 days in 1000-l fermenters, whereas solid substrate fermentation in specialized equipment of 100-kg substrate capacity yielded 8.1×10^{12} conidia/kg. Ravensberger (2011) estimated that new solid substrate equipment would cost €750,000 in 2007 versus €300,000 for a 1000-l liquid fermenter that had four times the production capacity. Ignoring quality of spores, their shelf life, losses to desiccation, and production of unwanted metabolites in submerged fermentation, that process may be more practical.

In contrast, for smaller-scale production serving local needs, solid substrate fermentation (e.g. in plastic bags as is practiced in Cuba, Brazil, and other Latin American countries) may be practical. About $5–10 \times 10^{16}$ spores would be needed for 5000 ha. Using the data in Table 11.4, 16,000–33,000 kg substrate (with a yield of 1.5×10^{12} per kilogram) would meet that need. If the highest yield obtained on a laboratory scale, 2×10^{13} per kilogram rice (Dorta et al., 1996), was operationally possible, then the substrate need would decrease to 1200–2400 kg, which is much more feasible.

For inoculative release of fungi in a biocontrol campaign (e.g. with Entomophthorales), such considerations are avoided; only small numbers of spores

TABLE 11.4 Estimates of Production Capacity Needed to Supply Enough Fungus Propagules to Treat 1.3×10^6 ha of Maize in the United States (5% of Total Potential Maize Market) Based on Documented Production Yields. The Total Spore Need Is 6.5×10^{18} spores, Based on a Use Rate of 5×10^{12} Spores/ha

Fermentation System	Yield per Unit	Fungus (Source)	Production Need
High-efficiency biphasic solid substrate fermentation	2.5×10^{13} conidia/kg substrate	*B. bassiana* GHA (Bradley et al., 1992)	2.6×10^5 kg
Low-efficiency (bag) solid substrate fermentation	1.5×10^{12} conidia/kg	*M. acridum* IMI330189 (Jenkins et al., 1998)	4.3×10^6 kg
Submerged fermentation	1×10^{12} blastospores/l	*I. fumosorosea* (Jackson et al., 1997)	6.5×10^6 l
Liquid surface culture	1×10^{14} conidia/m^2 of surface	*B. bassiana* (Bartlett and Jaronski, 1988)	6.5×10^4 m^2

are needed because the fungus will reproduce and proliferate, ideally causing an epizootic. In inundative applications, the fundamental challenge is to reduce fungus use rates. Although the typical application rate is on the order of $1–2 \times 10^{13}$ spores per hectare, lower application rates have been obtained by the selection of a more efficacious (yet still productive) strain. The official use rate for *M. acridum* against locusts is 5×10^{12} per hectare, which is already a lower rate than the generally accepted level for a mycoinsecticide, and good efficacy has been obtained with rates as low as 1.25×10^{12} per hectare. One-fourth the fungus per acre has a major impact on production needs.

In the past few years, efforts have been made towards creating fungal strains that are genetically modified for greater virulence (reviewed by St. Leger and Wang, 2010). Some of these transformations have greatly increased the efficacy of the fungus; for example, the incorporation of scorpion neurotoxin increased toxicity for hornworm 22-fold (Wang and St. Leger, 2007). If regulatory agencies will allow the use of such transformed fungi, application rates could be considerably lowered. Use rates per unit area have also been lowered by concentrating the spores into a narrow, targeted zone by modification of application equipment and methods to deliver more spores exactly where needed (Jaronski, 2010). If applied in a broadcast spray, 5×10^{12} conidia per hectare would result in a level of 5×10^4 conidia/cm^2 of surface area. With a 12.5-cm banded application of each row of plants, the conidial levels become 2.4×10^5/cm^2—a fivefold increase at the same rate per hectare, confined to the actual habitat of the target

insect, or a potential fivefold reduction in the rate of fungus per hectare if the 5×10^4 conidia/cm^2 of surface area was efficacious. For example, Wraight and Ramos (2002) were able to increase the conidial deposition on leaf undersides 6- to 30-fold by modifying the arrangement of spray nozzles. Similarly, spores can be placed in the path of insects, such as on fiber bands wrapped around tree trunks to control Asian longhorned beetle (Shanley et al., 2009) or in compact spray bands in front of migrating locusts, affording considerable economies. Therefore, the situation with mass production of entomopathogenic fungi is not a dismal one. Considerable potential exists for fungi to be significant tools in managing insect pest populations.

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