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# 13 Safety of Fungal Biocontrol Agents

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## Introduction

Fungi contain a diverse array of taxa with a great diversity of properties. They are relatively common and are important in regulating pest populations. There has been considerable interest in the use of fungi as microbial control agents of pest insects, nematodes, weeds and plant pathogens and some have been developed as commercial biocontrol products. However, fungi also contain many species that are pests in themselves, causing untold losses to crops, forests, stored products and buildings and also affecting animal and human health. Consequently the development and use of fungi as biocontrol agents requires an assessment of unintended effects associated with their use.

In this chapter we provide an overview of the potential hazards and safety concerns associated with biocontrol fungi. We restrict our discussions to fungi with biocontrol potential for plants, plant diseases and arthropods. We provide examples of how fungi can be detrimental as well as how they have been used safely as biocontrol agents. Finally, we shall review how regulations and registration requirements attempt to address and mitigate potential safety issues. Previous reviews on the safety of biocontrol fungi are those of Austwick (1980), Goettel *et al.* (1990), Prior (1990), Goettel and Johnson (1992) and Evans (1998, 2000). Reviews on the safety of microbial control agents in general are those of Flexner *et al.* (1986), Laird *et al.* (1990), Cook *et al.* (1996) and Goettel and Jaronski (1997). Guidelines for testing the pathogenicity and infectivity of entomopathogens to mammals have been reviewed by Siegel (1997) and guidelines for evaluating effects of entomopathogens on invertebrate non-target organisms have been reviewed by Hajek and Goettel (2000). Several authors have also addressed the protocols and guidelines that should be followed in order to introduce exotic agents for weed biocontrol (Wapshere, 1974, 1975, 1989; Klingman and Coulson, 1982).

## Some Examples of the Detrimental Effects of Fungi

In addressing the potential detrimental effects of fungi used for biological control, it is useful to reflect on the detrimental effects of fungi in general. This is especially so because, to date, the detrimental effects of fungi used specifically as biological control agents are either non-existent, have been minimal or have generally gone unnoticed. We have therefore included several examples of the detrimental effects of various fungal pathogens, both endemic and introduced, in order to illustrate the 'potential' detrimental effects of a biological control fungus in what we would consider a 'worst-case scenario'. We once again stress that these are examples of detrimental effects that have not arisen from attempts to use fungi as biocontrol agents.

### *Beauveriosis in the silkworm industry*

Prior to the early 20th century, silk production throughout the world suffered because of the devastating effects of several diseases in the silkworm, *Bombyx mori* (Steinhaus, 1975). In France and Italy especially, annual losses due to 'muscardine' were tremendous. For instance, silk production in Italy suffered losses of approximately 5 million kg year<sup>-1</sup> until *c.* 1925 (Bell, 1974). However, initially, the role of pathogens in disease was not understood. Agostino Bassi (1835, as cited by Bell, 1974, and Steinhaus, 1975) first demonstrated the germ theory of disease using mycosis in the silkworm. He showed that the muscardine disease was caused by a 'vegetable parasite', which grows and develops within the silkworm, eventually killing its host. He demonstrated how the 'seeds' produced on the surface of the cadaver were responsible for disease in new individuals and how these seeds could be destroyed by chemical and physical means. He recommended disinfection practices using lye, wine and brandy, boiling water, burning and exposure to sunlight. Thus he was also one of the founders of disinfection. The muscardine in question was a fungus, which was later named *Beauveria bassiana*, in his honour.

With time, strict hygienic practices and legislation were adopted and muscardine disease in silkworm insectaries was brought under control. For instance, in China and Japan, the application of *B. bassiana* and other microbial control agents was restricted to areas where no silkworms are bred, to avoid contamination of the mulberry leaves used to feed the silkworms (Hussey and Tinsley, 1981; Goettel *et al.*, 1990). And, through careful strain selection, it may even be possible to use *B. bassiana* to control a pest in the immediate vicinity of a silkworm rearing facility; in a pilot test in a Chinese 110 ha pine plantation, good control of pine caterpillars (*Dendrolimus* spp.) was achieved through six applications of 2100 kg of *B. bassiana*, with no significant effect on the silkworms within a rearing shed built within the plantation (Anon., 1981a, as cited by Goettel *et al.*, 1990). The strains of *B. bassiana* used against the pine caterpillars were 100 times less virulent to silkworms than strains isolated from silkworms themselves.

This example illustrates the potential that entomopathogenic fungi have in devastating insect colonies in insectaries. It also illustrates the importance of differences in the specificity of different fungal strains or isolates. Through modern disinfection and hygienic practices, diseases in insectaries can, for the most part, be kept under control.

### **Chalkbrood in honey-bees**

Reports of diseases in the honey-bee date as early as 700 BC (Steinhaus, 1975). The most important fungal disease of the honey-bee is chalkbrood, caused by the ascomycete *Ascosphaera apis* (Gilliam and Vandenberg, 1990). Although the disease is not generally considered serious, infections of certain colonies can be persistent and damaging. Chalkbrood is present on all continents where honey-bees are found (Bradbear, 1988; Anderson and Gibson, 1998). Although known in Europe since the early 20th century, the fungus has only been documented more recently in honey-bee colonies in North America and elsewhere.

The origins of the more recent findings remain a mystery and several possibilities exist: (i) the fungus was recently introduced into North America, possibly on imported pollen; (ii) the fungus has gone unnoticed until recently; or (iii) the fungus has occurred in feral and solitary bees and only recently invaded honey-bee colonies. Nevertheless, the fungus is spreading to honey-bee colonies throughout the world and is causing serious problems in some locations in some years. The disease is little understood and could be triggered by stress factors, such as chilling.

There is no chemotherapeutic agent registered for its control. However, Gilliam *et al.* (1988) demonstrated that genetically inherited hygienic behaviour is an important component of resistance of bee colonies to the disease. Colonies in which workers were able to detect and remove infected or dead bees were less susceptible to the disease.

Chalkbrood in honey-bees provides a rare example of a behavioural mechanism of disease resistance. It also demonstrates the difficulties of preventing the spread of a detrimental fungal pathogen. For instance, despite strict hygiene and importation restrictions of bees, bee equipment and bee products, chalkbrood has recently been detected in Australia and has rapidly spread over a wide geographical area within that country (Anderson and Gibson, 1998).

### **Plant pathogens**

Plague or 'pestilence' affecting cultivated crops has been recorded since biblical times, and fungal plant pathogens have been implicated in most of these catastrophes (Large, 1940; Agrios, 1997). However, the first scientifically documented and certainly one of the most dramatic examples of the detrimental effects of plant pathogens, or indeed of any pest, concerns the potato late blight in Europe during the 19th century. The disease or 'murrain' was first reported in 1845, but was initially ascribed to low temperatures, and it was not until several years later, after disastrous crop losses throughout Europe, that it was identified as being caused by a fungus, *Phytophthora infestans*, by the leading mycologist of the time, M.J. Berkeley, who further speculated that the pathogen had arrived from the New World (Large, 1940). This biotic invasion has been described by the latter author as 'a historic determinant of human affairs', since, in Ireland alone, at least 1,000,000 people died between 1846 and 1860 as a consequence of the potato famine, and more than 1.5 million emigrated. It also had immediate political implications in the UK because it led to the repeal of the Corn Laws and the fall of the government (Ramsbottom, 1953). To quote Disraeli: 'The mysterious malady of a single tuber changed the political history of the world.' As correctly deduced by Berkeley, the fungus had co-evolved with potato and its relatives in the

neotropics – the specific locale is now considered to be Mexico (Fry *et al.*, 1992) – and had arrived in Europe almost certainly through the introduction of infected germ-plasm.

Unfortunately, the story does not end with this initial invasion. The disease had a major resurgence in Europe in the 1980s. Prior to this recent event, it was determined that the European pathogen populations comprised only a single mating type and thus they were relatively uniform, often dominated by a single genotype. However, a second mating type was identified and its source was eventually traced to a second invasion from Mexico, probably in the 1970s (Fry *et al.*, 1992). Indeed, the threat from invasive plant pathogens should not be underestimated, because many of the world's major food crops are genetically vulnerable (Kingsolver *et al.*, 1983). In addition, fungal pathogens also pose an actual and potential threat to natural ecosystems, as well as to amenity trees and forestry. The catastrophic impact of successive invasions of Dutch elm disease, caused by *Ceratocystis ulmi*, on the UK and North American landscapes has been well documented and needs little introduction (Brasier, 1990; Liebold *et al.*, 1995). However, other tree diseases of fungal origin are less well known but have had equally detrimental socio-economic and environmental effects. For example, the Asian chestnut blight fungus, *Cryphonectria parasitica*, after its arrival on infected nursery stock in New York at the beginning of the 20th century, spread in several decades over large areas of the eastern seaboard, destroying almost all native American chestnut trees (*Castanea dentata*) by the early 1950s (Hepting, 1974). Before this invasion, American chestnut had dominated many forest ecosystems, forming up to 25% of the tree cover and constituting not only a high-value timber but also a vital source of food for wildlife (Anagnostakis, 1987). Whilst these pathogens have shown host specificity at the generic level and therefore have had an impact only on segments of the forest community, the root pathogen, *Phytophthora cinnamomi*, has a much broader host range, attacking plants from at least 48 different families, and has caused up to 75% losses in the native eucalyptus forests of western Australia (Weste and Marks, 1987). Since its suspected arrival in the 1920s, this plant pathogen has transformed the sclerophyll forests with a species-rich understorey into open woodland dominated by sedges (Burdon, 1987).

An even more recent threat from an invasive plant pathogen involves anthracnose disease of native dogwood (*Cornus* spp.) in the USA, caused by the fungus *Discula destructiva*, thought to have been imported on contaminated ornamentals from Asia in the 1970s (Daughtrey and Hibben, 1994). The devastation by this pathogen is worsening, as demonstrated by an increase in tree mortality in some areas from 33% in 1984 to nearly 80% 4 years later. Clearly, the long-term detrimental effects from the elimination of an important component tree on native forest ecosystems in North America will be immense, following changes in species composition and community dynamics (Hiers and Evans, 1997).

## The Potential Hazards and Safety Concerns

The safety of fungi being contemplated for use in biological control must be considered at many levels, with primary concern for direct effects on vertebrates, especially humans. In considering safety toward all organisms, vertebrates, invertebrates and plants not intentionally being affected by the biological control fungus are referred to as 'non-target organisms'. The North American Microbial Biocontrol Working Group

identifies the following potential safety issues: (i) competitive displacement of non-target organisms; (ii) allergenicity; (iii) toxigenicity to non-target organisms; and (iv) pathogenicity to non-target organisms (Cook *et al.*, 1996). In addition, indirect effects, such as those that could come about through the depletion of the target host itself, must be considered as a potential safety issue (Goettel and Hajek, 2000). The potential unintended effects will differ depending on the potential target and non-target organisms and the ecosystems they inhabit.

### Allergenicity

Fungi are capable of producing spores that cause allergies or allergic reactions; however, actual or potential fungal microbial control agents are not among the species that are responsible for the production of common allergens (Lalgé and Paris, 1991). Nevertheless, there are reports of allergic reactions to microbial control fungi, principally with those exposed during mass production and application (Austwick, 1980). For instance, a group of scientists working with *B. bassiana* reported moderate to severe allergic reactions, and consequently the US Environmental Protection Agency (EPA) lists this fungus as a dermal sensitizer (Saik *et al.*, 1990). Furthermore, it has been demonstrated that crude extracts of *Metarhizium anisopliae* contain one or more potent allergens (Ward *et al.*, 1998). Since all fungi are potentially allergenic, it is necessary to avoid exposing unprotected humans during production and application.

### Toxicity

Fungi secrete a wide variety of compounds, many of which are toxic to plants, invertebrates or vertebrates. *M. anisopliae* produces destruxins and cytochalasins; *B. bassiana* oosporein, beauvericin, bassianolide and beauveriolide, *Hirsutella thompsonii* hirsutellin, and *Trichoderma harzianum* peptaibols, to name a few. The role that many of these toxins play in pathogenesis is little understood; however, some may contribute to the ability of the fungus to overcome its host (see Chapter 12). However, toxin production varies according to isolate and does not necessarily play an important role in virulence in every case.

The production of toxins by a candidate fungal biocontrol agent should not necessarily immediately preclude its use in biocontrol. Considerations must be made as to possible effects from the presence of the toxin in the formulated product (e.g. danger to applicator), fate of the toxin after application (i.e. rate of degradation) and possible bioaccumulation of the toxin within the host or environment (e.g. danger to scavengers feeding on cadavers). For instance, the fungal pathogen *Fusarium nygamai* has been considered as a promising mycoherbicide for control of witch-weed (*Striga hermonthica*), a serious constraint on grain production in Africa (Abbasher and Sauerborn, 1992). However, recent work has shown that this fungus produces mycotoxins, including several novel compounds (Capasso *et al.*, 1996). Due to the potential threat to vertebrates, particularly humans and their animals, interest in developing this fungus as a mycoherbicide has waned.

## Pathogenicity

Pathogenicity towards the target host is usually the desired effect. However, pathogenicity towards non-target organisms could be an unintended effect. Fungi, including species intended for biological control, can infect a wide variety of hosts, which sometimes include mammals. For instance, there are reports of *B. bassiana* infecting captive American alligators and a giant tortoise that had been stressed by chilling (Heimpel, 1971; Saik *et al.*, 1990; Semalulu *et al.*, 1992). Results of laboratory assays demonstrate that a strain of this fungus can also be pathogenic to embryos of the inland silverside fish, *Menida beryllina*, and the grass shrimp *Palaemonetes pugio*; conidia attached and germinated and hyphae subsequently penetrated the chorionic membrane within 5 days (Genthner and Middaugh, 1992; Genthner *et al.*, 1997). Using a similar laboratory assay system, Genthner and Middaugh (1995) reported that, *M. anisopliae* conidia adversely affected both embryos and newly hatched larvae of the inland silverside fish. These effects included decreased cardiac output, chorionic rupture and teratogenic expressions in embryos and larvae.

More recently, several cases of human infection by *M. anisopliae* have been reported in both immunocompetent and immunoincompetent individuals, with one fatality in an immunoincompetent child (Burgner *et al.*, 1998; Revankar *et al.*, 1999). There are numerous case reports that the nematode-destroying fungus *Paecilomyces lilacinus* is a causative agent of human infections in both immunoincompetent and immunocompetent individuals (Itin *et al.*, 1998; Gutierrez-Rodero *et al.*, 1999). This species was listed in 1999 as an important emerging nosocomial fungal pathogen by the National Foundation for Infectious Diseases ([www.nfid.org/publications/clinicalupdates/fungal/noso.html](http://www.nfid.org/publications/clinicalupdates/fungal/noso.html)) and has been recovered from tortoises, lizards, snakes, crocodiles and alligators at the London Zoo (Austwick, 1983). Another entomopathogenic fungus, *Conidiobolus coronatus*, is commonly associated with lesions in both humans and horses (Saik *et al.*, 1990). Clearly, evaluation of potential fungal microbial control agents must include an evaluation of their virulence towards non-target organisms, especially vertebrates, with consideration given to potential human exposure scenarios.

For many pathogens of invertebrates and weeds, the ability to infect is usually constrained within a host group (e.g. many pathogens of weeds can only infect other plants and cannot infect animals). Among non-vertebrate non-target organisms, safety concerns first focus on safety towards invertebrates and crop amenity plants used by humans. In addition, plants and animals that constitute the flora and fauna of release areas but with little direct relation to humans could be affected and safety evaluations should consider direct effects at this level also.

## Depletion of hosts

The goal of any biological control programme is to lower the population of a pest. However, this reduction in the pest population may in turn detrimentally affect other non-target organisms that in one way or another depend on this pest. The extent of harm to the non-target population will very much depend on the extent and speed of the depletion of the host and on the length of time that the host has been in its targeted location (e.g. is it introduced or endemic?).

Over the years, the biocontrol of weeds – and specifically classical biological control

– has consistently been plagued by conflicts of interest, centring on the misconception that there will be a sudden depletion of the target weed host (Harris, 1985). Any exotic plant that has been deliberately imported for economic or ecological benefit or has subsequently acquired local added value, but which later invades and dominates native ecosystems or agriculture, may still have its ‘supporters’. Thus, any threat to that plant resulting from the introduction of a natural enemy, such as a fungal pathogen, can create controversy and seriously or irrevocably disrupt a weed biocontrol programme. As Harris (1985) concluded, classical biological control must be done as a matter of public interest and with enabling legislation since the long-term effects cannot be restricted to individual properties or release sites. Thus, even if a biocontrol programme is clearly in the interest of the general public and supported by it, this support must be unanimous and unambiguous because of the rule-of-law principle.

Although depletion of alien weed populations may well be popular with both farmers and conservationists alike, individuals or organizations who derive or perceive (more usually, misperceive) financial or ecological benefits from the presence of the ‘weed’ have the power to prevent the release of biocontrol agents. Because this process usually involves court cases, there are a number of examples in which lengthy and often costly battles have had to be fought (McFadyen, 1998). Such a conflict of interest – still one of the most contentious disputes in the history of biocontrol – occurred in Australia with the boraginaceous plant, *Echium plantagineum*. This plant is variously known as Paterson’s curse by farmers and Salvation Jane by bee-keepers, who rely on it as a dependable source of pollen during dry years. The case was eventually settled out of court after a considerable delay and cost to the biocontrol programme. On the positive side, however, it did result in the Australian Biological Control Act, which, for the first time, provided a legal basis for the introduction of exotic biocontrol agents (Cullen and Delfosse, 1985). Ironically, of course, classical biocontrol aims not to eliminate an alien plant – indeed, this can never be achieved with a co-evolved natural enemy – but to gradually reduce its competitive ability and thus restore the natural balance (see Chapter 6). In all probability, there will always be sufficient flowers from the remaining weed populations to satisfy the needs of the bee-keepers.

An even more extreme example, in the USA, involves salt-cedars (*Tamarix* spp.); alien shrubs from Eurasia, which have become the most serious threat to riparian ecosystems in western states, often completely replacing the native flora. Such has been the habitat change that the endangered southwestern willow flycatcher now relies almost entirely on salt-cedars for nesting (DeLoach *et al.*, 1996). Consequently, ornithologists, a powerful lobby in the USA, have thus far successfully blocked any attempts to implement a classical biocontrol strategy, despite the fact that a suite of potentially useful natural enemies has been identified in the plant’s native range (DeLoach *et al.*, 1996). Ironically, nine species of rare birds and at least five other species of vertebrates have been identified as being endangered as a direct result of the salt-cedar invasion. It has taken more than a decade of research to overturn these well-meaning but sadly misguided and even irresponsible objections. Further historical and ecological studies have now demonstrated that the flycatcher populations had actually declined since the arrival of salt-cedar and that experimental removal of the weed has resulted in increased growth and density of willows, the preferred vegetation type of this bird species. Biological control is expected gradually to reduce *Tamarix* populations, with a corresponding increase in native shrubs, but without the intervening loss (originally predicted by the ornithologists) of nesting habitats (DeLoach *et al.*, 1999).

This issue is even more contentious when the target host is indigenous. The

arguments centring around the recent introduction of an Australian pathotype 3 (= *Entomophaga praxibuli*) of the grasshopper-pathogenic fungus *Entomophaga grylli* into the USA illustrate this point (Carruthers and Onsager, 1993; Lockwood 1993a, b). Lockwood (1993a) speculated that suppression or even extinction of target as well as non-target acridids may result in the loss of biodiversity, proliferation of new weed species and otherwise innocuous acridid species, disruption of plant community structure, suppression of essential organisms vectored by grasshoppers and disruption of food-chains and other nutrient cycling processes. In rebuttal, Carruthers and Onsager (1993) pointed out that endemic *E. grylli* pathotypes 1 and 2 (= *Entomophaga calopteni* and *Entomophaga macleodii*) already periodically produce epizootics in grasshopper populations and reduce outbreak populations of grasshoppers. However, pathotype 3 from Australia, although biologically very similar to pathotype 1, differed in that it could produce conidia in *Melanoplus* spp., an attribute that was lacking in pathotype 1. This was seen as advantageous as it would allow infection of two major pest species by a single species within a single season.

Extensive evaluations of the *E. grylli* species complex in both the USA and Australia provided detailed information on the biology and epizootiology of the fungus, and models were used to predict the response to the introduction of the Australian pathotype into the USA (Carruthers and Onsager, 1993). A detailed proposal was submitted to the US regulatory agency and permits were granted for the field release of this pathotype into North Dakota and Alaska. Releases were made and the pathogen was monitored. Despite initial evidence that the fungus established and increased in prevalence (Carruthers and Onsager, 1993), current evidence suggests that the frequency of infection has declined to levels such that long-term survival of this pathotype in North America is questionable (Bidochka *et al.*, 1996). Only time will tell if the fungus will resurface in future years.

### **Competitive displacement**

Fungi introduced or applied as biocontrol agents have the potential to competitively occupy a niche, thereby adversely affecting one or more native organisms within that niche. This may be the intended effect with some fungal biocontrol agents. For instance, the saprophytic fungus *Phlebia gigantea* applied to freshly cut pine stumps competitively displaces *Heterobasidion annosum*, the cause of root rot of pine (Rishbeth, 1975). Unintended effects would occur if this competitive displacement were to seriously affect a non-target organism, perhaps even leading to its extinction or in some other way detrimentally affecting a component of the ecosystem. For example, fungal pathogens and insect parasitoids may compete within the tissue of the host insects (Goettel *et al.*, 1990; Vinson, 1990). The pathogen usually out-competes younger parasitoids, while older parasitoids are often capable of completing their development within fungus-infected insects.

Lockwood (1993a) suggested the possibility that the introduction of an exotic pathotype of *E. grylli* from Australia could suppress native North American grasshoppers, seriously affecting the 'natural' control of those species currently being regulated by the native *E. grylli* species through competitive displacement of the native *E. grylli* pathotypes. However, Carruthers and Onsager (1993) point out that there should be little concern if the introduced pathotype became the dominant grasshopper pathogen in rangeland and crop environments. Although exotic natural enemies may displace



native species, there are usually habitats in which these native species are able to coexist (Bennett, 1993).

## Specific Fungal Attributes and Use Patterns Related to Hazard

The potential hazards and the degree of difficulty in assessing hazards will very much depend not only on the pathogen in question, but also on its intended use. Fungal biocontrol agents can be used in augmentative, classical and conservation biological control. In augmentative approaches, the fungi are introduced either in low numbers (i.e. inoculative augmentation) or in very large numbers (i.e. inundative augmentation), essentially as pesticides. In the classical approach, fungi are introduced into geographical habitats where they have not previously occurred. The intent here is that they become established and provide self-perpetuating, long-term control. In the conservation approach, the habitat or management practices are manipulated in order to favour the naturally occurring fungi.

### *Host range*

As a group, fungi exhibit a very wide range of host specificity. Some species are very host-specific, while others are generalists and are known from a very wide array of hosts. Many species within Hyphomycetes are facultative pathogens and consequently have broad host ranges. However, individual strains generally exhibit specificity for a limited number of hosts (Glare and Milner, 1991). In contrast, many obligate pathogens, such as *E. grylli*, are restricted to several closely related host species (i.e. several species within Acrididae) (Carruthers and Onsager, 1993).

The range of species that a fungus can infect often differs between that found in the laboratory (physiological host range) and that found in nature (ecological host range) (Hajek and Butler, 2000). The physiological host range is determined from laboratory infection assays and demonstrates which hosts could potentially be infected under field conditions. The ecological host range can only be determined from field studies. Differences between physiological and ecological host ranges are thought to be the result of the complex biotic and abiotic conditions that occur in the field and have an impact on both pathogen and host susceptibility. Such conditions are not normally replicated in laboratory host-range tests. Consequently, in order to make laboratory-acquired data as applicable to the field situation as possible, every effort must be made to mimic the field situation (Butt and Goettel, 2000; Hajek and Goettel, 2000).

### *Dose-related susceptibility*

Host susceptibility to fungal pathogens is, for the most part, dose-dependent. A threshold for infection is presumed to exist whereby a minimum number of fungal propagules is required. Thereafter, increasing numbers of propagules increase the probability of a successful infection. This dose-mortality relationship provides a built-in safety factor in the inundative-augmentation use of many facultative fungal pathogens as microbial insecticides, because high doses are present for only a relatively brief time.

Even though infection may be dose-dependent, susceptibility and disease transmission are also very much dependent on many abiotic factors. For instance, most fungi depend on high relative humidity for sporulation. If humidity conditions during periods when large numbers of host cadavers are present are not conducive to sporulation, adequate numbers for infection of subsequent generations will not become available and the induced epizootic will subside.

### ***Persistence and dispersal***

Spore dispersal in most fungi is passive, relying on wind and water. In the Oomycetes, however, the spores are motile and are reliant on the presence of water (e.g. a film of water on a leaf surface). In the Entomophthorales, spores are forcibly discharged and can land centimetres from the host or be carried longer distances on air currents.

For the most part, most spore types are very sensitive to ultraviolet (UV) radiation and consequently spores exposed to sunlight are short-lived. The half-life of some spores exposed to direct sunlight can be a matter of minutes. Persistence is generally much increased in shaded habitats and in soil. In contrast, the thick-walled resting spores produced by species within the Oomycetes and Zygomycetes and some ascomycetous fungi are capable of persisting for many years under adverse abiotic conditions.

### ***Genetically altered fungi***

Some of the goals of genetic modification of fungi intended for biological control are to expand the host range, increase the speed of kill by incorporating more toxic modes of action and extend persistence (see Chapter 8). Through this direct genetic manipulation, and especially through the introduction of foreign genetic material from organisms within and outside the fungal kingdom, it may be possible, either directly or indirectly, to change many attributes of a given fungus, which could drastically change its safety. Genetically altered organisms are unique in that they have never existed in nature in their altered form and consequently their potential hazards can only be definitively evaluated once the pathogen has been released into the environment. Changes in the ecological attributes of a fungus which, for instance, favour its virulence against a target host may also inadvertently allow the fungus to persist in new niches and affect non-target hosts previously not encountered. Consequently, it would be prudent to engineer fungi in such a way as to minimize their ability to persist in nature. Nevertheless, there is still the ever-present concern that the genetically modified organism may pass its newly acquired traits to another microorganism.

Although there are several entomopathogenic fungi that have been or soon will be genetically altered, none to date have been released in the wild, and therefore any predictions as to their potential detrimental effects would be purely speculative. Permission has recently been granted to release a genetically altered strain of *M. anisopliae* (R. St Leger, personal communication), which overproduces the cuticle-degrading Pr1 protease required for pathogenesis. This strain causes more rapid death of its host and the subsequent melanization response of the host prevents the fungus from further colonizing it and subsequently sporulating on the cadaver (see Chapter 8).

### ***Non-indigenous vs. indigenous organisms***

Fungal pathogens have the ability to replicate, spread, persist and adapt to new environments. Regardless of whether pathogens are detrimental or beneficial within their area of endemicity, their population levels are more or less in 'ecological balance'. In other words, the pathogens themselves, as well as their hosts and other organisms dependent on the hosts, adapt to the ecosystem and coexist together. If inoculum levels of indigenous fungi are artificially raised, as in inundative augmentation, there can be a relatively short-term perturbation of the ecosystem, which is usually the desired effect on the target host. Inoculum levels eventually return to pre-augmentation levels, and the host-pathogen 'balance' is eventually restored. Consequently, Goettel (1995) argues that very little attention need be paid to the host range of an indigenous fungus that is to be used inundatively in its native area. Similar arguments are brought forth by Keller (1998) who suggests that the closer the use of a fungus is to the natural situation, the fewer the requirements that should be imposed on hazard identification.

However, when a pathogen is introduced into a new environment – that is, to an area where it was previously non-indigenous – three outcomes are possible. The pathogen can simply not adapt and die out. It may survive and reproduce, but with little consequence to the overall ecosystem. Or the microorganism can spread, unimpeded, sometimes with devastating consequences to its host. These devastating consequences are usually the desired effect in classical control if the effects are restricted to the target host. But, if the host range was not as predicted, or if the host is beneficial, the ecological or economic results can be devastating, as seen in the example of the accidental introduction of Dutch elm disease into North America (see examples in the section on plant pathogens).

### ***Mode of use: inundative vs. inoculative release***

These very different strategies differ significantly in concerns regarding non-target effects. For classical biological control, the establishment of natural enemies is generally considered permanent and irreversible, so predicting the host range in the area of release is critical before release. The intent of this strategy is that, after establishment, the fungus will increase in response to host increases; in particular, highly host-specific pathogens are sought for these programmes so that their life cycles are closely tied to host populations.

In inundative augmentation, it is assumed that the fungus is already resident in the release area and that organisms in that area will only experience higher levels for relatively short periods. Therefore, this strategy would only have a potentially temporary impact on the release area. Even if there were detrimental effects to non-target organisms, these effects would only be temporary and, in essence, no different from using a synthetic chemical insecticide, although fungal pathogens are invariably much more host-specific. The strategy for inundative or augmentative biocontrol typically involves the mass production and application of a formulated product (e.g. a myco-herbicide). This, of course, can pose significant hazards, especially as regards human exposure. Potential human health effects include allergenicity, pathogenicity and exposure to toxic metabolites. However, these hazards are minimal if the same precautions in the application of mycopesticides are used as those that have been adopted for the

application of chemical pesticides. Nevertheless, it is indisputable that basic knowledge on possible effects on humans needs to be evaluated prior to the registration of a product based on a fungal pathogen, as is the basic requirement for any microbial control agent today.

## Addressing the Hazards – Examples of Safe Use

Despite the many hazards that fungi possess as a whole, numerous species are being safely mass-produced and used for inundative augmentation to control pestiferous arthropods, nematodes and weeds without any apparent detrimental environmental or safety effects. Fungal pathogens have also been used for classical biological control of insects and weeds, although use of this latter strategy has been much more common with introductions of arthropods as natural enemies. Effects on the environment are considered before releases, irrespective of use strategy. Effects could differ by release strategy (e.g. introductions for classical biological control are permanent and this strategy usually focuses on perennial or natural systems while inundative augmentation usually involves release of high doses of a native pathogen in annual systems (see above)). The effects of outbreak pest populations on non-targets if no control is undertaken versus use of alternative controls (e.g. synthetic chemical pesticides) must always be weighed against potential effects of biological control agents on non-target organisms.

Hazards must be assessed and addressed at every step of the development and use of a fungal biocontrol agent. Steps must be undertaken in the manufacturing process to minimize human exposure, especially as concerns potential allergenicity or toxicity to workers, as is required in any industrial process involving allergens or toxic chemicals. This concern can be resolved by monitoring workers combined with production procedures that minimize human contact with the fungal agent. Formulations must be developed that maximize pathogen targeting and minimize drift or exposure to the applicators. Labels must clearly define the environments and targeted hosts where application is suitable. Potential dangers to non-target organisms must be addressed.

For preliminary estimates of host specificity, laboratory studies are frequently conducted exposing a diversity of species to a fungal pathogen, especially including predators and parasitoids that are already important in providing partial control of the pest. As previously mentioned, such assays should mimic the field situation as much as possible. Another type of preliminary data on host range is that gathered by collecting infected organisms in the field and identifying the cause of death. Although not always possible, the most accurate method for determining non-target impact is data from actual trials when fungi were released. However, few field studies have been conducted. Below we discuss issues and the relevant data that were used to determine safety of fungi that are currently used for biological control.

### ***Fungi against insects***

#### *Inundative*

At present, there are over a dozen commercial products based on nine principal fungal species registered worldwide for inundative use against invertebrate pests (Shah and

Goettel, 1999; see also Chapter 3). No apparent detrimental effects have been reported due to their use. The most common insect-pathogenic fungus produced for inundative augmentation is the hyphomycete *B. bassiana* (see Chapter 3). *B. bassiana* has been recorded from over 700 species of arthropods, many of which are non-target or beneficial hosts (Li, 1988; Goettel et al. 1990). However, most isolates are much more host-specific. For instance, isolate GHA, currently registered in several countries against an array of pests (Shah and Goettel, 1999) has been demonstrated as being innocuous under field conditions to many species that are included in the host list of *B. bassiana* (Goettel and Jaronski, 1997).

*B. bassiana* has been extensively tested, both in the early 1960s, by Nutrilite Products, Inc., and more recently, by Mycotech Corporation and Troy Biosciences, Inc. (Goettel and Jaronski, 1997). Tests have included repeated subcutaneous, intravenous and intraperitoneal injection of rats, as well as feeding studies. A human volunteer even taped 0.2 g of conidia on one arm for 8 h day<sup>-1</sup> every other day for 12 doses with no evidence of dermatitis. No incidents of human hypersensitivity reactions have occurred despite years of mass production of the fungus by Mycotech Corporation (Goettel and Jaronski, 1997). Clearance studies in mice reported that a low dose ( $2 \times 10^5$  spores) delivered subcutaneously cleared within 2 days (Saik et al., 1990; Semalulu et al., 1992). No mortality occurred in embryos and larvae of the fathead minnow, *Pemphales promelas* (Goettel and Jaronski, 1997). Although there is no evidence that *B. bassiana* is infectious in these laboratory studies, there are reports in the literature of *B. bassiana* infecting vertebrates, especially reptilians (see section on 'Pathogenicity' above). However, there has not been an upsurge in reptilian infections since *B. bassiana* was commercialized. In fact, Mycotech and Troy Biosciences obtained exemptions from the requirement of a tolerance for residues of their strains in or on all food commodities when applied or used as a ground spray and aerial foliar sprays on terrestrial crops (Anon., 1995, 1999).

Field studies conducted in rangeland treated with *B. bassiana* to control grasshoppers demonstrated no infection among 2500 non-target arthropods killed at the time of field collection and assessed for overt colonization by the fungus (Goettel et al., 1996). In lucerne, 10 days after application with *B. bassiana*, c. 20% of the leaf-cutting bees killed at time of collection demonstrated overt colonization by *B. bassiana*. However, there was no evidence that the fungus affected leaf-cutting bee larvae, diapausing prepupae or emerging next-generation adults. In lucerne, prevalence of *B. bassiana* colony-forming units in coccinellids and phalangids increased 2 days after application, but these effects were later demonstrated to be the result of ingested conidia within the digestive tract, rather than blastospores within the haemocoel, which would have been indicative of infection (M.S. Goettel, unpublished). It was concluded that the application of *B. bassiana* during their studies in rangeland and lucerne caused only minimal effects on non-target organisms.

*M. anisopliae* is another entomopathogen that is currently registered against several hosts, including cockroaches, grasshoppers, weevils and scarabs (Shah and Goettel, 1999; see also Chapter 3). This fungus has undergone extensive mammalian safety testing including inhalation and intraperitoneal injection in mice and rats, intraocular injection in rabbits, oral dosing of frogs and long-term feeding studies in rats (Burges, 1981; Shaddock et al., 1982; Saik et al., 1990) and birds (Smits et al., 1999). No adverse effects were reported and there was no evidence of infectivity. However, Mycotech observed extreme toxicity to mice by an isolate of *M. anisopliae* and one of *M. anisopliae* var. *acridum* (Goettel and Jaronski, 1997). In addition, another isolate

of *M. anisopliae* var. *acidum* was associated with severe dermal hyperallergenic response in humans by Mycotech (S. Jaronski, personal communication).

Interestingly, Genthner and Middaugh (1995) reported that, in their laboratory assay system, *M. anisopliae* conidia adversely affected both embryos and newly hatched larvae of the inland silverside fish (see section on 'Pathogenicity' above). It is difficult to judge the utility of the assay system of Genthner and Middaugh for predicting the hazard posed by a fungal control agent in the environment. Their system is highly sensitive and demonstrates the ability of certain fungi to penetrate the chorion, but rejecting a candidate agent based on their assay seems unwarranted. It is more useful to use their assay to label candidates that may need more extensive vertebrate safety testing.

Although several cases of human infection have been reported (see section on 'Pathogenicity' above), *M. anisopliae* has been registered and deemed safe when used according to the label instructions. If reports of human infections increase, it is inevitable that the fungus will need to be re-evaluated.

The hyphomycete *Beauveria brongniartii* has been developed for application against the scarab pest *Melolontha melolontha* in Switzerland (Baltensweiler and Cerutti, 1986). After aerial applications to the forest/pasture ecozone where adult beetles aggregate to mate, 10,165 insects and spiders were collected and reared to detect infection. Overall infection among non-targets was only 1.1%, with no infections in coccinellids and neuropterans but up to 9% infection among spiders.

### Classical

There are relatively few examples where fungi have been used for classical control of invertebrate pests. The most recent has been the attempted establishment of *E. grylli* from Australia for control of native North American grasshoppers. Although many predictions of detrimental effects were made, indications are that the fungus failed to establish (see section on 'Depletion of hosts' above). Another example is the introduction of the entomophthoralean fungus *Zoophthora radicans* into Australia from Israel to control the introduced spotted alfalfa aphid. Non-target effects were never seen, especially with regard to the hymenopteran parasitoid *Trioxys complanatus*, which was also introduced as part of this control programme (Glare and Milner, 1991).

Releases of the Asian entomophthoralean pathogen *Entomophaga maimaiga* were made in 1910–1911. However, the pathogen was not found to be established in the target North American populations of *Lymantria dispar* until 1989 (Hajek, 1999). It is unknown whether this pathogen became established during releases in 1910–1911 (it was not detected from 1911 to 1989) or from a more recent accidental introduction. Initial studies demonstrated that this fungus would only infect lepidopteran larvae. Further evaluations of the host range demonstrated low levels of infection in a number of lepidopteran families (Hajek *et al.* 1995). These studies were followed by collecting and rearing non-target Lepidoptera from foliage during epizootics caused by *E. maimaiga* in *L. dispar* populations (Hajek *et al.*, 1996). Among the > 1500 insects collected and reared, only two individuals of two common species were found to be infected by *E. maimaiga*. Because later instars of *L. dispar* spend significant amounts of time in the leaf litter, where large titres of *E. maimaiga* spores occur, lepidopteran larvae in the litter were also sampled. Although high levels of infection occurred among *L. dispar* larvae, only two other litter-dwelling lepidopteran larvae, of two different species, were infected by *E. maimaiga* (Hajek *et al.*, 2000). It was concluded that this pathogen is highly specific with very little risk to non-target organisms, including non-target lepidopterans.

Nevertheless, an ecologist questioned whether fungal epizootics causing declines in *L. dispar* populations might have an adverse impact that was indirect (i.e. due to rapid host depletion (see section on 'Depletion of hosts' above). With huge declines in *L. dispar* populations, would the other natural enemies linked with *L. dispar* then decline due to lack of hosts and not be able to respond quickly enough in order to control this pest if the fungus were not active? In this hypothesized scenario, the resulting unstable system would be even more prone to occasional outbreak populations of *L. dispar* (Valenti, 1998). This theory was proposed based on 1992 epizootics that devastated *L. dispar* populations, but to date (2000) there is absolutely no evidence that *E. maimaiga* drives other natural enemies of *L. dispar* to such low levels that they cannot respond if *L. dispar* begins once again to increase (A.E. Hajek, unpublished data). In fact, semi-field (Malakar *et al.*, 1999) and empirical field (Hajek, 1997) studies of interactions between *E. maimaiga* and the *L. dispar* nuclear polyhedrosis virus suggest minimal to no negative interactions between these two virulent pathogens.

### **Fungi against nematodes**

Many studies have been conducted investigating the use of fungi for control of plant-parasitic nematodes and, more recently, nematodes parasitizing livestock. In fact, nematode-attacking fungi are considered to be fairly common members of the soil community. Several fungal species attacking plant-parasitic nematodes are now mass-produced for control. Care must be taken so that fungi introduced to control plant-parasitic nematodes do not also affect insect-parasitic nematodes, beneficial fungi, such as mycorrhizae, or other beneficial members of the rhizosphere (see Chapter 5). To date, no detrimental effects on the rhizosphere microbial community have been documented, but few studies have been conducted to address non-target effects of nematophagous fungi. Due to the great diversity of soil inhabitants and the complexity of interactions within the soil, non-target studies are difficult to conduct. However, we can draw inferences from knowledge of the host specificity of nematophagous fungi. Nematode-attacking fungi utilize a diversity of types of associations with their hosts but these can generally be grouped as predatory or parasitic (Stirling, 1991). Predatory fungi have specialized structures, e.g. sticky rings and/or pegs for trapping nematodes. Among the predatory fungi, *Arthrobotrys* has received the most attention and methods for mass-producing and applying *Arthrobotrys irregularis* have been developed (Cayrol, 1983). Predatory fungi are not considered very specific to certain species of nematode prey (Barron, 1977; Kerry, 2000). Rosenzweig *et al.* (1985) found that, for nine nematodes tested, including free-living as well as plant and insect parasites, seven adhesive-producing nematode-trapping fungi were non-selective and were able to trap and consume all of the different nematodes. For a different fungus (*Monacrosporium ellipsosporium*), which traps nematodes using sticky knobs, ten of the 15 nematode species tested were trapped (Gaspard and Mankau, 1987).

More recently, emphasis has shifted to the use of parasitic fungi attacking sedentary stages of nematodes, e.g. saccate females and eggs. Among these parasitic fungi, much research has been directed toward two species: *P. lilacinus* and *Verticillium chlamydosporium* (Kerry, 2000). These fungi are opportunistic parasites, showing little host specificity, although isolates can differ in their ability to attack eggs of different nematode species. In addition, these fungi do not only attack nematodes; in particular, *V. chlamydosporium* is known to infect other organisms, including fungal spores and

eggs of snails and slugs, and it occurs in soil when root-knot and cyst nematodes are not present (Kerry and Crump, 1998; see also Chapter 5). Interestingly, although *V. chlamydosporium* populations do not depend only on populations of nematode hosts, a direct association has been found between the numbers of chlamydospores and the numbers of the plant-parasitic *Heterodera avenae* in the soil (Kerry and Crump, 1998).

*P. lilacinus* has been registered as a product for the control of nematodes. Mammalian safety tests included acute oral, dermal and pulmonary toxicity tests in rats and irritation studies in rabbits, and they indicated the relative safety of this microorganism (<http://www.ticorp.com.au/safety.htm>). However, there are numerous case reports that *P. lilacinus* is a causative agent of human infections (see section on 'Pathogenicity' above). This fungus is currently undergoing the tests necessary for registration in Australia. Given the numerous citations of its ability to infect humans, it would not be surprising if more extensive vertebrate testing is required for registration. It has been suggested that possibly the isolates of *P. lilacinus* collected from nematodes do not present a human health risk (see Chapter 5).

Perhaps the most unusual use of a fungus for nematode control is that of *Myrothecium verrucaria*. This soil-dwelling hyphomycete is mass-produced for the control of numerous species of endo- and ectoparasitic nematodes, but, after the fungus has been grown *in vitro*, it is killed before application (Warrior *et al.*, 1999). Anti-nematode effects are caused by multiple active ingredients that act synergistically to cause indirect effects; after application, the soil microcosm becomes an inhospitable habitat for nematodes, affecting motility, host/mate finding and egg development and increasing the parasitism of nematode eggs. Applications of this heat-killed fungus have been shown to enhance antagonism toward root-knot nematodes and are associated with structural and functional changes in the rhizosphere bacterial community (J.W. Kloepper, personal communication). Toxicological testing has demonstrated no effect of this product on aquatic invertebrates and tests have demonstrated no effects on an animal-parasitic nematode (*Nippostrongylus brasiliense*) or free-living species of nematodes (*Caenorhabditis elegans* and *Panagrellus redivivus*) (Warrior *et al.*, 1999).

## Fungi against weeds

### *Inundative*

As discussed previously, the fungal pathogens currently being used or evaluated as mycoherbicides can either have narrow or wide host ranges and this will be dependent upon the crop ecosystem in which that particular product is being targeted and/or the dispersal capacity of the constituent pathogen. One of the first products, Collego™, developed for use against the leguminous weed, *Aeschynomene virginica*, in rice ecosystems in the southern USA, was based on a supposedly host-specific strain or *forma specialis* of *Colletotrichum gloeosporioides* (Templeton, 1982). Subsequently, however, it was shown to have an expanded host range within the Leguminosae, attacking several economically important plants, including several bean species (TeBeest, 1988). Nevertheless, this spectrum has been assessed as posing no danger to agriculture, since the product is used exclusively within rice-based cropping systems, far removed from potentially susceptible crops, and, in addition, the pathogen has poor dispersal ability.

A similar strategy has been adopted for the use of the high-profile crop pathogen *Phytophthora palmivora* and, in what was a pioneering venture, a product (DeVine™)



was developed for the control of strangle-vine (*Morrenia odorata*) in citrus orchards in Florida (Ridings, 1986). Although the strain used is also pathogenic to cucurbitaceous crops, the product can be safely applied as long as there are no susceptible crops within 200 m of the treated orchard: appropriate labelling to this effect is, of course, mandatory (Charudattan, 1991).

A more recent and potentially more hazardous venture has involved the development of a mycoherbicide based on the silver-leaf fungus, *Chondrostereum purpureum*, which was once a notifiable disease in Europe and subject to strict legislation. This was targeted specifically at the invasive North American black cherry (*Prunus serotina*), but is also used to control not only this woody weed but also other exotic hardwood species in the Netherlands. The product, Biochon™, is applied as a mycelial suspension to cut stumps to prevent re-sprouting. The risk analysis of *C. purpureum*, which is a well-documented pathogen of plantation and ornamental *Prunus* spp., was based almost entirely on epidemiological data (De Jong *et al.*, 1990).

Conceptual and simulation models, supplemented by inoculation experiments, were developed to predict the risks posed to non-targets by artificially increased populations of pathogens (De Jong *et al.*, 1991). It was demonstrated that, although the risk to susceptible crops is high up to 500 m from the treated area, this falls dramatically thereafter and is negligible at 5000 m. Thus, the product has been approved for safe use as long as there are no *Prunus* orchards within this range.

### Classical

The inherent safety and stability of co-evolved natural enemies has been emphasized recently (Marohasy, 1996; McFadyen, 1998). After more than 100 years of experience of classical biocontrol of weeds, involving the release of over 600 exotic agents, there are only eight examples of damage to non-target plants. Five were anticipated, all were entirely predictable behavioural responses and not the result of host 'shift' and, most importantly, none had any significant economic or environmental impact. All these examples involved insect agents, whilst all of the 20 or more fungal pathogens so far used for classical weed biocontrol have proved to be extremely safe, with a high success rate (Evans, 2000). Nevertheless, there is still a general fear or mistrust of the concept of exploiting exotic plant pathogens by many countries, which is rarely expressed if the project involves insects for weed control. For various reasons, plant pathogens are adjudged to pose more of a risk, probably based on historical associations with invasive crop diseases (see section on 'Plant pathogens' above).

In contrast, Australia has shown some degree of plasticity or leniency in vetting or approving the release of exotic plant pathogens that have extended host ranges, attacking plant species other than the target weed, and these cases have been documented recently (Evans, 2000). An example is the introduction of a rust, *Uromyces heliotropii*, into Australia for control of the alien weed *Heliotropium europaeum*. Despite the fact that endemic *Heliotropium* spp. had proved to be susceptible in greenhouse tests, Hasan and Delfosse (1995) argued successfully that the Australian and alien *Heliotropium* spp. never overlapped in their ranges and that climatic conditions in the non-target areas were unsuitable anyway for rust establishment and development, and, therefore, that the risks were low. A similar, potential impasse was also faced and surmounted in the case of rubber-vine weed, *Cryptostegia grandiflora* (Asclepiadaceae), in Queensland. The co-evolved rust *Maravalia cryptostegiae*, from Madagascar, was shown to be pathogenic to a rare, endemic asclepiad (Evans and Tomley, 1994). However, it

was concluded that the risks involved were minimal and that the rust was safe to release, since the symptoms were not severe, and probably due to the severity of the testing protocol (Wapshere, 1989), and the only known habitat of the native species is itself endangered, as well as being both geographically distant and climatically distinct from the predicted range of the weed. Moreover, the threat posed by the weed to entire ecosystems far outweighed the potential loss of one species (Evans, 2000).

Even more recently, the microcyclic rust, *Puccinia melampodii*, from Mexico, has been approved for importation into Australia as part of an integrated strategy for management of the composite weed *Parthenium hysterophorus*, even though it has been found to sporulate on a related indigenous, albeit weedy, plant species, as well as on several sunflower and marigold cultivars (Evans, 2000). Once again, it was concluded by the Australian Quarantine and Plant Inspection Service that the actual and potential hazards involved in not attempting to control this allergenic weed, especially the threat to human health, were significantly greater than the perceived risks to non-target plants.

In summary, based on the protocols now in place and the experience gained, the classical introduction of fungal pathogens for the control of alien invasive plants is a safe, environmentally benign, economic and potentially sustainable strategy for long-term weed management.

## Regulations and Registration

We have seen how fungi can be successfully and safely used as both classical and inundative biological control agents. However, we have also seen that fungi as a whole can possess properties that make them potentially hazardous both to the user and to the environment in general. Consequently, the development and use of fungi as biocontrol agents requires an assessment of their potential hazards. In most countries, regulations and registration requirements serve two major purposes: (i) to ensure the safety of the agent; and (ii) to ensure efficacy. The major challenge facing the regulatory community is to adequately address safety issues without at the same time unduly slowing research and impeding the development and implementation of microbial control.

In many cases, the intended use of the organism and its origin will determine the type of regulatory oversight that will be required. For instance, in most countries, the importation of a non-indigenous organism intended for classical biological control will be regulated differently (and in many cases fall under different legislation) from the regulation of microorganisms intended for inundative use, be they indigenous or not. Here we attempt to provide an overview of the regulations and registration requirements for microbial control in general, while emphasizing as much as possible the specific requirements or concerns related to fungal microbial control agents.

### *Regulation of fungi as classical biocontrol agents*

Most countries regulate the importation and release of biological control agents, including pathogens, through legislation designed specifically for the exclusion of noxious agents. For example, the Animal and Plant Health Inspection Service (APHIS) of the US Department of Agriculture (USDA) until recently regulated the importation and release of microorganisms under the Federal Plant Pest Act and the Plant Quarantine

Act (OTA, 1995). Regulation of biological control agents through such plant pest statutes often posed difficulties, in that the acts addressed biological control agents in the context of noxious weeds or other concerns. For instance, in the USA, there were jurisdictional problems concerning the granting of permits for release of agents because the acts only covered the movement of agents and not their release (OTA, 1995). On 20 June 2000, a new Plant Protection Act was passed by the US Congress. This Act repealed ten statutes, including the Plant Quarantine Act and the Federal Plant Pest Act. For the first time, it recognizes biological control agents as beneficial tools, separating them from 'pests' in the context of the definitions (Henstridge, 2000; P. Henstridge, personal communication). It adds a new definition of biological control based on the definition under the International Plant Protection Convention. It also separates the definition entirely from the definition of plant pest. This changes the focus from negative to positive and places the correct emphasis on biocontrol as a beneficial control method. It contains language that emphasizes the need to avoid impeding commerce where there is no risk. For example, it includes provisions that clarify the Secretary's authority to allow for organisms or groups of organisms to be exempt from regulation once it is determined that they do not present a risk. In addition, there is a provision clarifying an individual's right to petition the Secretary to have an organism added or removed from regulation and contains language that emphasizes the need to facilitate commerce in beneficial organisms.

Australia is the only country that has enacted legislation – the Australian Biological Control Act – that deals specifically with biological control. Nevertheless, biological control agents are still regulated under other acts, such as the Quarantine Act and the Wildlife Protection Act; the Biological Control Act is invoked only as a last resort in controversial situations.

Recently, the Food and Agriculture Organization of the United Nations developed a code of conduct for the import and release of exotic biological control agents (FAO, 1996). The objectives of the code are to facilitate the regulation of exotic biological control agents through introducing internationally acceptable procedures and practices.

The most important part of the code as far as safety is concerned is paragraph 4.5. It states that the importer of biological agents must include:

...an analysis of the risks posed to possible nontarget organisms and to the environment generally and should entail available emergency procedures should the biological control agent after release display unexpected adverse properties. The dossier should also contain a report detailing laboratory tests, and or field host range of the candidate agent. Testing should be based on recommended procedures and approved by the authority. These tests should relate to the candidate agent only and different procedures should apply to any additives used in formulations of products which contain biological control agents.

The difficulty lies in that the tests and information that are appropriate and are adequate to ensure that these conditions are met are not apparent and are certainly open to interpretation. Nevertheless, this code addresses the importance of conducting a risk assessment prior to the introduction of an exotic agent.

When the concept of employing plant pathogens for weed control was first put into practice in the 1970s, it was almost taken for granted that the fungal agents had to have an extremely narrow host range and, preferably, that they should be specific to the target weed. As was discussed (see section on 'Addressing the Hazards' above), this is no longer a necessary prerequisite. For inoculative or classical biological con-

tol, the main emphasis is, and must always be, placed on high specificity to the weed target or its near relatives. Thus, the most intensive, time-consuming and expensive part of the programme involves determining the host range of the candidate pathogen, for which the centrifugal, phylogenetic screening protocol or methodology has been developed and subsequently modified (Wapshere, 1975, 1989). The strategy that underpins the classical biological control approach is based on the theory that only those natural enemies that have co-evolved with the target weed in its centre of origin or diversity will have the necessary attributes of high virulence and specificity for successful classical agents. The initial biocontrol programmes concentrated on testing plants of economic value in the release area instead of the more relevant concept of genetic relatedness. The centrifugal, phylogenetic screen now offers a scientific and dependable method of host-range testing with a proven track record (McFadyen, 1998). In fact, the aforementioned Australian Biological Control Act does not demand that the exotic agent selected for importation be host-specific, but only that it should not cause any significant harm to any person or to the environment. The Act thereby allows a certain degree of latitude, both in interpreting the results of host-range screening and in the predicted host range of the candidate agent (see section on 'Addressing the Hazards' above).

One of the main difficulties that remain as far as entomopathogenic fungi are concerned is the lack of acceptable evaluation methods that would adequately address host range (Hajek and Goettel, 2000). Laboratory bioassays, which are at present almost exclusively relied upon to establish host range as far as most regulations for the importation of exotic entomopathogens are concerned, measure the 'physiological host range'. However, it has been established that the physiological host range does not adequately predict the 'ecological host range', the range manifested under field conditions (Hajek and Butler, 2000). But, by the careful design and use of laboratory and semi-field assays that incorporate pertinent parameters and through detailed knowledge of the ecology and epizootiology within the area of endemicity of the pathogen in question, information for pertinent risk assessment for non-target organisms as a result of the introduction of entomopathogenic fungi can be obtained (Hajek and Goettel 2000).

Because agents intended for classical control are expected to have minimal contact with humans, they are generally exempt from mammalian safety testing. Concerns would arise if a potential agent were hyperallergenic and were spread aerially in large numbers during epizootics, but such fungi are not being pursued for introductions.

### ***Regulation of fungi as microbial pesticides***

Mycopesticides are often based on an indigenous rather than an exotic fungal pathogen. Hence, the selected pathogen is already 'in the system' but, for one reason or another, is not effective in controlling its host. The emphasis here is no longer on host specificity but on ensuring, through epidemiological analysis, that the mycopesticide poses no danger to neighbouring useful plants or insects and especially that it should not establish in a new, non-target host (see section on 'Addressing the Hazards' above). It is highly unlikely that an indigenous pathogen would establish itself in a new host as a result of inundative use. If some non-target organisms were to be affected, it would be expected that such an effect would be only temporary and only occur within the immediate area of application, as is currently the case with most chemical insecticides

used at present. Unfortunately, most registration requirements for indigenous microbial control agents still require relatively extensive laboratory host-range testing; this is a requirement that is not generally demanded of most chemical pesticides because, *a priori*, a wide host range is expected.

Most countries require registration of fungi destined to be used inundatively as microbial pesticides. And, as with agents destined for the classical control approach, one of the difficulties in regulation of these agents is that, once again, they are regulated by legislation initially designed for chemical pesticides. For instance, in the USA, microbial pesticides are regulated and registered by the EPA under the Federal Insecticide, Fungicide and Rodenticide Act. Even though the EPA developed special data requirements for microbial pesticides in the early 1980s, many problems existed until the mid-1990s when the EPA adopted a separate review system for microbial pesticides from that for conventional pesticides (OTA, 1995). Some problems with the methodology still exist.

An Organization for Economic Cooperation and Development (OECD) survey of data requirements for the registration of biological pesticides in OECD member countries found that there were real differences in certain data requirements among the respondent countries, most notably in physical-chemical properties, ecotoxicology and environmental fate. This situation exists even though a similar approach was used to structure data requirements (Table 13.1) and emphasis was placed on the same test areas or study categories (OECD, 1996). These differential data requirements have been one of the most detrimental aspects of regulations of microbial control products. In many cases, a company wishing to register a product in several countries would have to provide different registration packages for each country, each with different data requirements and formatting, thereby significantly increasing the costs of registration of products, many of which are useful for niche markets at best.

A promising development in recent years has been the move towards harmonization of regulatory requirements among countries. For instance, under the auspices of a North American Free Trade Agreement Technical Working Group on Pesticides, the USA, Canada and Mexico have worked together to harmonize data requirements for registration of microbial pest control products between their countries (NAFTA, 1998), making joint reviews possible (NAFTA, 1999). Similar attempts are being made to develop common registration requirements for the European Union (Neale and Newton, 1999). An even more ambitious project is to standardize and harmonize core

**Table 13.1.** Data requirements for the registration of a microbial pathogen. (Adapted from OECD, 1996.)

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|   |
|---|
| Identity  |
| Physical, chemical and biological properties          |
| Function, mode of action and handling                 |
| Manufacturing, quality control and analytical methods |
| Residues  |
| Efficacy <sup>a</sup>                                 |
| Toxicology  |
| Ecotoxicology   |
| Fate and behaviour in the environment                 |

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<sup>a</sup>USEPA requires efficacy data only for pesticide products used to control pests that threaten public health.

data requirements for the registration of microbial pesticides within the 29 countries that are members of the OECD (OECD, 1999).

The goal of vertebrate safety testing is to assess the hazard posed by a candidate microbial pest control agent (MPCA) by means of carefully selected laboratory tests. The World Health Organization (WHO) was the first organization to propose a tiered testing strategy to evaluate the hazard posed by MPCAs to mammals (Anon., 1981b). Elements of this proposal are incorporated into the current regulatory guidelines for Canada, the USA, and the European Union. These tests replaced the long-term assays that are used in assessing chemical insecticides with short-term (1 month) exposures that utilize invasive routes, such as intravenous and/or intraperitoneal injection, as well as feeding studies. This battery of tests is referred to as Tier 1 (US Code of Federal Regulations 40 CFR 158.740c). If questions arise during Tier 1 testing, the candidate then goes through a second battery of tests that are more extensive. A third tier of tests is also available, but it is unlikely that an MPCA that does not clear Tier 2 will go to that level, because it would most probably no longer be considered as a viable control product at this stage. Unfortunately, many of these data are unavailable to the public because they are considered proprietary. However, the results of some of these acute tests have been published in peer-reviewed publications. Routes of exposure in these published studies include inhalation, subcutaneous injection, intraperitoneal injection, intravenous injection, dermal and ocular irritancy testing, dermal sensitization studies and feeding studies (Anon., 1981b; Burges, 1981; Shadduck *et al.*, 1982; Siegel and Shadduck, 1987; Mier *et al.*, 1989, 1994; Kerwin *et al.*, 1990; Saik *et al.*, 1990; Semalulu *et al.*, 1992; Goettel and Jaronski, 1997; Ward *et al.*, 1998; Smits *et al.*, 1999).

The MPCA does not have to be harmless in all tests and at all concentrations, but, rather, the circumstances under which it produces infection or mortality must be elucidated. Historically, these tests have emphasized assessing the infectivity and pathogenicity of a candidate organism, but recently concerns have been raised about assessing the allergenicity of MPCAs and these tests may be added to the protocols necessary for registration (Ward *et al.*, 1998). Aspects of the biology (such as maximum temperature tolerated) of the MPCA are considered when designing tests as well as possibly vulnerable organ systems, based on literature reviews. This latter point may be complicated by the taxonomic status of an MPCA. Medical reports may only identify a vertebrate isolate to genus or, in some cases, the vertebrate isolate may have been misidentified (Siegel *et al.*, 1997). These questions can be addressed during safety testing by choosing a particular route of exposure, such as inhalation or intravenous injection, and/or by extensively characterizing the isolate proposed as the MPCA. Characterization includes both taxonomy (this can include differentiating between entomopathogenic and non-entomopathogenic strains) and identification of toxins and/or toxic metabolites.

Regulatory agencies utilize the data on hazard to determine risk (hazard  $\times$  exposure). It is unlikely that an MPCA that successfully passes these screens will cause problems when released in the field, but a possibility, perhaps remote, always exists that there may be susceptible species or a unique scenario in the field that may result in infection or mortality. The perspective necessary for evaluating both safety data and field reports was succinctly stated by Burges (1981):

a no-risk situation does not exist, certainly not with chemical pesticides, and even with biological agents one cannot absolutely prove a negative. Registration of a chemical is essentially a statement of usage in which risks are acceptable and the same must be applied to biological agents.

## Concluding statements

History has already demonstrated that fungi can be effectively and safely used in biological control. As we become more familiar with microbial control agents, and microbial ecology in general, regulations are being adopted to address the pertinent concerns. A key is that registration requirements for biocontrol fungi must remain flexible and address the hazards of the specific candidate in question and its proposed use on a case-by-case basis. The allowances for exemptions to many data requirements are of paramount importance. And hazards must also be weighed in consideration of the benefits of microbials, which, to date, almost always outweigh those of chemical pesticides.

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