Bioassays of Entomogenous Fungi

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Introduction

Many entomogenous fungi are relatively common and often induce epizootics and are therefore an important factor regulating insect populations. Most species attacking terrestrial insects belong to the Hyphomycetes and Entomophthorales while those attacking aquatic insects are generally from the Chytridiomycetes and Oomycetes. The host is usually invaded through the external cuticle, although infection through the digestive tract occurs with some species. Spores attach to the cuticle, germinate, and penetrate the integument by means of a combination of physical pressure and enzymatic degradation of the cuticle. The mycelium then ramifies throughout the host haemocoel. Host death is usually due to a combination of nutrient depletion, invasion of organs and the action of fungal toxins. Hyphae usually emerge from the cadaver and, under appropriate conditions, produce spores on the exterior of the host.

The importance of entomogenous fungi as biological control agents has been reviewed by Latge and Moletta (1988), McCoy *et al.* (1988), McCoy (1990), Ferron *et al.* (1991), Roberts and Hajek (1992), Tanada and Kaya (1993), and Hajek and St Leger (1994). Examples of common entomogenous fungi, including those of commercial importance, are given in Table 4.1, but a more detailed list is provided by Roberts (1989).

The search for commercially viable entomogenous fungi for use in integrated pest management programmes entails several steps. Fungal species and isolates must first be obtained from diseased insects or the environment, and identified. They must then be evaluated under laboratory conditions to identify the most promising candidates. Concomitantly, several problems

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Table 4.1. Some common entomogenous fungi and their hosts.

Entomogenous fungus	Invertebrate host
Division Zygomycotina	
Conidiobolus obscurus	Aphids
Entomophaga aulicae	Lepidopteran insects
Entomophaga grylli	Orthopteran insects
Entomophthora muscae	Dipteran insects
Entomophthora thripidum	Thrips
Erynia neoaphidis	Aphids
Massospora cicadina	Cicada
Neozygites fresenii	Aphids
Zoophthora radicans	Certain Hemiptera and Lepidoptera
Division Deuteromycotina	
Aschersonia aleyrodis	Whiteflies, scales
Beauveria bassiana	Wide host range
Beauveria brongniartii	Cockchafers and sugarcane borer
Culicinomyces spp.	Mosquitoes
Hirsutella thompsonii	Spider mites, citrus mites
Metarhizium album	Homopteran insects
Metarhizium anisopliae	Wide host range
Metarhizium flavoviride	Orthopteran insects
Nomuraea rileyi	Lepidoptera
Paecilomyces farinosus	Coleoptera, Lepidoptera
Paecilomyces fumosoroseus	Wide host range
Tolypocladium cylindrosporum	Mosquitoes
Verticillium lecanii	Wide host range

have to be addressed. The selected isolate must be economically mass produced, have adequate storage properties, and it must be efficacious under field conditions. Formulation is an important factor that can affect many of these properties. For instance, formulations can improve storage time and field efficacy by protecting against desiccation and harmful UV radiation. Some formulations can enhance fungal virulence by improving spore attachment to the host surface, diluting the fungistatic compounds in the epicuticular waxes and stimulating germination. Rapid germination and infection are a hallmark of virulent isolates. Finally, the inoculum must be targeted effectively because mortality is dose related.

Well designed bioassays are central to the successful development of entomogenous fungi. There exists a wide range of attributes among fungal isolates and species (Table 4.2). Bioassays are the tools for identifying the following key parameters: (i) host range, (ii) virulence, (iii) ecological competency (i.e. performance under field conditions), (iv) conditions impeding/enhancing epizootics, and (v) barriers to infection.

The development of bioassays requires a thorough understanding of both host and pathogen requirements. Failure to understand these can lead

Table 4.2. Comparison of the attributes of specialist and generalist insect pathogenic fungi.

Specialist	Generalist
Narrow host range	Wide host range
Mostly biotrophs	Mostly hemibiotrophs
Usually produce relatively few, large conidia	Produce copious small conidia
May produce more than one type of spore	Produce single type of spore
Conidia may be forcibly discharged from conidiophores	Conidia usually passively dispersed
Conidia coated in mucilage	Hydrophobic conidia
Few conidia required to cause rapid infection	Many conidia required to cause rapid infection
Subtilisins not detected	One or more subtilisins secreted
Little evidence of toxicosis	Toxins may play important role in host death
Colonize haemocoel as protoplasts or thin-walled hyphal bodies	Colonize haemocoel as blastospores or filamentous hyphae
Induce spectacular epizootics	Natural epizootics usually less obvious
Diseased insects usually located on aerial parts of plant	Diseased insects mostly found in the soil
Difficult to culture	Easy to culture

to inconsistent results, high control mortality, and poor assessment of fungal virulence. The production, formulation and application methods employed can also influence fungal viability, virulence and efficacy. Methods for isolation, cultivation and storage of entomogenous fungi and important aspects of host–pathogen relationships are briefly reviewed, focusing on specific factors which could influence the results of laboratory and field-based bioassays. The methods used for bioassay of these fungi against insects are discussed and examples are used to illustrate the different methods used, augmenting the recent reviews on bioassay techniques by Goettel and Inglis (1997), Kerwin and Petersen (1997) and Papierok and Hajek (1997).

Isolation of Entomogenous Fungi

Details on initial handling and diagnosis of diseased insects have been recently reviewed by Lacey and Brooks (1997). Pathogens can be retrieved directly from the surface of cadavers if the fungus has already sporulated. Most Hyphomycetes can be scraped directly off the cadaver (Goettel and Inglis, 1997), while insects infected with entomophthoralean fungi may be positioned to shower their conidia directly on to a nutrient surface

(Papierok and Hajek, 1997). If sporulation or external hyphal growth has not yet taken place, diseased insects can be incubated in a humid chamber such as a Petri dish lined with moist filter paper to encourage sporulation. Sporulating cadavers can be placed whole or dabbed on a selective medium for isolation of the pathogen.

Most selective media contain either a fungicide and/or antibiotics which encourage growth of entomogenous fungi and discourage growth of saprophytic fungi and bacteria. Some entomopathogenic fungi can be isolated indirectly from the soil by live baiting with insects such as larvae of *Galleria* spp. (Zimmermann, 1986), or directly by extraction using an aqueous solution, often in conjunction with a selective medium (e.g. Beilharz *et al*., 1982; Appendix 4.1) or discontinuous density gradients (Hajek and Wheeler, 1994). Aquatic fungi can be baited using a variety of substrates such as hemp seed (Kerwin and Petersen, 1997).

Once isolated, many fungi, especially from the Hyphomycetes, can be maintained *in vitro* on several media. Conidia and mycelium should be stored in cryovials under nitrogen, or freeze-dried and stored in sterile glass ampoules (Humber, 1997). Freshly harvested conidia can also be air dried and stored in a desiccator at 4°C or room temperature. Several hyphomycete fungi (e.g. *Verticillium lecanii*, *Metarhizium anisopliae*) can be stored as conidia bound to silica gel at −40°C. More details on isolation and storage of entomopathogenic fungi are given by Goettel and Inglis (1997), Humber (1997), Kerwin and Petersen (1997) and Papierok and Hajek (1997).

Production and Formulation

Once isolates have been identified, the next step is the production of stable, non-attenuated inoculum for use in evaluation bioassays. Minor changes in production, storage or formulation can greatly influence bioassay results. The amount of inoculum required for most bioassays is minimal and can sometimes even be obtained from cadavers. Although this is sometimes the only source of inoculum in fastidious fungi which do not readily grow on artificial media, it is preferable to obtain inoculum cultured on an artificial medium. The method of culture will largely depend on fungal species and the type of propagule required. More details on laboratory-scale production of entomopathogenic fungi are given by Goettel and Inglis (1997), Kerwin and Petersen (1997) and Papierok and Hajek (1997). More recent general reviews on mass production and formulation are those of Bartlett and Jaronski (1988), Baker and Henis (1990), Auld (1992), Bradley *et al.* (1992), Goettel and Roberts (1992), Feng *et al.* (1994), Jenkins and Goettel (1997) and Moore and Caudwell (1997).

Attenuation of virulence

Successive subculturing on artificial media often results in attenuation of virulence. Therefore, when possible, large quantities of inoculum should be produced using the initial isolate and stored (e.g. as dry conidia) for use in successive bioassays and studies. The rate of attenuation clearly depends on the isolate and species of pathogen.

Some fungal pathogens retain their virulence even after prolonged culture *in vitro* (e.g. *Culicinomyces clavisporus*, Sweeney, 1981; *Beauveria bassiana*, Samsinakova and Kalalova, 1983; *V. lecanii*, Hall, 1980). In contrast, some isolates rapidly loose virulence after only a few subcultures on artificial media. For instance, Nagaich (1973) noted that an isolate of *V. lecanii* pathogenic to aphids lost its virulence after the second or third subculturing. *Lagenidium giganteum* progressively lost the ability to form oospores and zoospores and to infect *Aedes aegypti* larvae after prolonged culture on a sterol-free agar medium (Lord and Roberts, 1986).

Virulence of attenuated isolates can often be regained with passage through an appropriate host. This has been demonstrated with several pathogens including *M. anisopliae* (Fargues and Robert, 1983), *Nomuraea rileyi* (Morrow *et al*., 1989), *Paecilomyces farinosus* (Prenerová, 1994), *B. bassiana* (Hall *et al.*, 1972; Wasti and Hartmann, 1975), *Lagenidium giganteum* (Lord and Roberts, 1986) and *Conidiobolus coronatus* (Hartmann and Wasti, 1974).

The effects of culture history on virulence poses a special problem if bioassays are used to compare virulence among isolates obtained from various sources and culture collections, as the precise culture history of such isolates is seldom known. In an attempt to address this problem, each isolate can first be passed through an insect host prior to culture on artificial media and use in bioassays. Vidal *et al.* (1997) first passed 30 isolates of *Paecilomyces fumosoroseus* through nymphs of *Bemisia argentifolii*, then carried out bioassays to compare their virulence against this host. Fargues *et al.* (1997b) passed isolates through a non-host prior to bioassays against a host; isolates of *Metarhizium flavoviride* were first passed through the waxmoth, *Galleria mellonella*, by injecting conidia into seventh-instar larvae prior to use in bioassays against the desert locust, *Schistocerca gregaria*. Although the waxmoth is not known to be a natural host of this pathogen, it was felt that growth and sporulation of the fungus on a natural substrate would help restore virulence. Although original, the utility of this technique in comparative bioassays of fungal isolates has not yet been determined. Further studies in this regard are warranted.

Production of infection propagules

There are four general methods for the production of fungal propagules on artificial media: (i) surface culture on solid media, (ii) fermentation on

Table 4.3. Production and storage information on selected entomogenous fungi.

Pathogen	Production method	Media	Form of inoculum
Aschersonia aleyrodis	1, 3	PDA or chopped millet	DM, B, C
Beauveria bassiana	1, 3	Most nutrient agar and liquid media	DM, B, C
Beauveria brongniartii	1, 3	Most nutrient agar and liquid media	DM, B, C
Coelomomyces spp.	2	Host rearing medium	S, infected copepods
Culicinomyces clavisporus	1, 3	Most nutrient agar and liquid media	DM, B, C
Entomophaga spp.	1, 2	Sabouraud dextrose, egg yolk, milk agar (SEMA)	C
Erynia neoaphidis	1, 2, 3	SEMA	DM, C
Hirsutella spp.	1, 3	Most nutrient agar and liquid media	Submerged C, B, C
Lagenidium giganteum	1	Different solid or liquid media that include a sterol	Z
Metarhizium anisopliae	1, 3	Most nutrient agar and liquid media	DM, B, C
Metarhizium flavoviride	1, 3	Most nutrient agar and liquid media	DM, B, C
Nomuraea rileyi	1, 3	Most nutrient agar and liquid media	DM, B, C
Paecilomyces farinosus	1, 3	Most nutrient agar and liquid media	DM, B, C
Tolypocladium spp.	1, 3	Most nutrient agar and liquid media	DM, B, C
Verticillium lecanii	1, 3	Most nutrient agar and liquid media	DM, B, C
Zoophthora radicans	1, 2, 3	SEMA	DM, C

Production method is: 1, surface or submerged culture; 2, live host; 3, semi-solid or diphasic culture. Form of inoculum: C, conidia; DM, dry mycelium; B, blastospores; Z, zoospores; S, sporangia.

semi-solid media, (iii) submerged fermentation and (iv) diphasic fermentation. Although production on solid media is considered as the most expensive, it is also the simplest and usually suffices for the production of the relatively small amounts of inoculum required for laboratory bioassays. The production methods of some important fungi are summarized in Table 4.3. Because few generalizations can be made regarding culture and production of propagules of the more fastidious fungi, we focus our attention here on those fungi that are more amenable.

Surface culture on solid media

Most facultative entomogenous fungi will grow on one or more defined or semi-defined agar-based medium (e.g. Czapek-Dox, Sabouraud) or on natural substrates (e.g. wheat, bran, rice, egg yolk, potato pulp). Specialist fungi are usually fastidious on artificial media and are usually best maintained on their respective hosts. A few can be grown *in vitro* but require a complex medium.

For example, *Lagenidium giganteum* can be cultivated on simple medium but requires sterols to induce oosporogenesis (Kerwin *et al.*, 1991). Entomophthoralean fungi grow well on Sabouraud dextrose or maltose agar fortified with coagulated egg yolk and milk (Papierok, 1978; Wilding, 1981).

Petri dishes and autoclavable plastic bags are recommended for small- and larger-scale production, respectively. However, other containers such as pans, glass bottles and inflated plastic tubing have been used (Samsinakova *et al.*, 1981; Goettel, 1984; Jenkins and Thomas, 1996). Agar-based media are usually used for routine culture. Alternatively, cheaper substrates such as rice or shelled barley can be used in autoclavable bags or other containers, especially when larger amounts of inoculum are required (Aregger, 1992; Jenkins and Thomas, 1996). Once the fungus has sporulated, conidia are harvested either by washing off using water or a buffer, direct scraping from the substrate surface (e.g. agar), or by sieving (e.g. rice). For some entomophthoralean fungi, the forcibly discharged conidia are allowed to shower directly on the host (Papierok and Hajek, 1997).

To obtain conidia virtually free of nutritive substrate contamination, non-cellulolytic fungi can be grown on a semi-permeable membrane such as cellophane (Goettel, 1984). Pans containing a nutritive substance such as bran are lined with the cellophane, placed in sterile bags, autoclaved, inoculated and incubated. After sporulation has taken place, the membrane with the adhering sporulating fungus is lifted from the nutritive substrate. Conidia can then be scraped from the cellophane surface.

Fermentation in semi-solid media

Production of fungi on semi-solid media involves impregnation of small particles with nutrients. Typically wheat bran is mixed with an inorganic substance such as vermiculite, although other substances can be used to provide a large surface area for growth. The mixture is then steam sterilized and the moisture content adjusted to 50–70%. The fermentation process takes place either in a bin or a rotating drum through which sterile, moist air is passed. Primary inoculum is usually grown in liquid medium. Toward the end of the fermentation cycle, the moist air is replaced by dry air to reduce the moisture content of the bran and to encourage sporulation. The temperature is controlled by regulating the circulating air temperature.

More recently, nutrient-impregnated membranes have been shown to reduce production costs of *M. anisopliae* conidia (Bailey and Rath, 1994). A range of membranes impregnated with skimmed milk were screened including blotting paper, fly screen, hessian, and gauze-type fabrics. Sporulation was profuse on Superwipe (an absorbent fibrous material) soaked in skimmed milk (20 g l^{-1}) supplemented with sucrose (2 g l^{-1}) or dextrose plus potassium nitrate. Spores could be washed off in a similar way to removal of conidia from grain.

Submerged and diphasic fermentation

Submerged fermentation can be used for production of blastospores and submerged conidia of selected isolates of entomogenous fungi. Dimorphic filamentous fungi like *M. anisopliae, B. bassiana, Beauveria brongniartii, V. lecanii, Paecilomyces farinosus* and *Nomuraea rileyi* produce relatively thin-walled blastospores in submerged culture that are infectious but difficult to preserve (Adamek, 1965; Samsinakova, 1966; Blanchere *et al.*, 1973; Ignoffo, 1981). Blastospores are produced in relatively large quantities during the log phase of growth. Most often they are spherical, oval or rod-shaped single cells which usually germinate within 2–6 h. Although several species of entomogenous fungi produce blastospores, there is considerable intraspecific variation. Some isolates produce blastospores more readily than others. The culture medium has a profound influence on blastospore production. There are several recipes for blastospore production (Appendix 4.2).

Blastospores sometimes are indistinguishable from submerged conidia. For example, some isolates of *M. flavoviride*, *M. anisopliae*, and *Hirsutella thompsonii* will produce conidia-shaped cells in submerged culture occasionally from phialide-like structures (van Winkelhoff and McCoy, 1984; Jenkins and Prior, 1993; T.M. Butt, unpublished observations). Van Winkelhof and McCoy (1984) noted that of 14 isolates of *H. thompsonii* only one produced true conidia. The others produced conidia-like cells.

Diphasic fermentation entails growth of fungi in liquid culture to the end of log phase followed by surface conidiation on a nutrient or inert carrier. This method has been developed for mass production of *B. bassiana* (Bradley *et al*., 1992) and *M. flavoviride* (Jenkins and Thomas, 1996). A similar approach was used in the production of dry marcescent entomphthoralean mycelium (McCabe and Soper, 1985).

Dry marcescent mycelium

The development of the dry marcescent process (McCabe and Soper, 1985) provides a convenient method for production of fungi, especially fastidious species like *Zoophthora radicans*. This process entails the production of the mycelium by submerged fermentation, harvesting by filtration, coating the harvested mycelium with a protective layer of sugar solution and then drying under controlled conditions. When hydrated, the mycelium quickly sporulates to produce infectious conidia. The dry marcescent process has been used successfully as a source of inoculum for *M. anisopliae* (Pereira and Roberts, 1990; Krueger *et al*., 1992), *C. clavisporus* (Roberts *et al*., 1987), *B. bassiana* (Rombach *et al*., 1988), *Z. radicans* and *Erynia neoaphidis* (Wraight *et al.*, 1990; Li *et al*., 1993).

Effects of culture conditions on virulence and ecological fitness

Culture conditions can greatly influence the virulence, longevity and ecological fitness of the resultant propagules. For example, St Leger *et al.* (1991) found that levels of enzymes on conidia from infected *Manduca sexta* larvae were higher than those cultured on an agar medium. Papierok (1982) found that conidia of four isolates of *Conidiobolus obscurus* produced *in vitro* were less virulent against aphids than those produced *in vivo*. Hallsworth and Magan (1994a, b) found that *B. bassiana*, *M. anisopliae* and *P. farinosus* accumulate polyols when grown on media with increasing ionic solute concentration and with different carbohydrate types at different concentrations. Inoculum with high reserves of polyols was shown to germinate and grow more rapidly at much lower water activities (A_w 0.90 = 90% RH) than those with small reserves of these polyols (Hallsworth and Magan, 1994c, 1995). Furthermore, in bioassays with *G. mellonella* larvae at different RHs, conidia with large amounts of glycerol and erythritol were more virulent than conidia grown on rich nutrient substrates (Hallsworth and Magan, 1994c). Culture conditions can also influence thermal tolerance. Increasing the sucrose content of the growth medium from 2 to 8% resulted in a reduction of thermal tolerance by conidia of *M. flavoviride* (McClatchie *et al.*, 1994).

Postharvest storage

The postharvest storage conditions greatly affect fungal viability and efficacy. Conidial moisture content is an important factor with respect to temperature tolerance and viability. Zimmermann (1982) showed that the tolerance of *M. anisopliae* for high temperatures increases with increasing desiccation, whilst Daoust and Roberts (1983) showed that at 37°C, two isolates of *M. anisopliae* retained most viability after long-term storage at either 0 or 96% RH. Drying conidia in the presence of desiccating agents like silica gel and $CaCl_2$ appears to improve their viability but direct contact with the desiccant can be detrimental (Daoust and Roberts, 1983).

Moore *et al.* (1995) found that dried conidia stored in oil formulations remained viable longer than those stored as a dried powder, especially if stored at relatively low temperatures (10–14°C compared with 28–32°C). Addition of silica gel to oil-formulated conidia appears to prolong their shelf life. Undried conidia of *M. flavoviride* lose viability rapidly, with germination dropping below 40% after 9 and 32 weeks at 17°C and 8°C, respectively. After 127 weeks in storage, germination remained at over 60 and 80% for the dried formulations at 17°C and 8°C, respectively (Moore *et al.*, 1996). These conidia were found to have retained virulence similar to that of freshly prepared formulations. Furthermore, conidia dried to 4–5% moisture content showed greater temperature tolerance than conidia with a higher moisture content (McClatchie *et al.*, 1994; Hedgecock *et al.*, 1995).

Formulation

Formulations can greatly improve the efficacy of entomopathogens both in protected and field crops. The type of formulation and selection of additives for a given formulation are critical to their stability. The basic components of most formulations include, in addition to the active ingredient (i.e. fungal spore), one or more of the following: a carrier, diluent, binder, dispersant, UV protectants and virulence-enhancing factors (Moore and Caudwell, 1997).

The most widely used carriers are oil and water. Because of their hydrophobic nature, conidia of some hyphomycete fungi readily suspend in oils, but oil itself can be toxic, especially when applied against small insects. Oils are reasonably effective in sticking spores to insect and plant surfaces (Inglis *et al.*, 1996a). In contrast, surfactants (e.g. Tween) need to be added to water to ensure conidial suspension, but these are toxic to conidia if used at high concentrations (e.g. >0.1% v/v). Incorporation of humectants (e.g. Silwet) can improve infection by providing moisture for germination and infection.

Recent studies show that more than 60% of the fungal inoculum can be removed from leaf surfaces by rain (Inglis *et al.*, 1995c; T.M. Butt, unpublished observations). Compounds increasing adhesion of spores to insect and plant surfaces need to be evaluated. Equally important, the formulation must not interfere with the infection process, and at best it should enhance disease transmission.

Photoinactivation has emerged as one of the major environmental factors affecting persistence and thus efficacy of entomogenous fungi. Ultraviolet radiation can sterilize surfaces of plants and insect cuticle (Carruthers *et al.*, 1992; Inglis *et al.*, 1993). Incorporation of UV blockers (e.g. Tinopal) in formulations can offer some protection against harmful UV radiation (Inglis *et al.*, 1995b).

Other Factors Affecting Virulence

How the culture, storage and formulation of fungi can influence their viability, virulence and field efficacy has been summarized in the previous section. In this section, other factors which could influence the results of laboratory and field bioassays are considered.

Most entomopathogenic fungi gain entry to the haemocoel by penetrating the host cuticle using a combination of hydrolytic enzymes and mechanical force (Goettel *et al.*, 1989; St Leger *et al.*, 1989a, b; Butt *et al.*, 1990, 1995; Schreiter *et al.*, 1994). The speed of kill, and to some extent the host range, are influenced by the number of infection propagules in contact with the cuticle. Mortality is dose related. There are vulnerable sites on the cuticle, such as the intersegmental membranes and sites under the elytra of

certain beetles. Basking in the sun, preening and ecdysis reduce the amount of viable inoculum on the insect surface. Handling of insects, rearing conditions and insect vigour influence their susceptibility to fungal infection. Fungal pathogens are greatly affected by abiotic factors such as temperature, light and humidity.

Dose-related mortality

Susceptibility of most insects is dependent on spore dosage. It is presumed that a threshold exists whereby a certain number of propagules are necessary to overcome the host, however, the exact nature of this relationship has not been determined. A positive correlation between the number of infective spores and mortality by mycosis has certainly been established for most insect/pathogen combinations, but there are exceptions. For instance, Goettel *et al.* (1993) reported a negative correlation between dose and mortality at concentrations greater that 10^4 ascospores of *Ascosphaera aggregata* per leaf-cutting bee, *Megachile rotundata,* larva. Therefore, care must be taken when interpreting results of very high application rates in some systems as the possibility of self-inhibition exists.

The dose–mortality relationship is the principal component in many bioassay designs (Chapter 7). Insects are treated at several increasing doses and the LD_{50} and its fiducial limits are then used to compare virulence or 'potency' against other isolates. The slope of the dose–mortality curve is also very useful when comparing virulence amongst different isolates.

Vulnerable sites on the cuticle

Not all areas of the insect cuticle are equally vulnerable to penetration by propagules of entomopathogenic fungi. The intersegmental membranes (Wraight *et al.*, 1990), areas under the elytra (Butt *et al.*, 1995) and the buccal cavity (Schabel, 1976) can be preferential sites of infection. Therefore, the location where the inoculum lands on the cuticle can also influence the probability of infection and the speed of kill. Consequently, targeting of the inoculum is an important consideration in the development of bioassay protocols.

Insect behaviour may affect ultimate sites of penetration. For instance, results of laboratory studies demonstrated that the most sensitive sites for penetration of *Beauveria brongniartii* on larvae of the cockchafer, *Melolontha melolontha*, were the mouth and anus (Delmas, 1973). However, Ferron (1978) found that in larvae of the same species collected in nature, the most frequent sites of infection occurred on the membranes between the head capsule and thorax or between the segments on appendages. This apparent contradiction is possibly due to the larval behaviour of burrowing

in soil; particles continuously scrape infectious inoculum off the exposed cuticle whereas the intersegmental membrane is protected from this mechanical action. This is a good example of how results obtained from laboratory bioassays must be treated with caution if used to predict the situation in the field.

Ecdysis and developmental stage

Not all stages in an insect's life cycle are equally susceptible to infection by entomogenous fungi. Pupal stages are often the most resistant stage, while adults can be the most susceptible. For instance, larvae of the thrips, *Frankliniella occidentalis* were found less susceptible to *V. lecanii* and *M. anisopliae* than adults, while later instars were less susceptible than earlier instars (Vestergaard *et al.*, 1995). Larvae of *Ostrinia nubilalis* were found to be most susceptible to infection by *B. bassiana* when exposed as first-instar larvae, while fourth instars were most tolerant (Feng *et al.*, 1985). Fransen *et al.* (1987) found that older instars of *Trialeurodes vaporariorum* were less susceptible to *Aschersonia aleyrodis*, while adults were seldom infected. Within adult stages, there could also be differences in susceptibility between different sexes and forms such as aphid alates and apterae (Oger and Latteur, 1985).

The time of inoculation prior to ecdysis, and the length of the intermoult period are important factors that may significantly affect bioassay results. Moulting may remove the penetrating fungus prior to the colonization of the insect, if it occurs shortly after inoculation (Vey and Fargues, 1977; Fargues and Rodriguez-Rueda, 1979). In contrast, Goettel (1988) found that larvae of the mosquito, *Aedes aegypti*, were more susceptible to *Tolypocladium cylindrosporum* during their moulting period.

Effect of diet on susceptibility

Successful infections are also dependent upon the host diet. For example, some insects maintained on artificial diet can be more susceptible to infection than insects fed a natural diet (Boucias *et al.*, 1984; Goettel *et al.*, 1993). Likewise, laboratory-reared insects can be more susceptible than field-collected ones (Bell and Hamalle, 1971). Insects which have been starved can also differ in their susceptibility compared with well-fed ones (Milner and Soper, 1981; Butt, unpublished observations).

Tritrophic interactions between host plants, insect pests and entomopathogens have been reported for fungi. The pathogenicity of the entomogenous fungus *B. bassiana* mediated by host plant species has been reported for both the Colorado potato beetle (Hare and Andreadis, 1983) and the chinch bug (Ramoska and Todd, 1985). Presumably, larvae growing

on more favourable plant species are better able to mount a successful defensive reaction to pathogens (Hare and Andreadis, 1983) or have a shorter intermoult period (see previous section). For chinch bugs, adults feeding on wheat or artificial diet and inoculated with *B. bassiana* demonstrated higher mortality and greater fungal development than adults feeding on maize or sorghum. These data were interpreted as showing that insects are benefiting from the fungistatic secondary chemicals in maize and sorghum (Ramoska and Todd, 1985).

An association has also been demonstrated between volatiles and fungal development. Crucifers contain glucosinolates, nitrogen- and sulphur-containing secondary metabolites. These are hydrolyzed by an enzyme to release biologically active compounds which, in addition to playing a major role in defending the plants from herbivores and fungal pathogens (Chew, 1988), also appear to interfere with the infection processes of insect-pathogenic fungi (Inyang *et al.*, 1999).

Sublethal Effects and Other Attributes

Measurement of the effectiveness of a pathogen against a host insect must be based on many factors, in addition to virulence. Not all insects treated with a fungus succumb to infection. Sublethal effects of entomopathogenic fungi have been insufficiently studied. It is usually presumed that those insects that do not succumb to infection do so at no expense. However, this is not necessarily so. For instance, Fargues *et al.* (1991) demonstrated that the fecundity of the Colorado potato beetle, *Leptinotarsa decemlineata*, surviving treatment was much lower than in beetles that were not treated. This study demonstrates that survival does not necessarily come without its price.

Many attributes of a pathogen are important in determining its ecological fitness. There exists a wide range of tolerance among fungal isolates to environmental factors such as sunlight (Fargues *et al.*, 1996) and temperature (Fargues *et al.*, 1997a, b) biotic attributes such as speed of germination (Papierok and Wilding, 1981) and ability to sporulate on the host cadaver (Hall, 1984). In addition to virulence, these are some of the important aspects that need to be considered when determining the effectiveness of an isolate for development as a microbial control agent. Determination of sublethal effects and other attributes is an important, yet much neglected area, which warrants further study.

Bioassay Procedures

Use of bioassay to assess the effects of entomopathogenic fungi in insects is essentially limitless. This, combined with the fact that there is a vast array of

entomopathogenic fungi with a great variety of hosts, means that there are no standardized bioassay methods as far as entomopathogenic fungi are concerned. Consequently, bioassays must be tailored according to host, pathogen and bioassay objective.

Bioassays can be used to determine and quantify host–pathogen relationships and the effect of biotic and abiotic parameters on these. Bioassays of entomopathogenic fungi have been used extensively in five important applications: (i) determination of virulence, (ii) comparison of virulence among isolates, (iii) determination of host range, (iv) determination of epizootic potential, and (v) studies on effects of biotic and abiotic factors such as host age, host plant, temperature, humidity and formulation.

The objective of a bioassay must be well defined before a bioassay protocol is adopted. Although bioassay procedures must be as efficacious as possible, they must also be designed to address the objectives and provide as meaningful results as possible to meet these. Choice, rearing and developmental stage of the host, infective propagule, formulation and inoculation method, conditions of post-inoculation incubation, method of mortality assessment (including mortality in controls), bioassay design and statistical analyses must all be carefully considered.

Special care must be taken when bioassays with non-target organisms (NTO) are used for risk assessment. It is common for entomopathogenic fungi to infect hosts in the laboratory which are never infected in the field. For instance, Hajek *et al.* (1996) demonstrated that data on host range of *Entomophaga maimaiga* from laboratory bioassays gave poor estimates for predicting non-target impact; the host range under field conditions was much narrower than that predicted from laboratory results. Laboratory assays demonstrated an LD_{50} of 2.2×10^5 conidia of *B. bassiana* per honey bee worker, whereas subsequent whole-hive exposures resulted in less than 1% mortality (M.R. Loeser, S.T. Jaronski, and J.M. Bromenshenk, cited in Goettel and Jaronski, 1997). The US Environmental Protection Agency (EPA) now accepts that infectivity tests with caged honey bees can be misleading and recommends that 30-day whole-hive tests be used instead (Goettel and Jaronski, 1997). Development of laboratory bioassays which better simulate the environment to which bees are exposed (e.g. internal hive temperatures are commonly held between 32 and 36°C) may provide a better and cheaper alternative to whole-hive assays.

Low mortalities in the field can be obtained even after application of highly virulent propagules. Inglis *et al.* (1997a) obtained low efficacy after applying conidia of *B. bassiana* on to native rangeland against grasshoppers, despite excellent targeting. Results of laboratory assays demonstrated high virulence, and high levels of infection were observed in caged field-collected grasshoppers maintained under glasshouse conditions with similar temperature and humidity to those experienced in the field. Subsequent studies revealed the importance of thermoregulation; grasshoppers bask in the sun, elevating their temperature to levels that prevent disease progress

(Inglis *et al.*, 1996b, 1997b). The development of a bioassay design whereby grasshoppers were allowed to thermoregulate allowed for more meaningful prediction of virulence under field conditions (Inglis *et al.,* 1996b, 1997b, c).

In bioassays designed to predict performance of the pathogen under field conditions, as many pertinent environmental (e.g. temperature, photoperiod) and other (e.g. inoculation method) parameters as possible must be taken into account and incorporated into the bioassay design. Unfortunately most bioassays are performed under static conditions (e.g. constant temperature, RH, photoperiod). Although such assays may be useful in comparing activity of different isolates, they often provide misleading information as far as performance under field conditions is concerned. However, bioassays performed under constant conditions studying single parameters can be very useful in identifying the pertinent parameters that need to be considered.

All bioassays should include a non-treatment control in order to monitor survival of insects under the post-inoculation incubation conditions. Such control insects should be treated with a carrier used for application of the inoculum. In dose–mortality assays, control mortality is then corrected for in the statistical analyses (Chapter 7). If bioassays are used for host range or safety to non-target organisms, it is imperative that a known susceptible host is also treated in parallel with the non-target organisms (i.e. positive control). Otherwise negative results are difficult to interpret unless evidence is provided regarding the virulence of the inoculum against a susceptible host under the same bioassay conditions.

Choice of sample size and range of doses is usually difficult when dealing with fungal pathogens due to the great variability in responses between different isolates and hosts. For dose–mortality assays, preliminary bioassays should be first conducted using a wide range of doses and relatively small numbers of hosts. A range of doses that would result in mortalities between 25 and 75% should then be chosen. The choice of sample size may be more problematic and will depend very much on the pathogen–host system. For instance, Oger and Latteur (1985) determined that, in bioassays of *Erynia neoaphidis* against the pea aphid, *Acyrthosiphon pisum*, the factor that most affected precision was the number of replicated assays. They found that a sample size of ten aphids for each of 10–20 doses replicated three or four times gave an adequate precision for comparative assays. However, more commonly, five doses should suffice, especially if at least three or four of the doses fall in the 25–75% mortality range. As in any scientific study, the whole bioassay must be repeated at a later date, preferably using another batch of insects and inoculum preparation in order to ensure reproducibility of results and thereby substantiate the conclusions. More discussion on choice of doses, sample size, bioassay design and repetition of experiments is presented in Chapter 7.

Inoculation

Method of inoculation is influenced primarily by the form of the inoculum and the size and fragility of the insect. Inoculum is most commonly administered to the surface of the cuticle either through direct methods such as dusting, dipping or spraying or through indirect methods such as the use of baits. Whatever the inoculation method, it is imperative that the viability of the inoculum be determined as close to the treatment time as possible. Otherwise, it is not possible to determine if lack of efficacy is due to low viability of the inoculum. If at all possible, viability assessments of the formulated product should be made. It may be necessary to compare viabilities between the active ingredient and formulated product in order to determine if the formulation adjuvants have a detrimental effect. Methods for viability assessments are summarized by Goettel and Inglis (1997).

Entomophthoralean fungi differ from hyphomycete fungi in several characteristics relevant to the development of bioassays. The former usually produce comparatively few, large, forcibly discharged, sticky conidia. The latter generally produce numerous, small, dry conidia. The methods for inoculating insects with entomophthoralean fungi are usually limited to either showering conidia on to anaesthetized insects or the host's food source such as leaf surfaces. The inoculum may be showered from mycosed cadavers, sporulating cultures or marcescent mycelium (Papierok and Hajek, 1997). In contrast, inoculum of hyphomycete fungi may be applied by the methods noted above.

The most important factor in choosing an inoculation method is to ensure presentation of a precise dose which will reduce variability and help ensure repeatable results. Consequently, crude methods of inoculation such as allowing an insect to walk on the surface of a sporulating culture should be avoided, although such methods may be useful in certain studies whose aims are, for instance, to establish new host records *per se*. It is preferable, however, if the amount of inoculum administered to each insect can be controlled as precisely as possible. This is usually accomplished through enumeration of the inoculum and administering a precise dose to each insect. In situations where it is difficult or impractical to determine the precise dose being administered, it is common practice to obtain estimates of the dose by recovering the propagules after application of the inoculum, either through washing or homogenizing the insects, and then estimating propagule concentrations or through direct enumeration or spread plating (Goettel and Inglis, 1997). Details on methods for enumeration of propagules are presented by Goettel and Inglis (1997) for Hyphomycetes, Kerwin and Petersen (1997) for water moulds, and Papierok and Hajek (1997) for Entomophthorales.

Introduction of inoculum through injection can be used in situations where the importance of the cuticular barrier is not an issue (i.e. immunological assays). A tuberculin syringe attached to a motorized microinjector is

usually used to treat many insects rapidly and effectively. The inoculum can be introduced *per os* or directly into the haemocoel by piercing the intersegmental membrane. Aquatic insects are usually treated by introducing known numbers of propagules into their rearing medium. Specific methods of inoculation are described in the examples presented below.

Bioassay chambers

A wide range of bioassay chambers has been used by various workers for disparate insect species. Bioassay chambers are usually chosen according to availability, price, convenience, ease of cleaning and requirements of the host. With entomopathogens, it is important that the chambers be adequately decontaminated prior to reuse. An alternative is to use disposable containers. Some commonly used bioassay chambers include inexpensive plastic or polystyrene coffee cups, ice cream cartons, cigar boxes, glass jars, plastic bins, buckets or bowls, portable cages, and nylon/cotton fine-mesh sleeves.

For assays with small insects, it is often possible to use single leaf petioles or excised leaves in small bioassay chambers such as Petri dishes. In such systems, it is important to delay leaf senescence as long as possible, by providing a nutrient or water source for the plant tissue. For instance, stems of single leaf petioles can be immersed in water (Fig. 4.1), kept wet with moistened cotton wool placed on parafilm (Mesquita *et al.*, 1996; Fig. 4.2) or placed directly on to a nutritive substrate. Vidal *et al.* (1997) cut out 3.5-cm diameter discs from ornamental sweet-potato leaves, disinfected them in a series of alcohol and sodium hypochlorite solutions, and placed them in small Petri plates containing a KNOP medium (in g l^{-1} water: 0.25 KCl, 0.25 KH_2PO_4, 0.25 $MgSO_4$, 0.02 $FeSO_4$, 10 agar) (Fig. 4.3).

Choice of bioassay chamber is critical in field-cage bioassays. Although these best 'mimic' field situations, the type of bioassay chamber can greatly influence the climate within. Even screened cages provide shading and protection from wind. In a field-cage experiment using screened cages, Inglis *et al.* (1997b) found minimal differences in temperature and relative humidity within and outside the cages, but there was approximately 55% shading within the cage due to the mesh screening.

Post-inoculation incubation conditions

Conditions of humidity, temperature and light can greatly influence bioassay results. Consequently, after inoculation, the insects should be incubated under controlled environmental conditions or transferred to field bioassay cages. Controlled environmental conditions are usually maintained using

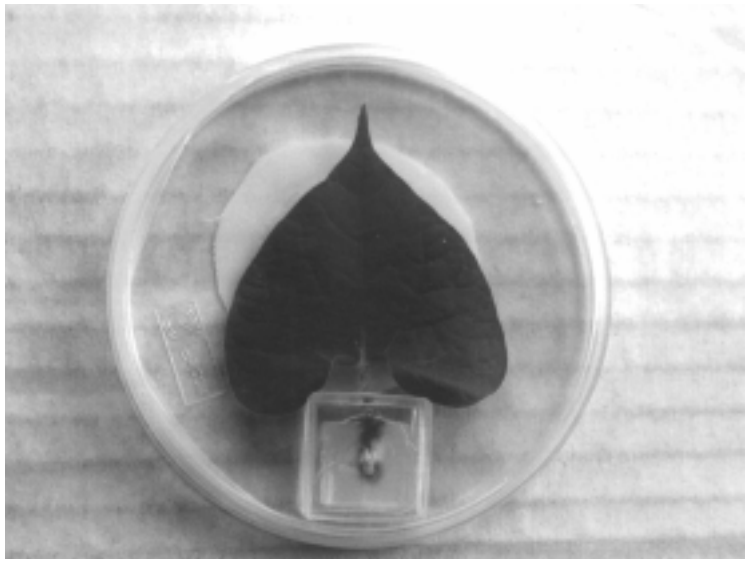

Fig. 4.1. Bioassay chamber containing single leaf with its water supply. Photo by courtesy of Lerry Lacey.

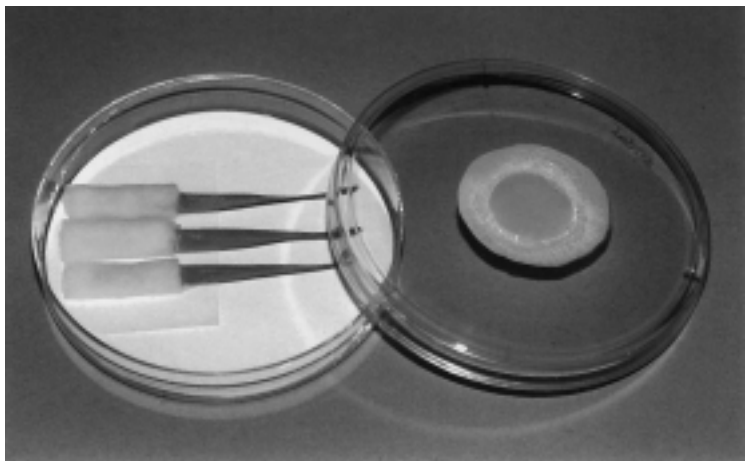

Fig. 4.2. Ventilated bioassay chamber containing single blades of barley leaves. Moisture is provided by soaked cotton battens placed on pieces of Parafilm. Photo by courtesy of Antonio Mesquita and Lerry Lacey.

environment chambers or incubators. Insects can often be pooled in assay chambers according to treatment group; however, it is preferable to incubate cannibalistic insects such as grasshoppers singly. The conditions chosen will vary according to the objectives of the bioassay, but they generally should be favourable for survival of non-inoculated insects.

Fig. 4.3. Ventilated bioassay chamber containing an excised sweet-potato leaf disc placed on a nutritive agar medium. Photo by courtesy of Claire Vidal and Lerry Lacey.

Bioassays must often be run for 1–2 weeks or even longer with slower-acting pathogens such as fungi, where LT_{50}s of 4–6 days are common. With such long time spans, control mortalities can often be problematic. Meaningless results are obtained if control mortalities are too high. Control mortalities are usually accounted for in the statistical analyses (Chapter 7), but as a general rule, results must be suspect if control mortalities are higher than 15–20%. If control mortalities greater than 20% are unavoidable, results must be interpreted with caution.

For some fungi, humidities approaching saturation must be maintained in order to obtain infection (Papierok and Hajek, 1997). Saturated and near-saturated conditions are usually provided using water agar or saturated filter paper within the bioassay chambers. Precise conditions of humidity can be maintained in the bioassay chambers by continuously circulating air through a humidifying medium of saturated salt solution (Fargues *et al.*, 1997b). Sealed chambers without air circulation should be avoided as the aerial humidity occurs at equilibrium only at the solution/air interface.

Temperature is one of the most easily controlled factors. Most bioassays comparing virulence among isolates use only one constant temperature, however, the results obtained could provide misleading information. For instance, Fargues *et al.* (1997b) compared the virulence of four isolates of *M. flavoviride* at four constant temperatures. No significant differences in virulence occurred among these isolates at temperatures of 25, 30 and 40°C. In contrast, there were significant differences in virulence among isolates at 35°C. This demonstrates the importance of using several temperatures when

screening for the most virulent isolates. Cyclical conditions of temperature approximating as much as possible the natural conditions should be considered for such screenings.

Mortality assessments

Mortality assessments should be made daily. In addition to computing median lethal doses, this allows computation of median lethal times, which can be very useful in making comparisons between different treatments (Chapter 7). If insects are not being incubated singly, dead insects must be removed as soon as possible and certainly prior to sporulation to prevent horizontal transmission and loss due to cannibalism. Incubation time varies according to the insect and fungus being evaluated. Generally, incubation should continue as long as insects continue to succumb to the pathogen.

Mycosis is usually verified by incubating dead insects at high RH (e.g. in Petri dishes containing moistened filter paper or water agar) to allow for fungal colonization and sporulation on the cadaver. It is important to also incubate cadavers from control insects to determine residual infection levels or if accidental contamination occurred.

Some Examples

Here we provide specific examples to illustrate some of the many different bioassay methods used with an array of fungal pathogens and insect hosts. It is not our intent to provide a detailed evaluation or critique on the methods used. Each bioassay needs to be adapted to the specific needs of the host/pathogen combination and according to the objectives of the bioassays themselves. We have divided the sections according to inoculation method.

Spray

Spray bioassays are used extensively, especially against small and fragile insects that are otherwise difficult to treat. Although sophisticated stationary and track sprayers are available for this purpose, simpler and less expensive systems can be developed using an artist's air-brush. Drop size and distribution must be carefully monitored.

Honey bees with M. flavoviride

As mentioned previously, laboratory assays for testing safety against honey bees can provide misleading results (Goettel and Jaronski, 1997). The assay

presented here was used to determine the safety of an oil formulation of *Metarhizium flavoviride* to adult honey bee workers (Ball *et al*., 1994). Similar protocols were used by Vandenberg (1990) and Butt *et al*. (1994) to test the safety of *B. bassiana* and *M. anisopliae* to honey bees.

1. Combs containing mature honey bee pupae were removed from colonies and maintained overnight in an incubator at 35°C. Groups of newly emerged bees (less than 18 h old) were transferred to small cages, supplied with concentrated sucrose, water and pollen, and maintained at 30°C for 1 week before use.
2. For the application of conidial suspensions, bees were briefly anaesthetized with carbon dioxide and transferred to spray cages made from 12.5 cm square perspex 6 mm thick with a hole 10 cm in diameter in the centre. The bees were sandwiched between two layers of 0.71 mm galvanized wire mesh with an aperture of 2.46 mm, 25 bees to each cage.
3. The bees were allowed to recover at room temperature and oil formulations of conidia were applied from a rotary atomizer attached to a track sprayer in a room maintained at 30°C. Sprays were calibrated to simulate field dose levels equivalent to twice and 20-fold expected field application rates. A solution of fluorescent tracer Uvitex OB (Ciba-Geigy) in Ondina oil was used to determine the volume of formulation deposited on the bees. The controls consisted of six cages of bees which were not sprayed and eight cages of bees sprayed with the oil carrier alone. Locust positive controls were also treated in parallel.
4. Immediately after treatment, bees were returned to their original cages without anaesthetization and maintained at 30°C. Bees that died within 24 h of transfer were omitted from the assay.
5. Cages were checked daily for 14 days and dead bees removed and incubated at room temperature on moist filter paper in plastic Petri dishes. Conidia of the fungus appeared within a few days over the surface of infected individuals.
6. A field dose killed 11% of the bees, twice field dose killed 30%, while a 20-fold dose killed 87%.

Whiteflies with Aschersonia aleyrodis

Spore suspensions are often sprayed directly on to leaves containing the host. The assay presented here was used by Fransen *et al*. (1987) to study differential mortality of different life stages of the glasshouse whitefly, *Trialeurodes vaporariorum* treated with conidia of *Aschersonia aleyrodis*. Difficulties with this protocol can be encountered when used with highly mobile insects, as the insects may differentially pick up inoculum post-application depending on their mobility.

1. Spores of *A. aleyrodis* were obtained from a 3-week culture grown on

coarse cornflour at 25°C. Spores were suspended in sterile distilled water and enumerated in a counting chamber.
2. Two millilitres containing 4×10^6 spores ml^{-1} were applied with a Potter spray tower at 34.5 kPa to the underside of a cucumber leaf bearing the whiteflies. Ten leaves per age class were treated with the spore suspension and two leaves were treated with distilled water as a control.
3. Spore viability was determined by spraying a spore suspension on to a water agar plate and counting the number of germlings 24 h after spraying at 25°C.
4. After the water had evaporated from the leaves, plants were covered with plastic bags to ensure saturated moisture conditions for the first 24 h at 20°C and 16 h photoperiod. Thereafter, the bags were removed and the plants were kept at 70 ± 10% RH.
5. Adult whiteflies had to be anaesthetized before inoculation, but difficulties were encountered. If adults did not revive quickly, they drowned in the spore suspension. To alleviate this problem, adults were exposed directly to spores on the surface of a sporulating culture for 24 h prior to transfer to leaves in clip cages.
6. Disease progress was recorded at 3- to 4-day intervals until 90% of the survivors had developed into adults.
7. It was found that older instars were less susceptible and that adults were seldom infected.

European corn borer with B. bassiana

Larger insects are often sprayed directly and then transferred to rearing containers. Using the bioassay method described here, Feng *et al.* (1985) determined age-specific dose–mortality effects of *B. bassiana* on the European corn borer (ECB), *Ostrinia nubilalis*.

1. Larvae were collected from overwintering sites (field corn stubble), transferred to a meridic diet and maintained for at least three generations to eliminate weak and diseased individuals.
2. Three isolates of *B. bassiana* obtained from a culture collection were first passaged through ECB larvae. Conidial suspensions were tower-sprayed on to larvae and incubated at 26°C. Conidia were then harvested from cadavers and inoculated on to Sabouraud dextrose agar (SDA). After incubation at 26°C for 20 days, conidia were harvested, dried and stored in vials at 4°C until use.
3. Spore suspensions were prepared and applied to groups of 20 newly moulted larvae of each instar in individual Petri dishes using a spray tower. Dose was assessed by spraying SDA Petri plates and counting colony-forming units (CFU) cm^{-2} that developed. Five doses of between 9.3×10^2 and 2.9×10^6 CFU cm^{-2} were applied for each isolate.

4. The Petri dishes in which the larvae were sprayed were covered. Larvae were fed an artificial diet with all fungicides removed, and incubated at 26°C for 24 h, after which time they were transferred to uncontaminated diet without fungicides and incubated for a further 24 h. After 48 h, standard artificial diet was provided. The larvae were examined daily for mortality.
5. The experiment was replicated four or five times using different batches of larvae and new conidial suspensions.
6. First-instar larvae were found most susceptible, there was little difference between 2nd, 3rd and 5th instars while 4th instars were most tolerant.

Immersion

Insects are often dosed by immersion into suspensions of spores for a specified time. Although this is usually a quick and convenient dosing method, precise measurement of dose is difficult. In order to ensure that each insect from a treatment group receives similar doses, groups of insects should be simultaneously dipped. Care must be taken that each insect remains precisely the same amount of time in the dipping suspension. If insects are dosed singly, then a separate suspension should be prepared for each insect. Otherwise, each subsequent insect dipped could receive less inoculum, especially since conidia of many entomogenous fungi are hydrophobic and therefore adhere preferentially to the insect cuticle.

Aphids with Verticillium lecanii

The difficulty of immersing small insects for a specified time can be overcome by draining off the inoculum rather than dipping the insects into the inoculum *per se*. This method was used by Hall (1976) to bioassay *Verticillium lecanii* against the aphid, *Macrosiphoniella sanborni* (see below). It can be adapted for use against almost any insect which can withstand submergence for a short period. However, this method is difficult to use when attempting to study effects of different carriers and formulations, as the inoculation method does not represent that which would be expected under operational conditions.

This method has been adopted for use with many insects and fungi. Some examples include bioassay of *B. bassiana* against larvae of the curculionid weevils in the genera *Sitona*, *Hylobius*, *Diaprepes*, *Chalcodermus* and *Pachnaeus* (McCoy *et al.*, 1985) and bioassays of *M. anisopliae* against beetle and aphid crucifer pests (Butt *et al.*, 1992, 1994).

1. Conidia were obtained from a single spore isolate of *V. lecanii* grown on SDA at 23°C for 7 days and then stored at −17°C. Conidia for bioassay were

produced by spread plating a spore suspension obtained from storage on to SDA and incubating at 23°C. After 7 days, spores were harvested using a bent glass rod and phosphate buffer containing 0.02% Triton X100 as a wetting agent. The spore suspension was purified by filtering through cheesecloth, centrifuging and washing four times in phosphate buffer. The concentration of spores was determined using a haemocytometer. A viability assessment was performed by incubating three drops of a suspension containing approximately 10^6 spores ml^{-1} on a thin layer of sterile SDA on a glass slide and incubating for 12 h at 23°C before examining using phase-contrast microscopy.

2. Adult alate aphids, obtained from a stock culture maintained on potted chrysanthemum plants, were transferred to chrysanthemum leaf discs in breeding cells. After 7 days, apterous progeny from the alatae were transferred to fresh leaf discs and were used for bioassay 8 days later.

3. Batches of mature aphids were placed in glass Petri dishes. Each batch was transferred on to filter paper in a 7.5-cm diameter Büchner funnel. Thirty millilitres of spore suspension were then gently poured over the aphids. After 2 s, the suspension was quickly drained by suction.

4. After inoculation, treated insects were placed singly on leaf discs in high humidity bioassay chambers, incubated at 20°C for 6 days, and examined daily for mortality. Dead insects were examined microscopically for signs of mycosis.

5. The $LC_{50}$was found to be 2.3×10^5 spores ml^{-1}. It was noted that aphids tolerated transfer to the assay cells much better if preconditioned in breeding cells than direct transfer from plants.

Dusting

Dusting is sometimes used to inoculate insects. Whereas some workers literally dust the insects others may simply allow healthy insects to walk over a sporulating culture (Bidochka *et al.*, 1993). Dusting allows inoculation of large numbers of insects at once. Care must be taken to ensure that the insects can withstand this procedure. If at all possible, attempts should be made to quantify the amount of inoculum received by each insect. Because it may be possible that death could be caused by suffocation due to obstruction of tracheal passages, controls should consist of killed spores plus the carrier. The great variance in dosage acquired should be noted from the example below. For this reason, generally, this method should be avoided for dose–mortality assays unless precautions are taken to ensure that the variation in the amount of dose administered within a dosage group is minimized.

Chinch bug with B. bassiana

Ramoska and Todd (1985) used a dusting method to study the effects of host plant on virulence of *B. bassiana* towards the chinch bug, *Blissus leucopterus leucopterus*. Although dose–mortality assays were not used, dosage levels received by each insect were estimated.

1. A culture of *B. bassiana* was grown on Sabouraud maltose agar for 3 weeks at 27°C. Conidia were harvested, dried, sieved and stored at 4°C until used.
2. Chinch bugs were inoculated by placing 25 adults at a time in a Petri dish containing dry conidia. Petri plates were then shaken to ensure full coverage of the insects. Insects were then removed to incubation chambers.
3. In order to quantify dose levels, two insects from each batch were removed and immersed in 10 ml of 5% v/v aqueous Tween 20 and vigorously agitated. Conidial density in the suspension was calculated using a haemocytometer. Dosage levels ranged from 0.9×10^5 to 1.8×10^8 conidia per insect.
4. Inoculated bugs were transferred on to host plant seedlings and a 43-cm, ventilated, clear plastic collar was placed over the plant which served to cage the insects. Twenty-five insects were placed into each of four replicate chambers.
5. After 2 weeks, the test chambers were dismantled and mortality was assessed. Dead bugs were transferred to Petri dishes containing moist paper towelling to assess fungal growth on the cadavers.
6. Results showed that feeding on sorghum and maize resulted in greater tolerance to the fungus compared with insects feeding on other food sources.

Japanese beetle with B. bassiana *and* M. anisopliae

Although dusting is generally not recommended for dose–mortality assays due to the high variability of inoculum received by insects in a dosage group, as seen in the example above, this inoculation method has been used successfully in dose–mortality assays with some insects. In the example presented here, Lacey *et al.* (1994) determined LT_{50} and LC_{50} estimates for isolates of *B. bassiana* and *M. anisopliae* in adults of the Japanese beetle, *Popillia japonica*.

1. One isolate each of *B. bassiana* and *M. anisopliae* was cultured on SDA, harvested with a rubber spatula, dried in an incubator overnight at 30°C, and then passed through a 250 µm sieve. Spore viability was determined by plating 100 µl of the conidial suspensions on SDA and counting the number of colonies formed after 48 h. Spore counts were estimated as 2×10^7 conidia mg^{-1} for *B. bassiana* and 3.6×10^7 conidia mg^{-1} for *M. anisopliae*.

2. Adult Japanese beetles were field-collected using baited traps and held briefly in the laboratory prior to treatment. One hundred individuals were counted out into 140-ml polystyrene cups, which were closed with perforated screw caps.
3. Five dosages ranging from 0.5 to 10 mg conidia were weighed out and added to the cups containing the adults. In a later modification of the procedure, lower dosages were used in which talcum powder at a ratio of 990 mg to 10 mg conidia was added as a carrier. Five replicate cups were prepared for each dosage. The cups were periodically rotated end-over-end for a 1-h period to help distribute the conidia.
4. Thirty beetles from each treatment were then removed and divided among three holding cages, which consisted of 950-ml plastic containers with perforated lids. Water and humidity was provided by dental wicks which protruded through the bottom of the cage into 100 ml reservoirs below. Adults were incubated at 22–24°C, checked for mortality, and provided with fresh blackberry leaves daily for up to 8 days. Four replicate tests were conducted on each of four separate dates.
5. Dead beetles were transferred to 950-ml plastic tubs containing 500 g moistened sterilized soil, incubated at 22–24°C for 1 week and then were examined for fungal outgrowth.
6. A dose–mortality response was obtained with LC_{50} estimates of 0.7 mg conidia per 100 adults for *M. anisopliae* and 0.026 mg conidia per 100 adults for *B. bassiana*.

Direct deposition on to individual insects

A precise droplet of inoculum can be placed directly on to the surface of the insect. This method can be used with larger insects that can tolerate handling. Often, some form of immobilization of the insect is required. Due to the hydrophobic nature of the cuticle, it is sometimes difficult to administer a drop of aqueous inoculum precisely. In such cases, it may be helpful to choose an inoculation site that would absorb the droplet by capillarity.

Cocoa weevil with B. bassiana

Prior *et al.* (1988) used the direct deposition method to compare the virulence of water and oil formulations of *B. bassiana* against the cocoa weevil pest, *Pantorhytes plutus*. Other examples of direct deposition bioassay include studies with *M. anisopliae* against flea beetles (Butt *et al.*, 1995).

1. *B. bassiana* was cultured for 2 weeks at 28°C on 2% malt agar or on autoclaved brown rice or oat grain in 250-ml conical flasks. Formulations were prepared by adding 100 ml of either filtered coconut oil or distilled

water with 0.01 ml of Tween 80 into the cereal cultures, stirring and filtering through a metal strainer. Serial dilutions were then made as necessary and conidial concentrations determined using a haemocytometer. Conidial viability was verified by streaking on to malt agar plates.
2. Adult weevils were field-collected, individually secured by pressing the dorsal abdomen lightly on to Blue tak® adhesive, and inoculated by applying 1 µl to the mouthparts. A Hamilton syringe was used to apply the oil formulation and an Agla microsyringe for the water formulation. For the water formulation, it was necessary to retain the insects secured until the drop of inoculum had dried. Otherwise the drop would run off.
3. Inoculated insects were transferred to 1-l plastic containers and fed every 3–4 days with pieces of cocoa stem. Mortality was checked daily and dead insects were transferred to plastic cups containing damp tissue for verification of fungal outgrowth. Only insects that showed visible outgrowth of the fungus were included in the analyses to determine the LD_{50}.
4. The oil formulation was found to be much more effective than the water formulation. LD_{50} estimates were 1.2×10^3 conidia per insect for the oil formulation and 4.3×10^4 for the water formulation.

Subterranean termite with B. bassiana *and* M. anisopliae

Lai *et al.* (1982) used the direct drop deposition method in bioassays to determine the virulence of six isolates of entomogenous fungi to the subterranean termite, *Coptotermes formosanus*.

1. Cultures of the fungi were kept on SDAY (SDA with yeast). Virulence was maintained by passage through termites every 3 months. For bioassay, 0.1 g of spores were scraped off 20-day-old culture plates. Conidia were then suspended in 10 ml of 0.1% Tween 80 to a final dilution of 1:100 using a magnetic stirrer. The suspension was filtered through two layers of Kimwipes®. A 0.5 µl aliquot of the spore suspension was placed on a microscope slide and the number of conidia in this drop counted under a phase-contrast microscope (Ko *et al.*, 1973). Dilutions were then performed as required.
2. Foraging termite workers were obtained from a field colony. One hundred workers were weighed in groups of ten in glass vials and the mean body weight was used to determine the dosage as expressed by the number of conidia per mg body weight.
3. Termites were anaesthetized with CO_2 for 10 s then transferred to a 100 × 20 mm Petri dish lined with filter paper.
4. A Hamilton microsyringe was used to apply 0.5 µl inoculum to the surface of the prothoracic area. This volume was enough to cover the insect without runoff. Mortality was reduced by avoiding direct contact with the syringe on the termite.

5. Insects were kept in inoculation chambers at 25°C and 56% RH with the filter paper and applicator sticks changed every 4 days to prevent secondary infection by saprophytes such as *Rhizopus* spp.
6. Thirty foraging workers were treated at each dosage level and caged in groups of ten. Three groups constituted a single replication and the experiment was repeated three times. Control groups were inoculated with the carrier alone.
7. Inoculated termites were incubated at room temperature for 15 days. Mortality data at 12 days was used for the probit analysis as control mortalities drastically increased thereafter. Samples of dead termites were homogenized on a microscope slide and examined for the presence of hyphal bodies.
8. Estimates of LD_{50} and LT_{50} revealed differences in virulence among isolates. Overall, isolates of *M. anisopliae* appeared more virulent than those of *B. bassiana*.

Inoculation of soil

Insects which either inhabit or are associated with soil during a part of their life cycle can be exposed to inoculum contained on the surface or within the soil. Soil texture, humidity and microbial flora can affect conidial viability and virulence and need to be considered. A discussion of procedures and precautions is presented by Goettel and Inglis (1997).

Pecan weevil with B. bassiana

Champlin *et al.* (1981) applied an aqueous suspension of *B. bassiana* conidia to the surface of soil to compare virulence of mutants against the pecan weevil, *Curculio caryae*.

1. Five *B. bassiana* mutants were obtained by ultraviolet irradiation of a wild-type culture of *B. bassiana*. Conidia from 14- to 21-day-old cultures grown on SDA + 3% yeast extract were obtained by washing with sterile 0.03% Triton X-100. Conidia were washed twice in sterile distilled water and the concentration estimated spectrophotometrically at 540 nm. Concentrations were determined by plating appropriate dilutions on SDA and counting CFUs.
2. Large plastic cups (14.5 cm deep, 11.5 cm diameter) containing autoclaved soil–sand mixture (10 : 1) were inoculated with 10 ml of spore suspension distributed over the entire 95 cm^2 surface in a dropwise manner using a pipette. After allowing the solution to be absorbed into the soil (to an estimated depth of *c.* 1.3 cm) 25 4th-instar larvae of field-collected pecan weevils were allowed to burrow down into the soil in each cup. Four dilutions of each mutant were prepared.

3. Cups were covered with Parafilm in which ten holes were punched, and incubated at 25°C. After 7 days, 5 ml of sterile water was added to each cup to maintain moisture. Two to three replicate treatments were performed for each mutant strain. Mortality was assessed 21 days post-inoculation. The percentage of insects that were mummified was used in the LC_{50} assessments.
4. The mutants exhibited different degrees of virulence with LC_{50}s ranging from 9.7×10^6 to 1.0×10^9 conidia ml^{-1}.

Ovipositing grasshoppers with B. bassiana

Conidia can be incorporated directly into the soil to bioassay virulence against insects which burrow or oviposit into soil (Fig. 4.4). Inglis *et al.* (1995a, 1998) used this method to determine the effects of conidial concentration, soil texture and soil sterilization on virulence of *B. bassiana* to ovipositing adults and emerging nymphs.

1. Conidia of *B. bassiana* were obtained commercially. Numbers of conidia g^{-1} were determined using serial dilutions in water and a haemocytometer. Conidia were mixed uniformly into autoclaved sand at a concentration of 10^8 conidia g^{-1} dry-weight sand. Water was added to obtain a water content of 9.2% (w/w) and 880 g of sand was added into each of three plastic containers ($10 \times 10 \times 7$ cm) per treatment. A 5 mm layer of dry sterile sand

Fig. 4.4. Grasshopper ovipositing into *B. bassiana*-augmented sand. Photo by courtesy of Doug Inglis.

was then placed on the surface. The moisture level within each cup was monitored daily by weighing and readjusted to 9.2% as necessary.

2. Two cores of sand were removed from each container using a 3.5-mm diameter cork borer. The sand was vortexed for 30 s at high speed in 10 ml of 0.05% Tween 80 in phosphate buffer. The suspension was diluted and spread on to a selective medium. The number of CFU was then determined after incubation in the dark at 25°C for 5–6 days.

3. A minimum of 30 virgin females and 20 virgin males of a non-diapausing laboratory strain of *Melanoplus sanguinipes* were placed into cages, 40 cm square. The cages had holes in the bottom so that the containers of the inoculated soil could be inserted with the surface of the sand being level with the cage bottom. The cages were maintained at a 25/20°C day/night temperature regime with a 16:8 h (light:dark) photoperiod.

4. Adults copulated and the pronota of the first seven females per cage to oviposit were marked with paint. The duration of each oviposition period was recorded, and upon completion, these adults were sacrificed, and the extent of abdominal infestation with conidia was quantified by excising the abdomens, sealing the cut end with molten Parafilm, and washing in 5 ml of buffer in 20 ml vials, vigorously shaken at 300 rpm for 2 h on a rotary shaker. The spore suspension was diluted, plated on selective medium and the number of CFU determined.

5. The remaining adults were maintained within the cages on a diet of bran and wheat leaves. At the end of 7 days, containers of sand were replaced with freshly inoculated sand for a further 7 days. At the time of removal, populations of conidia within the sand were assessed as described previously. Dead insects were removed daily and cadavers were surface sterilized in 0.5% sodium hypochlorite with 0.1% Tween 80 followed by two rinses in sterile water. The presence of hyphal bodies in the haemolymph or outgrowth of *B. bassiana* in cadavers held under moist conditions was recorded.

6. Each egg container was incubated at 25/20°C day/night with a 16:8 h (light:dark) photoperiod and nymphal emergence was recorded daily. At the time of first nymphal emergence, densities of viable conidia were enumerated as previously described. Ten newly emerged nymphs per replicate were collected and anaesthetized in CO_2. Nymphs were homogenized and the CFU were determined on a selective medium.

7. Remaining nymphs were maintained in cages on a diet of wheat seedlings for a period of 10 days. Dead nymphs were removed daily and placed on moistened filter paper within Petri plates. After 10 days, egg pods were sifted from the sand and the number of unhatched eggs per female was determined.

8. All experiments were repeated and analysed as completely randomized designs. Extensive mortality attributed to the fungus occurred in ovipositing females, associated males and in emergent nymphs.

Inoculation through contact with contaminated substrates

Insects are sometimes inoculated by allowing them to walk over the surface of a substrate, such as filter paper, which was pretreated with a known concentration of inoculum. Although such methods are an improvement over allowing insects to walk over sporulating cultures, there are still difficulties in ensuring that each insect receives precise and equitable doses. For instance, when using this method, it would be expected that the more mobile insects would acquire more propagules than the less active individuals. Nevertheless, this inoculation method may have utility in some situations.

Aphids with M. anisopliae

Butt *et al.* (1994) used spore-impregnated filter paper to assay *M. anisopliae* against *Lipaphis erysimi* and *Myzus persicae*. Similar methods have also been used to assay pathogens against thrips (Butt, unpublished) and corn earworm, *Heliothis zea* larvae (Champlin *et al.*, 1981).

1. Conidia from 8–12-day-old sporulating cultures of two *M. anisopliae* isolates were harvested in a 0.03% solution of Tween 80 and diluted to the desired concentrations.
2. *Myzus persicae* and *Lipaphis erysimi* were placed for 15 s on filter paper impregnated with conidia by vacuum filtration of a 10 ml conidial suspension of 1×10^7 or 1×10^{10} conidia ml^{-1}. Aphids were then transferred to a healthy Chinese cabbage leaf in a ventilated perspex box ($5.5 \times 11.5 \times 17.5$ cm) lined with moist tissue paper. Control insects were treated similarly with 0.03% Tween 80. The aphids were incubated at 23°C in a 16:8 h (light:dark) photoperiod and humid conditions were maintained for the first 24 h by placing the boxes between wet paper towels.
3. Mortality of both *M. persicae* and *L. erysimi* was 100% within 4 days post-inoculation at 1×10^7 or 1×10^{10} conidia ml^{-1} with little or no control mortality (0–3%). The earliest deaths were recorded on the first day after inoculation and sporulation occurred 1 to 2 days after death. Young, healthy aphids which contacted mycosed insects also succumbed to the *M. anisopliae* isolates.

Bait

Inoculum can be incorporated directly into the diet and presented to the insects as a bait. Although this method is most often used with fungi that infect through the gut (e.g. *Ascosphaera aggregata*), it can also be used as

a method for inoculation of fungi that invade through the external integument. While feeding, insects contaminate their mouthparts and body with the pathogen propagules.

Leaf-cutting bees with Ascosphaera aggregata

Ascosphaera aggregata is one of the few species of entomogenous fungi which infects the host through the gut. Consequently, a bioassay method has been developed whereby the inoculum is introduced on an artificial diet to study the susceptibility of different ages of larvae of leaf-cutting bees, *Megachile rotundata*, to this fungus (Vandenberg, 1992). In a later study, a similar bioassay technique was used to demonstrate that larval susceptibility was much reduced when larvae were fed a natural diet (Goettel *et al*., 1993; Fig. 4.5). This study demonstrates the importance in choice of diet if results are used to predict events under natural conditions.

1. A pollen/agar-based diet was prepared and dispensed asceptically into wells of sterile flexible microtitre plates. Sections of 16 wells were cut and placed in 60 × 15-mm sterile plastic Petri dishes. Eggs were obtained from field-collected bee cells and were transferred to the sterile diet.
2. Ascospores were obtained by scraping field-collected cadavers and stored at −20°C. Inoculum was prepared by suspending spores in sterile buffer and grinding between two microscope slides to break up the spore balls.

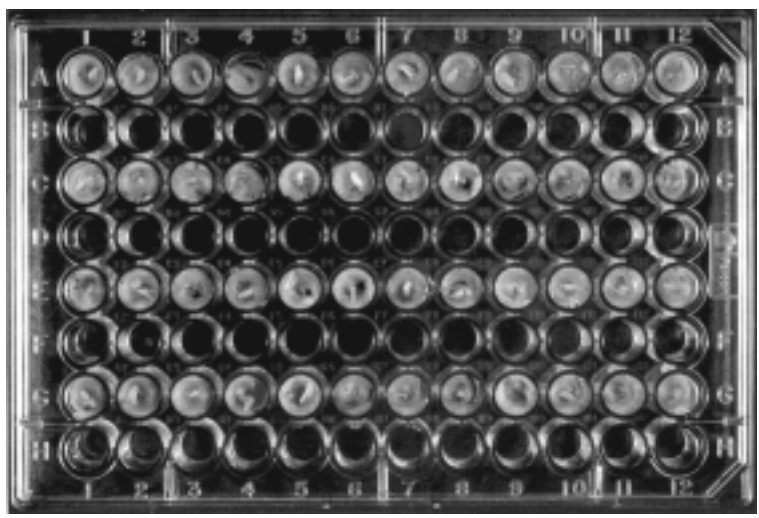

Fig. 4.5. Ninety-six well microtitre plate used for bioassay with leaf-cutting bees. Artificial media are separated with empty wells to help prevent possible cross-contamination. Photo by courtesy of Grant Duke.

Appropriate dilutions were made and concentrations determined using a haemocytometer.

3. Bees were inoculated within 1 h of inoculum preparation by applying 2 µl of the spore suspension to the diet surface adjacent to the mouthparts of the newly emerged larvae. Larvae were inoculated at 1, 2, 3 or 5 days of age. A total of 15 assays were carried out.

4. Larvae were checked daily for mortality. Unhatched eggs and larvae which died within the first 24 h were not included in the analyses. Uncertain diagnoses were verified by microscopy or fungal isolation into pure culture.

5. A dose–mortality relationship was found. There was an increase in LD_{50} with increasing age. The estimated LD_{50} values ranged between 120 and 1698 spores per bee, depending on age of larvae at time of inoculation.

Grasshoppers with B. bassiana

A leaf surface treatment bioassay has been used successfully to inoculate numerous fungi against several insect hosts (Ignoffo *et al*., 1983 and references therein; Inyang *et al*., 1998). Inglis *et al*. (1996a) used an oil-bait bioassay method to compare the virulence of several isolates of *B. bassiana* against the grasshopper, *M. sanguinipes*. In subsequent studies, this method was used to demonstrate the effect of bait substrate and formulation on virulence of this fungus (Inglis *et al*., 1996c). It was demonstrated that the efficacy of this method depends on the extent to which nymphs become surface-contaminated with conidia during ingestion.

1. Conidia of several isolates of *B. bassiana* were obtained from cultures grown in the dark at 25°C on potato dextrose agar (PDA) for 7–10 days. Conidial viability was assessed on PDA amended with 0.005% Benlate (Dupont), 0.04% penicillin and 0.1% streptomycin after 24 h incubation at 25°C. Conidia were scraped from the surface of the PDA and suspended in sunflower oil. Conidial densities were determined using a haemocytometer and adjusted as necessary to obtain final concentrations of 1×10^5, 3.2×10^4, 1×10^4, 3.2×10^3 and 1×10^3 viable conidia.

2. Nymphs hatched from eggs laid by field-collected adults were reared on a diet of bran and wheat leaves. Third-instar nymphs were individually placed in sterile 20-ml vials stoppered with a sterile polyurethane foam plug, and starved for 12 h.

3. Five-microlitre aliquots of conidial suspensions were pipetted on to 5-mm diameter lettuce discs. A control consisted of oil applied to the discs alone. The inoculated discs were then pierced in the centre with a pin and suspended approximately 2 cm into the vial from the foam plug, and presented to the starving nymphs (Fig. 4.6). Nymphs were held at 25°C under incandescent and fluorescent lights for 12 h. Nymphs that underwent

ecdysis or did not consume the disc after this period were excluded from the experiment.

4. Groups of 12 to 15 nymphs per treatment were transferred to 21 × 28 × 15 cm Plexiglass containers equipped with a perforated metal floor to reduce contact with frass (Fig. 4.7). Cages were incubated at a 25/20°C day/night and 16 : 8 h (light : dark) photoperiod regime and the nymphs were maintained on a diet of wheat leaves. Alternatively, in some assays, grasshoppers were kept singly in plastic cups (Inglis *et al*., 1996b; Fig. 4.8).

5. The experiment was arranged as a randomized complete block design with four blocks conducted in time. The total number of nymphs per isolate–dose combination ranged from 46 to 61 nymphs. Nymphs that died and subsequently produced hyphal growth of *B. bassiana* on moistened filter paper were considered to have died of mycosis.

6. The oil-bait bioassay method facilitated the rapid inoculation of grasshopper nymphs. Within 1 h, 350 nymphs could be easily inoculated using this method. A dose–mortality relationship was demonstrated and substantial differences in virulence between isolates were found.

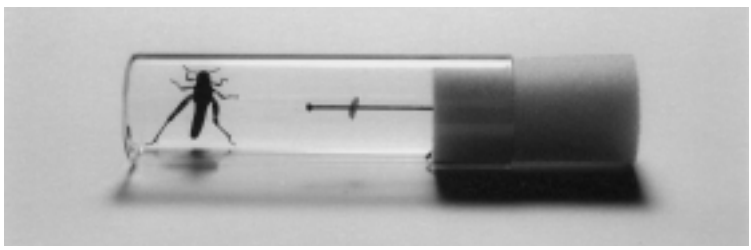

Fig. 4.6. Inoculation method for grasshoppers. An inoculated leaf disc is pinned to the inside of a foam plug and presented to a starved nymph within a shell vial. Photo by courtesy of Doug Inglis.

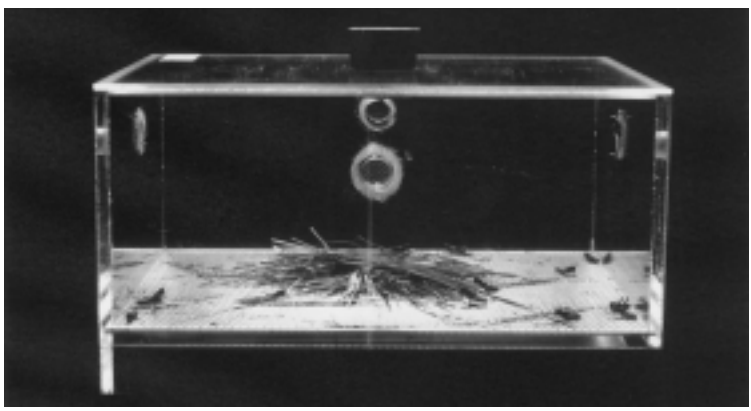

Fig. 4.7. A Plexiglass bioassay chamber used to incubate groups of inoculated grasshoppers. Photo by courtesy of Doug Inglis.

Fig. 4.8. A plastic container used to incubate single grasshopper nymphs. Photo by courtesy of Doug Inglis.

Inoculation using forcibly discharged conidia

Most fungi in the Entomophthorales produce forcibly-discharged conidia. These conidia are usually relatively short-lived and it is often not possible to harvest and enumerate them before using as inoculum. Consequently, many bioassays with these fungi use methods to inoculate the host directly from sporulating cultures or cadavers. In using such methods, much attention must be paid to the specific conditions that are required to induce spore discharge (Papierok and Hajek, 1997). Depending on the species involved, spores can be obtained from cultures maintained on agar medium, sporulating cadavers or hydrated marcescent mycelium.

Potato leafhopper with Zoophthora radicans

Wraight *et al.* (1990) used the forcibly discharged conidia of *Zoophthora radicans* from cultures and infected cadavers to inoculate the potato leafhopper, *Empoasca fabae*.

1. Dry mycelium of *Z. radicans* was prepared according to McCabe and Soper (1985), milled and sieved to retain particles between 1 and 0.5 mm. The mycelial particles were spread evenly on to water agar in Petri dishes and incubated at 21–22°C for approximately 12 h to obtain abundant sporulation.
2. Bioassay chambers consisted of 35-mm diameter plastic Petri dishes. Most of the upper surface of the lid was excised, leaving a narrow strip across the

centre for attachment with a small screw to a flat Plexiglass base. A cowpea leaf was then sandwiched between the base and modified lid. Each leaf was misted with water and each chamber was covered with a matching, unmodified lid.
3. Newly moulted, 5th-instar nymphs of *E. fabae* from a laboratory colony on cowpea were anaesthetized with CO_2 and randomly collected in groups of five. Individuals were placed dorsal side up on the wet leaf surface in a bioassay chamber. The chamber lid was then replaced with a lid containing the sporulating fungus. The leafhoppers were continuously exposed to the sporulating culture for 7 min. During this exposure period, the culture was continuously rotated.
4. After inoculation, each group of insects was transferred to clean chambers with fresh leaves. Each chamber was sealed in plastic bags and incubated at 20–22°C and 90–100% RH.
5. The LD_{50} was estimated at 4.1 spores per leafhopper.

Aquatic insects

Bioassay of aquatic insects is usually accomplished by introducing the inoculum directly into the water. However, use of high concentrations of inoculum in static aqueous systems may have adverse effects on water quality. Therefore, at times it is necessary to replenish the water depending on host species. Also, continuous exposure to propagules of some fungi such as *Tolypocladium cylindrosporum* may not be ideal, because the effective dose may vary according to length of exposure, as hosts continually reingest excreted conidia that remain viable (Goettel, 1987). This problem can be overcome by using a limited exposure time (Nadeau and Boisvert, 1994).

Mosquitoes with Culicinomyces clavisporus

Sweeney (1983) used a static bioassay method to determine the time–mortality responses of mosquito larvae inoculated with *Culicinomyces clavisporus*.

1. *C. clavisporus* was cultured in a broth of 1.25% corn steep liquor, 0.2% glucose and 0.1% yeast extract for 7 days at 20–24°C. Conidia were separated from the mycelium by filtration through fine gauze, then pelleted by centrifugation followed by two washes with sterile water. Conidia were counted using a haemocytometer and adjusted to the desired concentration.
2. Within 4–6 h of emergence, 5th-instar larvae of *Anopheles hilli* were placed in groups of 40 into plastic trays (18 × 12 × 5 cm) with 200 ml of water. Conidia were added on the following day.

3. The trays were incubated at 25°C and larvae were fed daily with powdered animal food pellets. Dead larvae were removed daily and the experiments were terminated after 12 days.
4. Nine separate experiments were performed with six to eight concentrations of conidia in each experiment. Five separate trays were dosed at each concentration and five trays were kept as a control.
5. A dose–mortality relationship was found and time to death decreased with increasing dose.

Mosquitoes with Coelomomyces

Toohey *et al.* (1982) used a bioassay to determine the intermediate copepod host in Fiji for a *Coelomomyces* sp. The fungus is a pathogen of mosquitoes which requires an alternate host to complete its development.

1. Cultures of five species of copepods and three species of mosquito were reared in the laboratory in rain water in transparent or opaque cups (6 × 10 cm).
2. Field- and laboratory-reared *Coelomomyces*-infected mosquito larvae which had been dead for less than 24 h were used as the inoculum.
3. Inoculum and 150–200 copepods of various ages were placed into each bioassay cup containing 200 ml of boiled treehole water. Ten to 12 days later, 20 first-instar *Aedes* larvae were placed in each cup. Cups were examined three times a week and dead larvae, pupae and adults were removed and examined microscopically for signs of infection. If infection was not apparent, a second group of larvae were added. Crushed mouse chow was added periodically for food.
4. Controls consisted of a set of three cups, one with only copepods, a second only with inoculum and the third of both copepods and larvae. There were at least five replicates for each copepod species tested and a total of 20 controls for all the species tested.
5. Only one species of copepod, *Elaphoidella taroi*, was found to be the intermediate host.

Novel bioassay methods

As stated previously, bioassays must be adapted to suit the host, pathogen and objectives of the bioassay. At times, the approach taken is very novel and sometimes even controversial. Novel approaches must balance efficacy and usefulness of the results.

Silverleaf whitefly with P. fumosoroseus, V. lecanii *and* B. bassiana

Landa *et al.* (1994) used a novel approach to bioassay entomopathogenic fungi against the whitefly, *Bemisia argentifolii*. The bioassay is based on rapid characterization of the growth rate and development of the fungi on whitefly nymphs. It can be used in determining effects of environmental factors, adjuvants and pesticides on development of these fungi in whiteflies. This method could be adapted for use with many other small insects.

1. Isolates of *P. fumosoroseus*, *V. lecanii* and *B. bassiana* were cultured on PDA at 25°C in constant light for 7–10 days. Conidia were harvested by rinsing the cultures with 0.05% aqueous Tween 80. The suspension was mixed using a vortex mixer and conidia were enumerated using a haemocytometer and adjusted to a final concentration of 1.0×10^7 conidia ml^{-1}.
2. Test materials (adjuvants, pesticides) were then added to the conidial suspensions. Drops of the test suspension were then placed on sterile microscope slides, 30 drops in three rows per slide. Laboratory-reared, early 4th-instar nymphs were singly placed in the centre of each drop and a total of 25 nymphs were placed on each slide. The remaining five drops were used as controls.
3. The slides were dried in a laminar airflow hood, placed in plastic Petri dishes with a sterile wet filter paper on the bottom and incubated for 7 days at 25°C under constant light. Each fungus was assayed using ten slides.
4. The influence of the bioassay protocol on the development of nymphs was assessed. Early 4th-instar nymphs were incubated on microscope slides in the diluted Tween 80 only for 7 days. The number of emerged adults was assessed daily.
5. A rating system, named the Fungus Growth Development Index (FGDI), was used to assess the degree of fungal development on the insect host. Ratings were made at either daily intervals or at 24, 72 and 120 h. An FGDI of 0.5 represented the first sign of viability of conidia, 1.5 for colonization of the host and 2.5 for initial sporulation on the cadaver.

Semi-field and field-based assays

Field trials are essential to demonstrate that fungal isolates identified as virulent in laboratory trials are efficacious in the field. Initial trials may be done using small cages enclosed in gauze or potted plants enclosed in a nylon sleeve which allow for easy monitoring of insect pests. Trials may be done in 'walk in' cages containing potted plants to ease the collection of insects and assess the efficacy of the pathogen under field conditions. However, most small-scale trials are done in randomized plots (3 m × 3 m) alongside,

or occasionally within, a growing crop. Although it may be more difficult to find the target insect there are various ways of assessing the impact of the pathogen. For example, insects may be collected randomly within each plot and incubated in humid chambers favouring fungal development. This could reveal more about targeting of the pathogen and its potential impact on the pest population. Alternatively, incubating healthy insects with plant parts collected from trial sites can reveal a considerable amount about the persistence (distribution on the plant and viability) of inoculum under field conditions. Assessing plant damage, or the number of larvae infesting leaves or flowers in control and treated plots is another way of assessing the impact of the pathogen. Fewer larvae would be expected to be found in plots where the pathogen was deployed. Field trials may not only be conducted on growing field crops but also rooted cuttings (Dorschner *et al.*, 1991).

Field trials against subterranean pests are technically more difficult for several reasons. First, the soil is a natural reservoir of many insect-pathogenic fungi so it would not be surprising to find target pests in control plots killed by fungi related to introduced pathogens. Second, targeting of the pathogen is not easy. Most often, inoculum is applied as a drench or ploughed into the soil using specialized equipment, but some workers have even used helicopters to treat large areas of pasture (e.g. Keller *et al.*, 1989).

Field bioassay of B. bassiana *against grasshoppers*

Inglis *et al.* (1997b) used a field cage bioassay to study the influence of environmental conditions on mycosis of grasshoppers caused by *B. bassiana*.

1. Conidia of *B. bassiana*, obtained from Mycotech Corp., were suspended in 1.5% (w/v) oil emulsion amended with 4% clay and applied to 12 ha of rangeland at a rate of 112 l ha^{-1}. Grasshoppers were collected in sweep nets immediately after treatment and placed in cages (41 × 61 × 48 cm) (Fig. 4.9), 100 hoppers per cage. Treatments consisted of cages: (i) placed in a glasshouse located at the laboratory, (ii) exposed to full sunlight, (iii) shaded from sunlight by a black plastic screen, and (iv) protected from UVB radiation by a UVB-absorbing plastic film (Fig. 4.9). Field cages were arranged as a randomized complete block with four sub-blocks, each containing three cage treatments per sub-block.
2. Grasshoppers were maintained on a diet of wheat seedlings and rangeland grasses. Cadavers were removed daily and assessed for mycosis by placing on moist filter paper.
3. Higher prevalence and more rapid development of the disease were observed in grasshoppers kept in shaded cages than in cages exposed to full sunlight or protected from UVB radiation.

Fig. 4.9. Field cages used to study the effects of solar radiation and shade on virulence of *B. bassiana* against grasshoppers under field conditions. Photo by courtesy of Doug Inglis.

Honey bee mediated infection of pollen beetle (Meligethes aeneus) *by* M. anisopliae

Butt *et al.* (1998) evaluated dissemination of fungal inoculum by honey bees against pollen beetles in oilseed rape (= canola) using field-caged insects. This method has also been shown to control seed weevil (*Ceutorhynchus assimilis*) and has the potential to control most floral pests including thrips (T.M. Butt, unpublished observations).

1. Trials were carried out in winter oilseed rape between late April and late May, and in spring oilseed rape between mid-June and late July.
2. Nine insect-proof cages (2.7 × 2.7 × 1.8 m high) were erected over the flowering crop infested with adult pollen beetles. Small colonies of honey bees were placed in the corner of each of six of the cages; each consisted of about six British Standard combs of bees and brood housed in a single British Standard Modified National hive body. Three of the hives had modified entrances containing an inoculum dispenser similar to that used by Peng *et al.* (1992). This consisted of a Perspex tray to contain the inoculum, through which the bees walked on leaving the hive. Bees returned to the hive *via* an entrance below the dispenser to prevent inoculum being brought into the hive. Inoculum was replenished at 48-h intervals. The three treatments (bees without inoculum, bees with inoculum, and no bees or inoculum) were randomized.
3. Ninety pollen beetles were collected from each cage at intervals of 3–6 days in winter rape and 7 days in spring rape, and were placed in groups of 30 in ventilated Perspex boxes (5.5 × 11.5 × 17.5 cm) lined with moist

tissue paper and incubated at 23°C and 16:8 h (light:dark) photoperiod. Three freshly cut inflorescences of rape were placed in each box as food. Mortality was recorded daily for 14 days. Dead beetles were removed and placed in a Petri dish lined with moist filter paper to encourage external conidiation of the fungus.

4. The first mortalities due to *M. anisopliae* were 3–5 days and 2–6 days after the sample was taken in winter and spring rape, respectively. The final mortalities for samples 1 and 2 were approximately 60% on winter rape and 99% and 69%, respectively, on spring rape. These results suggest that honey bees are effective in delivering conidia of *M. anisopliae* to flowers of oilseed rape and in the subsequent control of pollen beetles.

Checklist of Bioassay Preconditions and Requirements

There are several aspects which need to be checked to ensure effective bioassays with fungal pathogens.

1. It is important to ensure that the pathogen:
- has not lost virulence during culturing,
- inoculum is viable and percentage germination is determined,
- application method is satisfactory.

2. The target insect must be:
- healthy,
- not overcrowded or stressed,
- isolated if carnivorous or cannibalistic.

3. The bioassay chamber must:
- allow survival of control insects,
- not contain harmful substances, such as formaldehyde in food.

4. All bioassays should have:
- large enough sample size and enough replicates per treatment to make the results meaningful,
- the assays repeated at least once,
- field plots which are randomized,
- internal environments in the field cages which approximate to the external environment,
- sampling procedures which reflect the field fitness of pathogens.

Concluding Remarks

Bioassays are central to the successful development of fungi as microbial control agents. Although useful in providing valuable information on the

insect–pathogen–environment interactions, the validity of bioassay results depend on the bioassay design, execution, analysis and interpretation of results. The ultimate challenge is to develop bioassays that can be used to predict field efficacy. It is therefore imperative that pertinent environmental parameters be incorporated into bioassay designs. For instance, knowledge of an LD_{50} or LT_{50} obtained from comparative laboratory assays of numerous isolates under static conditions provides minimal useful information as far as predicting the potential efficacy of a strain under field conditions is concerned.

Bioassay designs must be constantly improved to provide more meaningful information. The advent of increasingly sophisticated equipment such as incubators, environmental monitoring and inoculum application devices has allowed for the development of more complex bioassay designs which provide more pertinent results. Computerized statistical analyses have made it possible to model environmental parameters and process data with greater ease. As our understanding of the pertinent parameters important in fungal epizootiology increases, bioassays must be adapted so that they will provide information applicable for prediction of efficacy under field conditions.

We have provided some of the important parameters that need to be considered in the development and execution of a bioassay with an entomopathogenic fungus. We have also provided numerous examples of bioassays to illustrate the many methods and bioassay designs that have been used with an array of fungal and target species combinations. It is hoped this provides the reader with adequate information that should stimulate and facilitate the design of novel and pertinent bioassays which will provide useful information for the understanding of fungal biology, host–pathogen interactions, epizootiology and ultimately aid in the development of these microorganisms as microbial control agents of pest insects.

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Appendix 4.1: Selective Media for Isolation of Entomogenous Fungi

Veen's agar medium (1 l) (Veen and Ferron, 1966)

35 g Mycological agar (Difco) or 10 g Oxoid neutralized soya peptone, 10 g dextrose, 15 g No. 1. agar (or Bacto-agar), 1 g chloramphenicol (store 4°C), and 0.5 g cycloheximide (= Actidione; store 4°C). Add 1 l distilled water, stir, and cover. Autoclave for 10–15 min at 18–20 psi. Cool to *c.* 52°C and pour plates in laminar flow cabinet.

Oatmeal dodine agar (Beilharz *et al.*, 1982)

1. Antibiotic stock solution: add 4 g penicillin G (Sigma) and 10g streptomycin sulphate (Sigma) to 40 ml sterile distilled water under sterile conditions. Store at 4°C.
2. Crystal violet stock solution: add 0.1 g crystal violet (Sigma) to 200 ml distilled water. Store in the dark.
3. Add 17.5 g oatmeal agar (Difco) and 2.5 g agar (Fisons) slowly to 0.5 l distilled water while stirring vigorously and heat to boil.
4. Add 0.5 ml of the fungicide dodine (*N*-dodecylguanidine monoacetate; Cyprex 65WP, American Cyanamid Co.) and 5 ml crystal violet stock solution to the medium.
5. Autoclave for 20 min at 15 psi.
6. Allow medium to cool to 50–55°C and add 2 ml of antibiotic stock solution under sterile conditions.
7. Swirl flask well to ensure thorough mixing of compounds and pour while warm. There should be enough media for twenty 9-cm diameter Petri dishes.

Selective agar medium (1 l) (Kerry *et al.*, 1993)

37.5 mg carbendazim, 37.5 mg thiabendazole, 75 mg rose bengal, 17.5 g NaCl, 50 mg each of streptomycin sulphate, aureomycin and chloramphenicol, 3 ml Triton X-100, and 17 g corn meal agar (Oxoid) in 1 l distilled water. This medium is appropriate for selecting some *Paecilomyces* spp. and *Verticillium* spp. from soil.

***Paecilomyces lilicanus* medium** (Mitchell *et al.*, 1987)

To prepare 1 l of medium, mix the following: 39 g PDA, 10–30 g NaCl, 1 g Tergitol, 500 mg pentachloronitrobenzene, 500 mg benomyl, 100 mg streptomycin sulphate, and 50 mg chlorotetracycline hydrochloride.

Wheat germ based selective agar medium (1 l) (Sneh, 1991)

1. Prepare an aqueous extract of wheat germ – mix 30 g wheat germ in 1 l water, autoclave for 10 min and filter through four layers of cheesecloth.
2. Mix wheat germ extract (1 l) with 0.25 g chloramphenicol (heat stable) + 0.8 mg benlate (50% benomyl), 0.3 g dodine (65% *n*-dodecyl-guanidine acetate), 10 mg crystal violet and 15 g agar.
3. Autoclave and pour into plates.

Copper-based selective agar medium (1 l) (Baath, 1991)

2% malt extract (Oxoid), 1.5% Agar (Difco) amended with 2–4 mg $CuSO_4 \cdot 5H_2O$ per litre. *Cordyceps militaris* and *Paecilomyces farinosus* are tolerant of high Cu levels (400 mg l^{-1}), followed by *Metarhizium anisopliae* and *Beauveria bassiana*. Most other soil-borne fungi including nematophagous species of *Verticillium* were less tolerant.

Appendix 4.2: General Culture Media

Medium	Ingredients	g l^{-1}
Straw agar medium	Supernatant of boiled straw	40
	Agar (Difco)	8
	Aureomycin	0.05
	Streptomycin	0.05
	Chloramphenicol	0.05
Soya peptone medium	Soya peptone	10
	K_2HPO_4	0.3
	$MgSO_4 \cdot 7H_2O$	0.3
	NaCl	0.15
	$CaCl_2 \cdot 6H_2O$	0.3
	$MnSO_4 \cdot 6H_2O$	0.008
	$CuSO_4 \cdot 5H_2O$	0.0002
	$FeSO_4 \cdot 7H_2O$	0.002
Minimum medium	K_2HPO_4	0.3
	$MgSO_4 \cdot 7H_2O$	0.3
	NaCl	0.15
	$CaCl_2 \cdot 6H_2O$	0.3
	$MnSO_4 \cdot 6H_2O$	0.008
	$CuSO_4 \cdot 5H_2O$	0.0002
	$FeSO_4 \cdot 7H_2O$	0.002
	Agar	20.0
MC medium	Potassium phosphate dibasic	36
	Sodium phosphate heptahydrate	1.1
	Magnesium sulphate heptahydrate	0.6
	Potassium chloride	1
	Glucose	10
	Ammonium nitrate	0.7
	Yeast extract	5
	Agar	20
Sabouraud dextrose agar (SDA)	Mycopeptone	10
	Dextrose	40
	Agar	15
Oatmeal agar (OA)	Oatmeal	30
	Agar	20
Potato dextrose agar (PDA)	PDA (Oxoid)	39
Malt extract agar (MEA)	Malt extract	30
	Mycological peptone	5
	Agar (technical grade)	15

Continued

Medium	Ingredients	g l^{-1}
Sabouraud dextrose agar with yeast (SDAY)	Dextrose	40
	Neopeptone	10
	Yeast extract	10
	Agar	15
V8 Juice	V8	200
	$CaCO_3$	3
	Agar	20
Blastospore-producing medium	Corn steep liquor	20
	Sucrose	30
	KH_2PO_4	2.26
	$Na_2HPO_4 \cdot 12H_2O$	3.8
	$MgSO_4 \cdot 7H_2O$	0.123
	$FeSO_4 \cdot 7H_2O$	0.023
	$ZnSO_4$	0.020
	K_2SO_4	0.174
	$CaCl_2 \cdot 2H_2O$	0.147
PYG with supplements	Peptone	1.25
	Glucose	3.0
	Yeast extract	1.25
	Agar	20
	Vegetable oil (e.g. soybean, maize)	1–2 ml
	Sterol (e.g. cholesterol, ergosterol)	0.01–0.1
	Lecithin	0.05–0.1
	$CaCl_2 \cdot 2H_2O$	0.07
Blastospore-producing medium	Glucose	25
	Soluble starch	25
	Corn steep	20
	NaCl	5
	$CaCO_3$	5

Note: most solid media can be used as liquid media by excluding the agar. Conversely, adding agar can convert a liquid medium to a solid medium. The pH of most media ranges between 5 and 9 with most workers adjusting to pH 7 with 1 M NaOH or HCl.