ASPECTS OF FUNGAL PATHOGENESIS IN *Lygus lineolaris* (PALISOT DE BEAUVOIS) (HETEROPTERA, MIRIDAE)

by

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To my Dad and in loving memory of my Mom.

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Abstract

The pathogenicity of native fungal isolates, *Beauveria bassiana*, *Metarhizium anisopliae*, and *Verticillium lecanii*, and a commercial formulation of *B. bassiana*, BotaniGard (GHA strain) against adult *Lygus lineolaris* Palisot de Beauvois (Hemiptera, Miridae) was investigated with particular emphasis on the influence of body region and topography on spatial conidial deposition patterns and germination of conidia as they related to the fate of the insect. Mycosis was induced by all isolates tested, and not surprisingly, BotaniGard was the most virulent. Electron microscopic assessment of the cuticle revealed that conidia were capable of attachment and subsequent germination for all isolates tested. Relatively few conidia were observed having attached compared to the total number sprayed on the insect. Furthermore, the number of germinating conidia was a small proportion of those that adhered, suggesting that germination may be a limitation for infection. Significant differences were detected between infected versus uninfected insects based on germinating conidial counts in relation to body region and both conidial and germinating conidial counts for surface topography. Appressorial development in germinating conidia was also affected by surface topography. Despite the fact that the native fungal isolates used in laboratory assays had been collected in Nova Scotia, a 2-year survey of *L. lineolaris* populations in apple orchards and fields, did not detect incidence of natural fungal infection. Although, the direct spraying of BotaniGard and *M. anisopliae* in laboratory trials resulted in high mortality, field applications will not likely realize the same level of success. Successful biological control using fungal pathogens against *L. lineolaris* requires contact between the insect host and the pathogen. Since both body region and surface topography of the insect cuticle influenced the spatial deposition patterns of conidia and germination, promoting conditions that favour disease development at the conidia-cuticle interface may play a more critical role in the future use of fungal pathogens against *L. lineolaris*. This finding emphasizes the need for novel application strategies that will increase the likelihood that conidia will be delivered in a sufficient dose, to the right location on the insect cuticle, while under conditions that will favour early development of the pathogen.

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Chapter 1: General Introduction

Increased public demand for safe alternatives to chemical controls for insect pests emphasizes the importance of continued research towards a strategy that takes full advantage of the potential of biological control agents. Naturally occurring fungal pathogens are important in the regulation of insect pest populations (Ferron, 1978; Hajek and St. Leger, 1994; Lacey and Goettel, 1995; Lacey et al., 2001; Roberts and Yendol, 1971; Steinhaus, 1949), and in the past 50 years, have become increasingly more important for their potential as biological control agents. These novel agents may provide a viable alternative to the repeated applications of broad-spectrum insecticides currently used to manage *Lygus lineolaris* (Palisot de Beauvois) (Hemiptera, Miridae), a pest of fruit crops (e.g. apple, strawberry, and raspberry) in Nova Scotia.

Fungal pathogens are ubiquitous and coexist with other natural enemies of pests such as predators, parasitoids and other pathogens. Fungi that attack living insects are represented in over 100 genera across every major fungal subdivision. Although, the deuteromycete genera comprise the majority of entomopathogenic fungi, many of these genera contain only one to a few species that attack living insects (Inglis et al., 2001; Roberts & Humber, 1981). Within the deuteromycetous fungi, most species that kill insects can be found within the class Hyphomycetes (Roberts and Humber, 1981). Of the entomopathogenic hyphomycetous fungi, only a small number (e.g. *Beauveria bassiana* (Balsamo) Vuillemin, *Metarhizium anisopliae* (Metschinkoff) Sorokin) are considered to have potential for use in pest management as biological control agents in greenhouse and outdoor crop systems (Ferron, 1978; Roberts and Humber, 1981).

The biological control potential of fungal pathogens is due, in part, to their ability to

penetrate directly through the insect cuticle (McCoy et al., 1988). This mode of action is particularly useful for control of phloem-feeding insects (Smits, 1997) like those belonging to the Orders Hemiptera and Homoptera. However, the potential for infection also depends on the capability of the fungus to survive exposure to external factors such as temperature extremes, ultra-violet (UV), and low relative humidity (RH). A number of factors influence the development and eventual commercialization of a fungal pathogen as a biological control agent including mode of infection, speed of kill, host specificity, safety to non-target organisms, ease of mass production and application (McCoy, 1990; Smits, 1997). I provide a general overview of the potential for fungal pathogens as biological control agents and discuss (1) the influence of the pathogen-cuticle interface on infection; and (2) the limitations of extrapolating from laboratory findings to field studies.

Excluding *Bacillus thuringiensis* var. *kurstaki* (Btk), a bacterial entomopathogen, few microbial-based insecticides have successfully achieved widespread commercial application despite their recognized potential for insect control (Georgis, 1997; Wraight and Carruthers, 1999). Early research in this field often produced narrow spectrum biopesticide products that were more expensive than chemical insecticides and had mixed success in insect control (Lisansky, 1989, 1997). However, approximately 20 hyphomycetous fungal pathogens have been successfully developed as commercial products and are available for use in the OECD countries in Asia, Australia, Europe, and North America (Kabaluk and Gazdik, 2004). *Beauveria bassiana* is used against Colorado potato beetle, *Leptinotarsa decemlineata* (Say) (Coleoptera, Chrysomelidae)

(Butt et al., 1999) and grasshoppers (Johnson and Goettel, 1993); *Verticillium lecanii* (Zimmermann) Viégas is currently used in greenhouses for control of aphids, thrips and whiteflies (Askary et al., 1999; Butt et al., 1999), and *Metarhizium anisopliae* is used against vine weevil (Butt et al., 1999). None of these products has realized the success of Btk as a biological control agent and commercial production of fungal formulations remains in the domain of niche markets, not yet having fulfilled their promise to significantly reduce the amounts of pesticide used (Evans, 1999; Gaugler, 1997; Lacey et al., 2001; Wraight and Carruthers, 1999).

Even the limited role that fungal pathogens currently play is now threatened with the new classes of insecticides, such as neonicotinoids and insect growth regulators, being developed for use in agriculture (Georgis, 1997; Wraight and Carruthers, 1999). Like other insecticides, these novel chemicals kill or suppress feeding more quickly than pathogens. Mean mortality in adult *L. lineolaris*, placed on cotton plants and isolated in sleeve cages, was significantly higher (64.6%) after 3 days for insects and plants treated with the neonicotinoid imidacloprid (Provado 1.6F) at a rate of 0.053 kg AI/ha, compared with 43.8% mortality after 7 days for those insects on cotton sprayed with a commercial formulation of *B. bassiana* (Mycotrol WP) at a rate of 1.12 kg/ha (Brown et al., 1997). Furthermore, like pathogens, neonicotinoids pose less risk to applicators and wildlife and are less toxic to natural enemies of pests and to pollinators than conventional broad-spectrum insecticides (Zalom et al., 2005). These novel chemicals often cost the grower less than microbial based-insecticides, although they are considerably more expensive than existing organophosphates and pyrethroids. Limited

commercial success of pathogen-based control agents has been partly attributed to inconsistent performance or reduced efficacy compared with insecticides, slower rates of kill, high production costs, and vulnerability to a myriad of abiotic and biotic factors (Lacey et al., 2001; Lisansky, 1989, 1997).

Although entomopathogenic fungi are often considered widespread in their distribution (Tanada and Kaya, 1993; Zimmermann, 1993), they are often present at low levels in insect populations (Hajek and Goettel, 2000; Lacey and Brooks, 1997). As a result, attempts to control insect pest populations in highly managed systems (e.g. agricultural) are often conducted using high doses of fungal entomopathogens that have broad host ranges (Hajek and Goettel, 2000). The most successful inundative releases of insect pathogens were with Btk (Pedigo, 1996a), which are able to curb insect feeding quickly, even though mortality occurs over a period of several days. However, if pest densities exceed thresholds during the season then repeated applications are required (Pedigo, 1996b). The dosage required to control insect pests with repeated applications could directly affect non-target organisms, including beneficials. Although, insects that are in direct contact with the pathogen could be at high risk for infection, the likelihood that the fungi will become established within the non-target insect population is low because high doses are usually present for only a short period of time (Goettel et al., 2001). This is likely due to the number of factors including the extensive use of fungicides (Puterka, 1999) and exposure to UV radiation that affect the development of infection and persistence in the environment. Goettel et al. (2001) cautioned that an increase in levels of indigenous fungi could result in a temporary disruption to the

ecosystem similar to chemical controls, but overall, any negative effects have either been minimal, not detected, or did not exist. Regardless, the need to examine the impact to non-target species and the pathogen's persistence when assessing the performance of a fungal entomopathogen against a particular insect pest cannot be emphasized enough, especially with the increased use of beneficials (e.g. parasitoids and predators) in Integrated Pest Management (IPM) programs (Hajek and Goettel, 2000). Initial assessment of the potential impact of fungal pathogens on non-target hosts can be done through laboratory investigations of host-specificity and through identification of endemic disease levels in field collected insects (Goettel et al., 2001). Results from greenhouse studies revealed significantly fewer green leafhopper, *Empoasca decipiens* (Homoptera, Cicadellidae), nymphs emerged from parasitized eggs on broad bean plants that were sprayed with 10 ml conidial suspensions (1×10^7 conidia per ml) of *M. anisopliae* and *Paecilomyces fumosoroseus* (Wise) Brown and Smith (Deuteromycotina, Hyphomycetes) versus the controls (Tounou et al., 2003). The egg parasitoid, *Anagrus atomus* (Hymenoptera, Mymaridae), of *E. decipiens* was not as susceptible (no effect on adult emergence and longevity) as its host after exposure to the same treated plants. Furthermore, when provided a choice between treated versus untreated *E. decipiens* eggs for oviposition, wasps attacked fewer eggs treated with a fungal pathogen (Tounou et al., 2003). However evaluation in a field setting is often more challenging with endemic levels of infection in insect populations often low for the target pest and even lower for non-target species (Hajek and Goettel, 2000). There have been only two published reports (Broadbent, 2000; Steinkraus, 1996) of fungal infected *L. lineolaris* in

the field and in both instances the pathogen was *B. bassiana*.

Much has been published on the potential of fungal pathogens for biological control of insects (Ferron, 1978, 1985; Hajek and St. Leger, 1994; Hall and Papierok, 1982; Inglis et al., 2001; Lacey and Goettel, 1995; McCoy, 1990; Roberts and Humber, 1981; Wraight and Carruthers, 1999). Since the last century, many resources have been put into the development of fungal pathogens as alternatives to chemical pesticides but success has been limited: widespread acceptance and use has been poor. Cook (1990) argues that enormous potential exists but that progress is too slow. By 1990, only five biological control agents used to control plant diseases were registered as minor-use products in the U.S.A (Cook, 1990). Unfortunately, this has resulted in a somewhat pessimistic outlook towards the actual potential of microbial control agents, even though, the lack of successful exploitation of entomopathogenic fungi as biological control agents has been attributed to a number of problems that can be overcome through continued research and its application (Charnley et al., 1997; Georgis, 1997; Hall and Papierok, 1982). These can include the lack of basic knowledge of the relationship between host and pathogen, labour-intensive techniques needed to assess spore (conidia) counts on insect cuticle, and others previously stated (e.g. external factors like unfavourable temperatures, UV, and low relative humidity).

Fungi invade insects through the cuticle, in contrast to chemical insecticides that enter insects through one or more of three possible modes that include cuticular contact, ingestion and fumigation (Evans, 1999). Breaching the cuticle involves a series of stages that include conidial attachment, germination, conidial differentiation (with or without

formation of appressoria[1]) and penetration of the cuticle by enzymatic and mechanical processes (St. Leger, 1991). Enzymes break down the protein, lipid and chitin in the cuticle (St. Leger, 1991). Once germinated conidia have penetrated, the pathogen proliferates inside the insect tissues and haemocoel spreading throughout the body of the host. At the time of the insect's death, the pathogenic phase ends and the fungus continues to develop saprophytically (Ferron, 1978). Under warm, humid conditions, hyphae will then emerge through the cuticle of the insect cadaver, and sporulation will take place producing millions of conidia. Death of the insect host is a result of one or a combination of factors including nutrient deprivation in the hemolymph, the invasion of tissues, and/or the release of toxins from the fungus (Roberts and Humber, 1981; Butt and Goettel, 2000).

Environmental conditions on the insect cuticle are particularly relevant as adhesion and germination are critical to the pathogens' success (Boucias and Pendland, 1991; Farques, 1984; Hajek and Eastburn, 2003). In addition, spore adhesion is affected by the chemical composition of the spore wall and of the epicuticle of the host and the initial attachment of hydrophobic conidia to the insect cuticle involves electrostatic mechanisms (Boucias and Latgé, 1986; Boucias et al., 1988, Boucias and Pendland, 1991). A number of the entomopathogenic Hyphomycetes, including *B.bassiana* and *M. anisopliae*, produce hydrophobic conidia (Boucias and Latgé, 1986; Boucias and

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1 the term appressorium (pl. appressoria) refers to "a flat swelling that forms on the end of a germ tube or vegetative hypha and which adheres to the surface of the host before penetrating it with an infection hypha that originates from the bottom of the swelling (M. Ulloa and R.T. Hanlin, 2000)."

Pendland, 1991), whereas others such as *V. lecanii* and *Hirsutella thompsonii* Fisher produce hydrophilic conidia usually in a droplet of mucilage (Boucias and Latgé, 1986). Although, hydrophobic conidia are capable of attaching to all body regions (Boucias et al., 1988), various cuticle characteristics (e.g. setae, inter-segmental seams) can influence the likelihood of conidial attachment at specific sites. Successful adhesion is considered the first stage in the infection process.

Surface topography and cuticular region influence attachment, germination, and thus, probability of infection and speed of kill. Additional factors that interact with features of the cuticle and also affect adhesion, include concentration of inoculum, mode of exposure, physical removal by host behaviours (e.g. grooming) and local microclimate (Boucias and Pendland, 1991; Butt and Goettel, 2000; Charnley, 1984; Lacey and Goettel, 1995). The exact site of infection can also be affected by the developmental stage and diet of the insect (Butt and Goettel, 2000). However, adhesion does not guarantee infection. For example, Peng et al. (2002), reported that the occurrence of *H. thompsonii* conidia on the cuticle did not lead to infection for several life stages of the honey bee. A number of vulnerable sites on the insect cuticle have been identified, such as inter-segmental membranes and folds (Fernandez et al., 2001; Hajek et al., 2002; Wraight et al., 1990), and areas with large numbers of setae that include antennal tips and apical parts of the tibia and tarsi (Sosa-Gomez et al., 1997). Fewer *M. anisopliae* conidia adhered to the smoother, heavily sclerotized regions such as the head and thorax of the southern green stink bug, *Nezara viridula* (Hemiptera, Pentatomidae) (Sosa-Gomez et al., 1997). In contrast, Wraight et al. (1990), found no variation between the

head, thorax or abdomen of the leafhopper *Empoasca fabae* (Homoptera, Cicadellidae) for attachment or germination of *Erynia radicans* (Zygomycetes, Entomophthorales) conidia. Zacharuk (1970) also reported that *M. anisopliae* conidia can attach to all regions of the cuticle in wireworm larvae, but remain firmly attached to the cuticle only if protected by inter-segmental folds. Direct spraying of spores onto the insect may help lodge conidia in vulnerable areas (Boucias et al., 1988).

The critical determinant of infection is thought to be the number of conidia that come into contact with the insect cuticle because of positive correlations between the number of spores and death from infection for most insect/pathogen combinations (Butt and Goettel, 2000). However, few researchers have explored the reasons why 100% mycosis is seldom achieved experimentally even in the laboratory. Inadequate understanding of host-pathogen relationships is thought to be partly responsible for the inconsistent results often observed in trials with fungal pathogens (Lacey et al., 2001; Samson et al., 1988). In bioassays, it is thought that likelihood of infection is dependent on spore dosage and that a threshold exists for the actual spore numbers needed to cause infection (Butt and Goettel, 2000; Goettel et al., 2000). If the dose is high enough then it is assumed that an effective number of spores will adhere to the insect and germinate. Good coverage will overcome initial barriers such as insufficient deposition on vulnerable regions of the cuticle (Goettel et al., 2000). At low concentrations, conidia were observed near setae on corn earworm larvae, *Heliothis zea* (Lepidoptera, Noctuidae), whereas at high concentrations there was attachment over the entire body surface (Pekrul and Grula, 1979). Conidial densities on the cuticle are further influenced

by host specificity: e.g. densities of *Entomophaga maimaiga* (Zygomycetes, Entomophthorales) conidia differed within the same cuticular regions for host versus non-host lepidopteran larvae (Hajek and Eastburn, 2003). Differences between regions and distribution of conidia on the insect cuticle can also influence whether or not germination and penetration occur and patterns are often affected by spore application method. In trials where *B. bassiana* was sprayed on Colorado potato beetle larvae, most conidia were observed on the dorsal abdominal surface whereas conidia were more abundant on the ventral abdominal surface and mouthparts when larvae were exposed to treated foliage (Fernandez et al., 2001). Days to death were reduced for stem borer larvae, *Chilo partellus* (Lepidoptera, Pyralidae), that were sprayed directly versus those that were exposed to treated leaves (Tefera and Pringle, 2003).

Usually, research results are correlative and the susceptibility of the host must be inferred from observable phenomena such as external sporulation after exposure to the pathogen in bioassays. A particular challenge in field trials is that some infected insects often exhibit changes in behaviour that reduce the chance of being observed or collected. Methods to minimize these problems (e.g. isolation cages) often introduce others such as alterations of the microclimate. Currently, traditional indices of host susceptibility such as mortality, mycosis, median lethal time (LT_{50}) leading to 50% mortality, median lethal concentration (LC_{50}) leading to 50% mortality, and median lethal dose (LD_{50}) leading to 50% mortality, are assumed to be adequate predictors of the potential of a pathogen to control the target insect host. A review of 15 studies published between 1990 and 2004, that assessed the potential of entomopathogens for

biological control, concluded that in every case, results were based solely on, one or more of the above-mentioned indices (e.g. Akbar, et al., 2004; Bidochka et al., 1993; Tefera and Pringle, 2003). Mycosis was determined simply by observing sporulation on cadavers after incubation in moist chambers. This approach remains the convention. However, depending on the research question being asked, LT_{50}, LC_{50} and LD_{50} measurements in laboratory bioassays may not be meaningful in field settings (Butt and Goettel, 2000; Hall and Papierok, 1982; Jaques, 1983). For example, simple reporting of mortality rates, ignores the impact of sub-lethal effects such as reduced fecundity or feeding suppression on crop damage. Moreover, conclusions drawn from field trials, often rest upon implicit assumptions that are seldom challenged (e.g. that high inoculum concentrations will compensate for the negative effects of adverse abiotic and biotic conditions). In practice, use of a particular spore concentration may lead to different results in different trials. For example, field trials using *B. bassiana* against *Lygus* spp. gave little control despite application of 2.47×10^5 conidia/cm^2, approximately six times the LC_{90} determined in the laboratory for adult *Lygus hesperus* (Hemiptera, Miridae). Field results were strongly influenced by timing of spray (prebloom versus bloom in alfalfa), life stage, host density, and pattern of inoculum coverage on the foliage because < 46% of conidia were recovered from top and middle alfalfa foliage (Noma and Strickler, 1999). Thus, taking better account of host and pathogen biology, crop characteristics, inoculation method, and environmental conditions in field trials should result in more reliable rates recommended for field use and generate greater confidence in their reliability (Evans, 1999).

There are a multitude of interactions between pathogen, host and environment taking place continuously in complex habitats (Inglis et al., 2001). This is not the case in laboratory assays because these trials can be deliberately designed to control factors such as fluctuations in temperature and relative humidity (Fargues et al., 1997), UV exposure and substrate (Rangel et al., 2004). Historically, the primary objective of laboratory bioassays has been to estimate lethal doses or concentrations, an often less than useful predictor of pathogen impact in the field (Butt and Goettel, 2000) and to determine the physiological host range (laboratory determined range of species that a fungus can infect) (Hajek and Goettel, 2000). Moreover, quantification of key relationships at the microscopic level, such as the number and spatial distribution of conidia needed to cause infection, are required to estimate the relationship between dosage of conidia applied and field efficacy (Evans, 1999; Goettel et al., 2000). Within the last two decades, researchers have used microscopy to examine the impact of processes operating at the conidia-cuticle interface on disease development but their studies have not yet improved our ability to predict pathogen efficacy under field conditions (Hajek and Eastburn, 2003, Quintela and McCoy, 1998; Sosa-Gómez et al., 1997, Wraight et al., 1990). Unfortunately, most measures and techniques (e.g. control over environmental conditions and use of the microscopes) employed in laboratory trials cannot be replicated in a large scale field trial resulting in a lack of quantitative knowledge of relationships among key variables and inadequate understanding of the process of pathogenesis. This limited progress towards the incorporation of laboratory results into field trial designs may have hindered the adoption of fungal pathogens into

the biological control market (Bateman, 1999). When this was done with a population model it was discovered that the varied susceptibility of gypsy moth larvae, *Lymantria dispar* (Lepidoptera, Liparidae) to the nuclear polyhedrosis virus played a critical role in the recurrence of outbreaks (Stone, 2004). Nonetheless, even given these difficulties, continued efforts towards better understanding the biology and ecophysiology of pathogenic fungi may lead to more effective use of fungi as potential biological control agents (Charnley et al., 1997).

The overall objective of this study was to investigate the potential of fungal pathogens (Deuteromycotina, Hyphomycetes) for use as biological control agents against *L. lineolaris* in apple orchards in Nova Scotia. Three approaches (two experimental and field observations) were undertaken to meet the objective of this study. Laboratory bioassays were conducted to evaluate the pathogenicity of the native fungal isolates of *M. anisopliae*, *B. bassiana*, and *V. lecanii* and a commercial formulation of *B. bassiana*, BotaniGard, against adult *L. lineolaris*, as measured by mortality and infection levels. Additional assays were then conducted for the purpose of investigating factors that may influence the development of infection at the conidia-cuticle interface (e.g. attachment and germination) in adult *L. lineolaris* exposed to *M. anisopliae* conidia only. Using a variable pressure scanning electron microscope (VPSEM), the influence of body region and topography of the insect cuticle on spatial deposition patterns of conidia and germinating conidia and how this relates to the development of disease were examined. The impact of fungal pathogens in field populations was also assessed in a 2-year survey of endemic infection levels of *L.*

lineolaris, conducted in Nova Scotian apple orchards and adjacent fields.

Chapter 2: Pathogenicity of *Beauveria bassiana*, *Metarhizium anisopliae* and *Verticillium lecanii* (Deuteromycotina, Hyphomycetes) to *Lygus lineolaris* (Hemiptera, Miridae).

Abstract

The pathogenicity of three Nova Scotian fungal isolates, *Beauveria bassiana*, *Metarhizium anisopliae*, and *Verticillium lecanii*, and a commercial formulation of *B. bassiana*, BotaniGard (GHA strain) on adult tarnished plant bug, *Lygus lineolaris* (Hemiptera, Miridae), was determined experimentally by spraying conidial suspensions on insects. Spore concentrations applied to insects ranged from 1.7×10^4 conidia/insect in a vial to 3.0×10^5 conidia/insect in a vial. Direct application of conidial suspension on adult *L. lineolaris* resulted in mortality, and in some treatments the number of insects that died was high, but in all cases only a proportion of dead individuals were determined to have been killed by fungus. Mortality for BotaniGard ranged from 85 to 100%, regardless of concentration. The estimated mean time for 50% mortality (LT_{50}) ranged from 4 to 6 d, indicating that BotaniGard was the most virulent of the pathogens tested against adult *L. lineolaris*. Among the native isolates tested, mortality induced by *M. anisopliae* and *B. bassiana* were significantly different from the procedural controls, reducing *L. lineolaris* numbers by 52.5 to 61.5%. Electron microscopic assessment of the dorsal cuticle of adults post inoculation revealed that conidia from all treatments, were capable of attachment and subsequent germination. However, overall percentage germination was low regardless of pathogen and concentration of conidial suspension. Results support continued evaluation of BotaniGard and the native isolate, *M. anisopliae*, as potential microbial control agents against adult *L. lineolaris*.

1. Introduction

The tarnished plant bug, *Lygus lineolaris*, is an economically important pest in North America (Henry and Lattin, 1987; Kelton, 1975; Michaud *et al.*, 1989; Parker and Hauschild, 1975), with over 350 known host plants (Young, 1986). Management of this insect pest continues to be a challenge with control of *L. lineolaris* in Nova Scotia primarily based on repeated applications of broad-spectrum insecticides, especially organophosphates and pyrethroids (Hardman et al., 2004). Unfortunately pyrethroids, the most effective registered insecticides, are highly toxic to natural enemies of other pests including mites. Organophosphate insecticides are likewise considered undesirable because of their hazard to applicators and to wildlife (Hardman et al., 2004). Both, horticultural and field crop industries need safer and less disruptive alternatives to broad-spectrum insecticides to prevent significant *Lygus* damage to their commodities. In Canada, there is active research into the development of effective biological control of *L. lineolaris* with parasitoids (Sohati et al., 1989), predators (Arnoldi et al., 1991), and pathogens (Broadbent, 2000).

The hyphomycetous fungi, *Beauveria bassiana*, *Metarhizium anisopliae*, and *Verticillium lecanii* are widely distributed species (Tanada and Kaya, 1993; Zimmermann, 1993), that produce conidia capable of direct penetration of the insect exoskeleton (Charnley, 1984). Both *B. bassiana* and *M. anisopliae* are considered generalist pathogens, capable of causing infection across a variety of insect groups including Isoptera, Orthoptera, Homoptera, Hemiptera, Lepidoptera, Hymenoptera and Coleoptera (McCoy et al., 1988; Tanada and Kaya, 1993; Zimmermann, 1993). *V.*

lecanii has been used as a biological control agent of homopteran insects, primarily aphids and scales (Hall, 1981). Despite the ubiquitous distribution of the fungal pathogens, naturally occurring infections have only been recorded on two occasions in field collected *L. lineolaris*: once in the Albany area in New York (Broadbent, 2000) and once in Arkansas (Steinkraus, 1996). In both cases, *L. lineolaris* were infected with *B. bassiana*. The strain isolated in Arkansas, ARSEF 3769, is highly infectious to both *L. lineolaris* nymphs and adults (Steinkraus, 1996). Similar results were found for the strain isolated in New York (Broadbent, 2000).

Over the years, *B. bassiana*, and to a lesser extent, *M. anisopliae*, have been assessed for their potential as biological control agents against *Lygus* bugs (Bajan and Bilewicz-Pawińska, 1971; Bidochka et al., 1993; Leland and Behle, 2005; Liu et al., 2002; Noma and Strickler, 1999; Steinkraus and Tugwell, 1997). In 1999, the development of *Beauveria* isolates for commercial application was considered a research need for biological control of *L. lineolaris* in Canada (Foottit and Mason, 2000). Commercial fungal strains, primarily *B. bassiana*, have enjoyed some success against *L. lineolaris*. Snodgrass and Elzen (1994) reported a 53.8% reduction in nymphs on cotton after spraying the commercial formulation of *B. bassiana*, Naturalis-L (Troy Biosciences, Phoenix, Arizona). However, adult *L. lineolaris* populations were only reduced by an average of 20.2% during a three week period after receiving the same treatment. In contrast, Steinkraus (1996), reported 100% mycosis for adult *L. lineolaris* that were exposed to Mycotrol WP (Mycotech, Butte, Montana), a commercial product containing *B. bassiana*, through direct spray or contact with treated cotton plants. Liu et

al. (2002) also reported high mortality (86.7 ± 2.0%) to second instar nymphs after exposure to *B. bassiana* 726, commercially known as Mycotrol. However, inconsistent results generated in field trials using fungal pathogens against *L. lineolaris* suggest that key challenges remain for their practical use as a biological control agent. Field trials with fungal pathogens have not had an impact on *L. lineolaris* numbers (Kovach, 1996), despite 75-92.1% mortality achieved in screening trials using *M. anisopliae* isolates (Liu et al., 2002), and 90% mortality achieved with mixed *Lygus* spp. in laboratory trials with this pathogen (Bidochka et al. 1993). Virulence can vary among the different strains of a fungal species and is often measured by the response of the insect host with exposure to the pathogen, using the median lethal concentration (LC_{50}) or the median lethal time (LT_{50}) values (Tanada and Kaya, 1993). Features of virulence that vary can include speed of kill, dose required to infect its host, ability to replicate in the host, and toxin production (Tanada and Kaya, 1993). The ability of a pathogen to infect its host has often been assessed through screening trials where groups of insects are exposed to varying concentrations of conidial suspensions and then monitored for death and subsequent infection. When fungal-induced mortality is not observed, one can only surmise that this is due to variation in some aspect of the pathogen's ability to infect its host. The performance of a pathogen can be better understood if researchers could follow the fate of a spore from the time the insect is exposed through to death of the host, whether from fungal or non-fungal causes. A number of studies have evaluated virulence based on factors such as conidial attachment, rate of germination and site of infection (Altre et al., 1999; Hajek and Eastburn, 2003; Hajek et al., 2002; Peng et al.,

2002; Quintela and McCoy, 1998; Sosa-Gómez et al., 1997; Vestergaard et al., 1999).

The objectives of the present study were to determine the direct-contact pathogenicity of BotaniGard® (Mycotech, Butte, Montana), a commercial product with the active ingredient *B. bassiana* (strain GHA) to adult *L. lineolaris*. BotaniGard is recommended for use on a variety of insect pests including phytophagous Hemiptera, such as chinch bugs and lace bugs. Three additional isolates of *M. anisopliae*, *B. bassiana*, and *V. lecanii*, that were native to the Annapolis Valley, Nova Scotia were also assessed for their ability to cause disease based on time of death, mortality and infection levels. Local isolates may be better suited to infecting *L. lineolaris* in Nova Scotia, because they are adapted to local climatic conditions. To understand further the relationship between conidial dose and the development of infection, insects exposed to fungal pathogens were examined microscopically to determine the ability of conidia, for each isolate tested, to attach and germinate on the dorsal cuticle.

2. Materials and Methods

2.1 *Source of pest species*

Adult *Lygus lineolaris* were collected in October 2001, from wildflower field sites located at the Atlantic Food and Horticulture Research Centre (AFHRC) in Kentville, Nova Scotia (45.063° N, 64.488° W). Composition of groundcover was predominantly broad leaf plants including dandelion, *Taraxacum officinale*, common plantain, *Plantago major*, and sheep sorrel, *Rumex acetosella*. Insects were maintained at 21°C, 70% relative humidity (RH) and 16 h light:8 dark photoperiod in wooden framed

isolation cages (25 insects per cage) with nylon screening (47 x 47 x 68 cm) in a temperature controlled rearing room, located at the AFHRC. Cages were lined with white paper, to facilitate finding dead individuals, and three potted green bean, *Phaseolus vulgaris*, plants (Derby variety, Veseys Seeds, York, Prince Edward Island) per cage were provided as food. Plants of the same physiological age and condition were used in all trials and watered daily. *L. lineolaris* were held in isolation cages for 24 hours before being used in bioassays. After this time, only healthy individuals (defined as *L. lineolaris* that flew up to the top of the cage or plant when disturbed) were selected for use in the fungal bioassays.

2.2 *Source and preparation of fungus*

Trials included native *M. anisopliae*, *B. bassiana*, and *V. lecanii* isolates and BotaniGard® 22WP, a commercial formulation of *B. bassiana* strain GHA (Mycotech Corp., Butte, Montana). Native isolates were chosen for this trial based on past performance in other trials (D. Strongman, personal communication) and because they had been isolated locally and would be adapted to regional climatic conditions. Pathogens were isolated by Dr. D. B. Strongman (Saint Mary's University, Halifax, Nova Scotia) from blueberry leaf tier, *Acleris curvalana* Kearfott (Lepidoptera, Tortricidae) and sawfly, *Neopareophora litura* (Klug) (Hymenoptera, Tenthredinidae), larvae collected from blueberry fields in Parrsboro and Sheffield Mills, Nova Scotia (Table 2.1).

Table 2.1. Description of native fungal isolates used in pathogenicity bioassays conducted in October 2001. Fungal strains isolated from field collected insects by Dr. D. B. Strongman, Saint Mary's University, Halifax, Nova Scotia.

Fungal Species†	Identification #	Location	Year	Host Species
Metarhizium anisopliae	MET-01	Sheffield Mills, NS	1999	Sawfly larvae
Beauveria bassiana	1756	Parrsboro, NS	1991	Leaftier pupa
Verticillium lecanii	A2011	Parrsboro, NS	1992	Leaftier pupa

† Isolates were made on malt extract agar and sub-cultured on two occasions before grown for bioassays. Conidia were stored on ceramic beads in cryo-vials and maintained at -80°C since isolation.

Conidial suspensions of BotaniGard were prepared following manufacturer's instructions and the number of conidia per gram of product was measured using a Neubauer-Improved haemocytometer (Hausser Scientific, Horsham, PA). Fungal cultures using the native isolates were grown on malt extract agar (MEA) (Difco, Detroit, MI) supplemented with antibiotics (in g l^{-1}: 20 g agar, 10 g malt extract, 0.5 g Penicillin G, 0.5 g Streptomycin sulphate) in petri dishes (110 x 15 mm). Incubation conditions were 25°C, 85% RH in total darkness. Conidia were harvested and stored at -80°C on ceramic beads in cryo-vials (Pro-Lab Diagnostics, Fisher Scientific, Ottawa, ON) containing 10% glycerol. These frozen suspensions were the source of inoculum for each successive bioassay so no serial transfers were required. Conidia were harvested from 10-day old cultures using 3 mL sterile water containing 0.05% Tween 80 (Fisher Scientific, Ottawa, ON). Conidial concentrations in the inoculum suspension were determined using a Neubauer-Improved haemocytometer and adjusted to the desired final concentration with sterile distilled water. All fungal suspensions were applied to experimental insects within 24 h of preparation.

In addition, conidial viability was verified by plating 100 µl of the conidial suspension on each of three 9 cm petri dishes containing MEA media and incubated in the dark at 25°C. After 24 hours, a drop of 10% lactophenol cotton blue stain and a coverslip was placed on three areas in each petri dish. The number of germinated conidia and total number of conidia were counted in five fields of view at 400x under phase contrast illumination for each plate and used to calculate mean germination rate (Goettel and Inglis, 1997). A conidium was considered viable if the germination tube

was twice the length of the spore.

2.3 *Pathogenicity bioassays*

2.3.1 *Pathogenicity of a commercial formulation of Beauveria bassiana, BotaniGard, to adult Lygus lineolaris*

Trials were conducted in October 2001 to investigate the effect of conidial concentrations on mortality, days to mortality (LT_{50}), and mycosis. Individual insects were treated with either a high (0.3 ml of a 10^6 stock solution to give 3.0×10^5 conidia/vial) or low (0.3 ml of a 10^5 stock solution to give 3.0×10^4 conidia/vial) dose of BotaniGard in conidial suspension. A randomized complete block design (RCBD) was used to account for variation by block differences, where time of trial was the blocking factor (Underwood, 1997). The entire trial was repeated three times (Trial 1, 10-24 October; Trial 2, 17-31 October; and Trial 3, 31 October to 14 November, 2001) and both treatments were tested in each of the three blocks (Table 2.2). Regimes included the fungal treatment groups (where insects were sprayed with conidial suspension) and procedural control (application of 0.05% aqueous Tween 80). A single host in a vial (2.9 mL) was considered one replicate, with 51 to 76 replicates in the treated groups and 53 to 69 replicates in the control group (Table 2.2). A small wad of absorbent cotton was packed into the bottom of each vial and then the vial with cotton was autoclaved. The cotton absorbed excess spray solution to prevent the insects from drowning. Autoclaved sponge plugs were used to prevent the escape of the insect but permit air exchange. Replicate vials were assigned arbitrarily to each

Table 2.2. Concentrations and number of insects for trials conducted in 2001 to assess the susceptibility of *Lygus lineolaris* after exposure to two concentrations of BotaniGard, a commercial formulation of *Beauveria bassiana*.

Bioassay	Trial	Trial date	Concentration (conidia/ml)	*n* size
B. bassiana (high)	1	10-24 Oct.	1.0×10^6	51
B. bassiana (high)	2	17-31 Oct.	1.0×10^6	76
B. bassiana (high)	3	31 Oct.-13 Nov.	1.0×10^6	67
B. bassiana (low)	1	10-24 Oct.	1.0×10^5	54
B. bassiana (low)	2	17-31 Oct.	1.0×10^5	72
B. bassiana (low)	3	31 Oct.-13 Nov.	1.0×10^5	67
Control	1	10-24 Oct.	0	53
Control	2	17-31 Oct.	0	69
Control	3	31 Oct.-13 Nov.	0	68

regime. Conidial solutions were applied, at a rate of 0.3 ml of suspension per insect host, with a micro-atomizer (Kontes Glass Company, New Jersey) connected to a source of compressed nitrogen and regulated at a pressure in the range of 20.5 to 34.5 kPa. Conidia for each isolate tested were also applied to an inert substrate, autoclaved dialysis tubing (7 x 4 mm), placed singly in ten autoclaved vials with cotton on the bottom emulating vials used to hold insects. Both insects and dialysis tubing in vials were incubated at 25°C, 70% RH and in the dark. After 24 h, individual insects were transferred to sterile petri-dishes (110 x 15 mm), supplied with a piece of green bean (approx. 15 mm) and examined every 24 hours for 14 days after treatment. Pieces of bean were replaced every three days. Four to six individuals, (both live and dead) arbitrarily chosen, were removed from each treatment and control group and tested at 24, 48, 72, 96 and 120 h post-inoculation, in Trials 1 and 3. Insects were immediately frozen at -86°C for electron microscopy trials. Individual hosts were examined for changes in behaviour and evidence of disease development using a binocular microscope and daily mortality was recorded. Dead insects were removed from the bioassay and placed on sterile moistened filter paper (Whatman No. 1) within a petri-dish (110 x 15 mm) and incubated for three to five days to assess fungal growth. For all trials, individuals were recorded as killed by fungus if there were signs of mycosis, as determined by the development of fungus in cultures or on the cadavers. Re-cultures, on MEA and Sabouraud's dextrose agar (in g l^{-1}: 10 g peptone, 40 g dextrose, 15 g agar) supplemented with 1% yeast extract (SDAY), and wet mounts were made from colonized individuals to observe fungal morphology (hyphal bodies and/or

conidia).

2.3.2 *Pathogenicity of native isolates Metarhizium anisopliae, Beauveria bassiana and Verticillium lecanii to adult Lygus lineolaris*

Bioassays were conducted using varying concentrations of the native isolates of *M. anisopliae*, *B. bassiana* and *V. lecanii*. Final concentrations of the conidial suspensions were low because relatively low numbers of conidia were harvested from cultures. Individual insects were treated with 0.3 ml of a stock conidial solution (Table 2.3). A completely randomized design (CRD), was used to assign individuals to the different regimes, where regimes included the fungal treatment groups (insects were sprayed with conidial suspension) and procedural control (application of 0.05% aqueous Tween 80). A single host in a vial (2.9 mL) was considered one replicate, with 52 to 75 replicates in the treated groups and 53 to 69 replicates in the control group (Table 2.3). Bioassay procedures were identical to those discussed previously for assessment of BotaniGard. The entire experiment was replicated three times (Trial 1, 10-24 October; Trial 2, 17-31 October; and Trial 3, 31 October to 14 November, 2001).

2.4 *Statistical analysis for pathogenicity bioassays*

Mean cumulative mortality and mycosis values were calculated by dividing the number of dead or infected by the total number of live and dead or infected individuals. The SAS LIFETEST procedure (SAS Institute 1990) was used to calculate non-parametric estimates of mortality and mycosis trends over time of exposure with

Table 2.3. Concentrations and number of insects for trials conducted in 2001 to assess the susceptibility of *Lygus lineolaris* after exposure to varying concentrations of *Metarhizium anisopliae*, *Beauveria bassiana*, and *Verticillium lecanii*. Fungi were isolated from dead insects collected in Nova Scotia (Table 2.1).

Bioassay	Trial	Trial date	Concentration (conidia/ml)	*n*-size
M. anisopliae	1	10-24 Oct.	5.7×10^4	59
M. anisopliae	2	17-31 Oct.	1.0×10^5	65
M. anisopliae	3	31 Oct. - 13 Nov.	1.6×10^5	66
B. bassiana	1	10-24 Oct.	4.3×10^4	60
B. bassiana	2	17-31 Oct.	6.7×10^4	75
B. bassiana	3	31 Oct. - 13 Nov.	6.3×10^4	68
V. lecanii	1	10-24 Oct.	1.3×10^5	52
V. lecanii	2	17-31 Oct.	1.3×10^5	57
V. lecanii	3	31 Oct. - 13 Nov.	1.1×10^5	65
Control	1	10-24 Oct.	0	53
Control	2	17-31 Oct.	0	69
Control	3	31 Oct. - 13 Nov.	0	68

their corresponding estimated variances (Marcus and Eaves, 2000). Log-rank and Wilcoxon statistics were also generated for testing homogeneity of mortality and mycosis distributions between treated and control groups. Both statistics were considered as Log-Rank placed more weight on the larger survival times, whereas, the Wilcoxon is sensitive to shorter survival times. Median lethal time (LT_{50} values) leading to 50% mortality or mycosis were generated, for treated and control insects, as summary measures of the cumulative probability of mortality or mycosis. Comparison of LT_{50} values were used for BotaniGard bioassays to see if there was significant effect of the blocking factor, time period when the experiment was conducted. Graphs were plotted using Slide Write Plus 6.1 graphics (Advanced Graphics Software for Windows, V. 6.10, 2002).

2.5 *Observations of attachment, germination, and spatial distribution of conidia*

Preliminary observations of attachment, germination, and spatial distribution of conidia on the cuticle of the insect host involved assessment of 23 adult *L. lineolaris*, for all isolates tested in Trial 1, using a LEO 1450 variable pressure scanning electron microscope (VPSEM) (LEO Electron Microscopy Ltd., Cambridge, U.K.) interfaced with a Gatan C1002 cold stage attachment (Gatan Incorporated, Abingdon, U.K.), located at Saint Mary's University, Halifax, Nova Scotia. Insects were taken directly from frozen storage and mounted dorsal side up, using Tissue Tek adhesive (Fisher Scientific, Ottawa, ON), and inserted into the VPSEM. Conventional fixation techniques and sputter coating are not required for examination of insects if using a

cold stage. Only the dorsal cuticular surface was examined as it was assumed that the dorsal surface would most likely come into contact with conidia since they were applied by spray from above in the assays. The distribution of conidia was evaluated by cuticular region. One to three observations were taken from each of seven regions on the dorsal surface of the cuticle: head, pronotum, mesoscutum, scutellum, right wing, left wing, and wing membrane, as shown in Table 2.4. Care was taken to ensure that observations were taken from approximately the same area on each insect sampled. Conidia on the dorsal surfaces of cadavers were photographed at 400x while being examined under the VPSEM. Insects were held under vacuum for up to 2 h. Digitized images were examined at a later date to make counts and determine location of conidia and germinating conidia on the insect cuticle. An acetate template (219 μm x 293 μm to scale), further sub-divided into 16 grids, was created to overlay on captured digital images of each observation made and was used to quantify conidial density and production of germination tubes for up to 17 observations per adult insect (Table 2.4). An observation was defined as all conidia or germinating conidia found within a 219 x 293 μm quadrat. Conidia were considered to have germinated if the germ tube was at least the length of the conidium. The number of ungerminated and germinated conidia associated with the location where conidia were attached were recorded.

2.6 *Statistical analysis for scanning electron microscopy*

Conidial and germinating conidial densities per unit area (mm^2) for each body

Table 2.4. Area calculations made for body regions (excluding wing membrane) observed on the dorsal cuticular surface of adult *Lygus lineolaris* exposed to fungal pathogens in bioassays conducted in October and November 2001.

Body region	Total region area (mm^2)	Area of region vs surface area (%)	No. of observations	Total area observed (mm^2)	Area observed/total surface (%)
Head	0.30	2.6	2	0.12	1.1
Pronotum	2.61	23.2	3	0.18	2.1
Mesoscutum	0.45	4.0	2	0.12	1.1
Scutellum	0.55	4.9	3	0.18	2.1
Right wing	3.66	32.6	3	0.18	1.6
Left wing	3.66	32.6	3	0.18	1.6

region were computed for individual insects (Table 2.4). The number of conidia for each observation was summed and divided over the total number of observations taken for the entire region. To correct for bias caused by body regions that were different in size, the area of each body region was estimated using a number of geometric shapes to assume the approximate area (e.g. tetrahedron and triangles for the pronotum). The total area observed in a region was then divided by the total region area to establish a correction factor for differing sizes of regions (Table 2.4). Mean densities for conidia and germinating conidia per unit area observed for each body region for individual insects were then multiplied by the correction factor to estimate counts of conidia and germinating conidia for an entire region. Comparison of LT_{50} values were used for BotaniGard bioassays to assess the effects of spore and germinating spore counts on survival time in days after exposure.

3. Results

3.1 *Pathogenicity bioassays*

3.1.1 *Pathogenicity of a commercial formulation of Beauveria bassiana, BotaniGard, to adult Lygus lineolaris*

Individual adult *L. lineolaris* were dosed with two concentrations of BotaniGard: a high rate (0.3 ml of a 10^6 stock solution to give 3.0 x 10^5 conidia/vial) and low (0.3 ml of a 10^5 stock solution to give 3.0 x 10^4 conidia/vial). Data sets generated from the three blocks in time (trials) were not combined because mortality and mycosis determined for insects treated at the higher concentration in Trial 1 were shown to be

significantly different from Trial 2 (log-rank test, $\chi^2 = 2.26$, df = 2, $P < 0.13$). Similarly, mortality and mycosis observed in insects treated at the lower concentration were significantly different between Trials 1 and 2 (log-rank test, $\chi^2 = 30.42$, df = 2, $P < 0.001$) and Trials 2 and 3 (log-rank test, $\chi^2 = 28.67$, df = 2, $P < 0.001$). Associated LT_{50} values ranged from 4 to 6 days for both mortality and mycosis observed in insects exposed to both concentrations of BotaniGard (Table 2.5). Final mortalities were similar for both insects treated at either concentration in all three trials, ranging from 85 to 100% (Figures 2.1 A-C), however, mortality increased more rapidly with the higher concentration in Trials 1 and 3. Mycosis for insects treated with BotaniGard followed similar trends to those for mortality, with infection levels ranging from 73 to 96% for low and high concentrations (Figure 2.2 A-C). Procedural control mortality averaged 23.9 ± 3.2%, for insects across all three trials (Table 2.5). The highest concentration, for all three trials, produced mortality significantly different from the procedural control group (Trial 1, log-rank test, $\chi^2 = 105.87$, df = 1, $P < 0.001$; Trial 2, $\chi^2 = 114.79$, df = 1, $P < 0.001$; Trial 3, $\chi^2 = 108.66$, df = 1, $P < 0.001$). Mortality for insects treated at the lower dose was also significantly different from the controls for Trial 1 (log-rank test, $\chi^2 = 46.50$, df = 1, $P < 0.001$; Trial 2, $\chi^2 = 109.52$, df = 1, $P < 0.001$; Trial 3, $\chi^2 = 46.70$, df = 1, $P < 0.001$). For mortality observed in the procedural control group in Trials 1, 2 and 3, mean days to death ranged from 9 to 12 days (Table 2.5), compared to 4 to 7 days for treated insects. Mean days to death (4 to 6 days) for infected insects was similar to that observed for all insects that had died. Infection was not observed in the control group with the exception of three individuals in Trial 1,

Table 2.5. Mortalities of *Lygus lineolaris* in Trials 1-3, after exposure to two concentrations of BotaniGard, a commercial formulation of *Beauveria bassiana*. Within a treatment, different letters for LT_{50} values denote a significant difference between trials, for a given concentration, using the SAS procedure LIFETEST (SAS Institute 1994). Confidence limits with no value for determined LT_{50} values were inestimable.

Treatment (conidia/vial)	**Trial**	***n*-size**	**Cumulative mortality (%)†**	**Mean time to death (days ± SE)§**	**LT_{50}**	**95% Confidence Interval (days)**	**Mycosis (%)**	**Mean time to mycosis (days ± SE)‡**
B. bassiana (3×10^5)	1	51	100	4.04 ± 0.20	0.17	-	96.1	4.21 ± 0.22
B. bassiana (3×10^5)	2	76	100	4.36 ± 0.13	0.17	4 - 5	89.5	4.51 ± 0.13
B. bassiana (3×10^5)	3	67	98.5	5.18 ± 0.26	5 b	-	91	5.39 ± 0.24
B. bassiana (3×10^4)	1	54	88.9	7.24 ± 0.40	0.25	6 - 7	81.1	6.51 ± 0.32
B. bassiana (3×10^4)	2	72	100	4.72 ± 0.22	4 b	4 - 5	90.3	4.74 ± 0.22
B. bassiana (3×10^4)	3	67	85.1	7.25 ± 0.43	0.25	5 - 7	73.1	6.65 ± 0.39
Control	1	53	30.2	12.58 ± 0.40	-	-	5.7	6.67 ± 0.33
Control	2	69	20.9	9.98 ± 0.30	-	-	0	-
Control	3	68	20.6	10.96 ± 0.30	-	-	0	-

† Cumulative mortality represents death due to infection and unknown causes combined
§ Mean days to death included death due to infection and unknown causes combined
‡ Mean days to mycosis includes death due to infection only

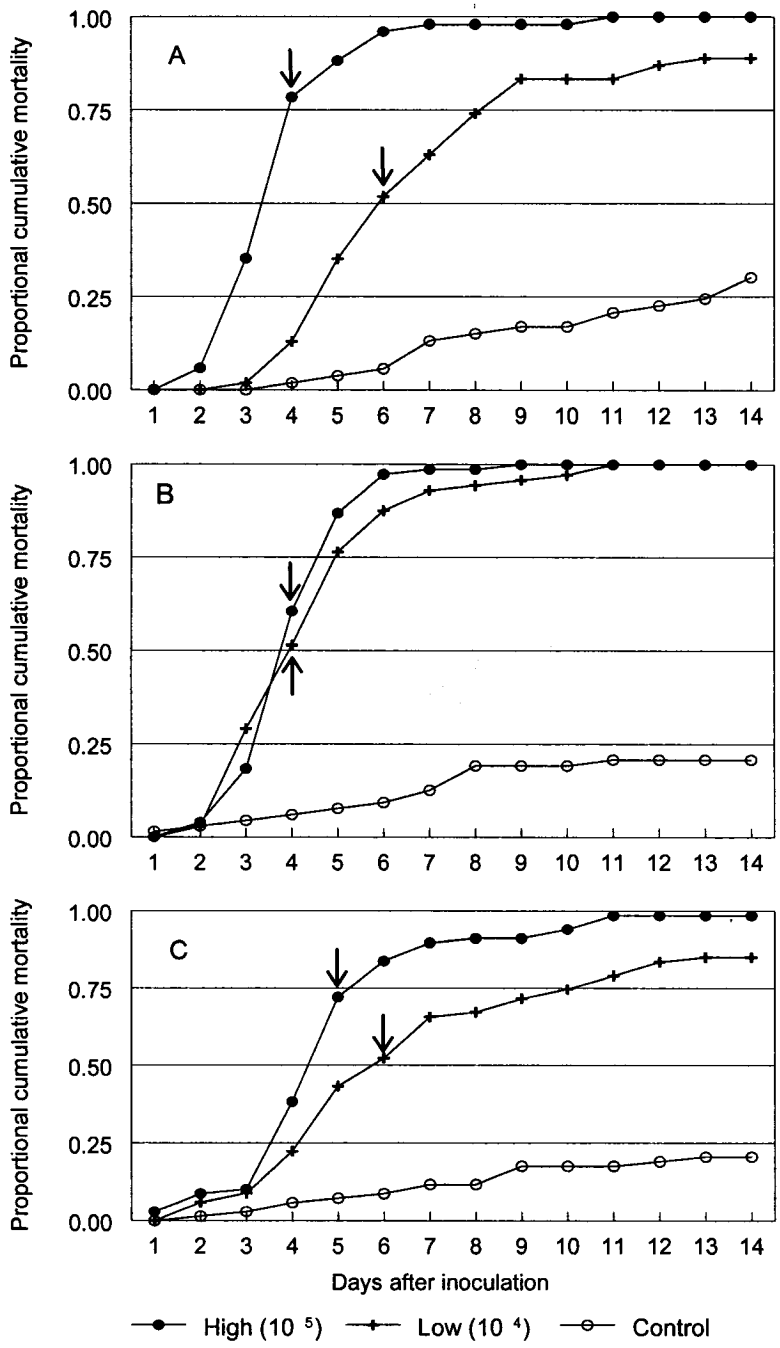

Figure 2.1. Cumulative total mortality (fungal and non-fungal) of adult *Lygus lineolaris* after spray application of BotaniGard at high and low doses/insect. Legend for bioassays: Trial 1 (A), Trial 2 (B), and Trial 3 (C). Arrows show LT_{50} values for high and low concentrations of BotaniGard.

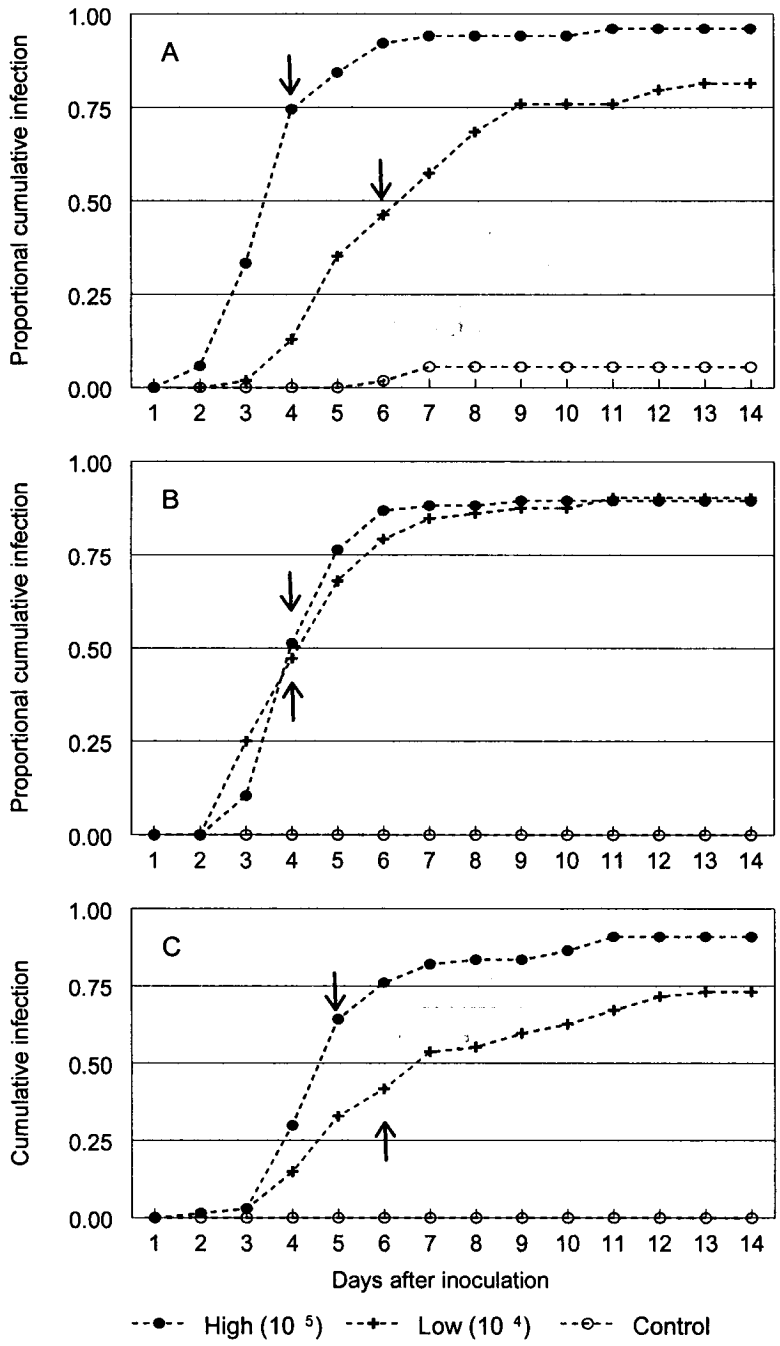

Figure 2.2. Cumulative proportion of adult *Lygus lineolaris* that died of fungal infection (infected insects/total dead insects) after spray application of BotaniGard at high and low doses/insect. Legend for bioassays: Trial 1(A), Trial 2 (B), and Trial 3 (C). Arrows show LT_{50} values determined for infected insects only, for both concentrations of BotaniGard.

where the pathogen was confirmed as *B. bassiana*. Infection was likely due to cross-contamination when fungal treatments were being applied.

Based on the log-rank and Wilcoxon tests, comparison of the two concentrations revealed significant differences for all trials except for Trial 2 (log-rank test, $\chi^2 = 2.29$, wilcoxon, $\chi^2 = 0.37$, df = 1, $P > 0.05$). Calculated LT_{50} values showed that 50% of infected insects dying at 4 or 5 days versus 4 or 6 days, respectively (Table 2.5). No differences were detected between concentrations, based on the associated confidence intervals for LT_{50} values generated for Trial 2. Days to death were similar for both concentrations, with the first mortality observed on day 2 (high concentration) and 3 (low concentration) in Trial one, day 2 in Trial 2, and day 1 (high) and 2 (low) in Trial 3 (Figure 2.1 A-C). The procedure LIFETEST was not always able to estimate confidence intervals for the LT_{50} values that corresponded to both concentrations (Table 2.5). A wide range of values used to calculate survival distributions would cause large or inestimable confidence intervals. With an increase in conidial concentration, the mean survival time (mean number of days from treatment to insect death ± SE) was less for all three trials (Table 2.5).

Overall, the number of insects that were still alive 14 days after fungal treatment was low, with no survivors exposed at the high concentration in both Trials 1 and 2. Furthermore, all insects treated at the low concentration in Trial 2 had died by day 11 (Figure 2.1 B). At both concentrations, all were dead by 13 days after inoculation. Although, individual insects exposed to the low concentration were treated with one-tenth of the high rate, cumulative mortality and mycosis were similar (Figures 2.1, 2.2 A-C).

For example, by day 7 in Trial 2, 93 % of insects treated at the lowest concentration had died and 85% had died of infection. Insects treated at the highest concentration showed 99% mortality and 88% mycosis at the same time interval. Fungal development was observed on infected insects within 2-3 days of their recorded death and external sporulation occurred soon after.

3.1.2 *Pathogenicity of native isolates of Metarhizium anisopliae, Beauveria bassiana and Verticillium lecanii* to *adult Lygus lineolaris*

Individual adult *L. lineolaris* were exposed in Trials 1, 2 and 3 to varying concentrations of *M. anisopliae*, *B. bassiana*, and *V. lecanii*, as shown in Table 2.6. Total mortality across the three trials was highest for *M. anisopliae*, intermediate for *B. bassiana*, and lowest for *V. lecanii*, respectively (Figures 2.3 A-C). Death due to mycosis was highest for insects treated with *M. anisopliae* (Figure 2.4). Mortality for insects treated with *M. anisopliae* and *B. bassiana*, for all three trials, were significantly different from those in the procedural control group (Table 2.6). Mean days to death (8 to 10 days) for insects that died after exposure to *M. anisopliae* and *B. bassiana* were lower than that observed in the control group, for all three trials. Infected insects died, on average, 7 to 8 days after exposure to *M. anisopliae* and 9 to 11.5 days for insects treated with *B. bassiana* (Table 2.6). Mortality was significantly different between Trial 1 and 3 (log-rank test, $\chi^2 = 4.43$, df = 1, $P < 0.035$) and at the smaller survival times observed between Trials 1 and 2 (Wilcoxon test, $\chi^2 = 5.18$, df = 1, $P < 0.023$) for insects sprayed with *M. anisopliae*. This is likely due to the differences in concentrations (Table 2.6).

Table 2.6. Mortalities of *Lygus lineolaris* for Trials 1-3, after exposure to each of three native fungal isolates, *Metarhizium anisopliae*, *Beauveria bassiana*, and *Verticillium lecanii*. LT_{50} values and associated confidence limits for each trial, for a given fungal isolate, were generated using the SAS procedure LIFETEST (SAS Institute 1994). Within a treatment, different letters for LT_{50} values denote a significant difference between trials, for a given concentration. Confidence limits with no value for determined LT_{50} values were inestimable.

Treatment (conidia/vial)	**Trial**	***n*-size**	**Cumulative mortality (%)†**	**Mean time to death (days ± SE)§**	**LT_{50}**	**95% Confidence Interval (days)**	**Mycosis (%)**	**Mean time to mycosis (days ± SE)‡**
M. anisopliae (1.7 x 10^4)	1	59	76.3	9.97 ± 0.46	10 a	8 - 12	52.5	4.21 ± 0.22
M. anisopliae (3.1 x 10^4)	2	65	80	8.20 ± 0.40	7 b	7 - 9	61.5	4.51 ± 0.13
M. anisopliae (4.8 x 10^4)	3	66	80.3	8.05 ± 0.50	7 b	6 - 9	59.1	5.39 ± 0.24
B. bassiana (1.3 x 10^4)	1	60	58.3	10.37 ± 0.53	11.5a	10 (L)	31.7	6.51 ± 0.32
B. bassiana (2.0 x 10^4)	2	75	62.7	9.25 ± 0.34	10 a	8 - 12	21.3	4.74 ± 0.22
B. bassiana (1.9 x 10^4)	3	68	67.7	9.34 ± 0.50	9 a	8 - 12	30.9	6.65 ± 0.39
V. lecanii (3.8 x 10^4)	1	52	36.5	11.92 ± 0.47	-	-	23.1	-
V. lecanii (4.0 x 10^4)	2	57	45.6	10.46 ± 0.46	-	10 (L)	5.3	-
V. lecanii (3.4 x 10^4)	3	65	46.2	10.97 ± 0.53	-	-	3.1	-
Control	1	53	30.2	12.58 ± 0.40	-	-	5.7	6.67 ± 0.33
Control	2	69	20.9	9.98 ± 0.30	-	-	0	-
Control	3	68	20.6	12.26 ± 0.52	-	-	0	-

† Cumulative mortality represents death due to infection and unknown causes combined
(L) Represents lower limit of confidence interval
‡ Mean days to mycosis includes death due to infection only

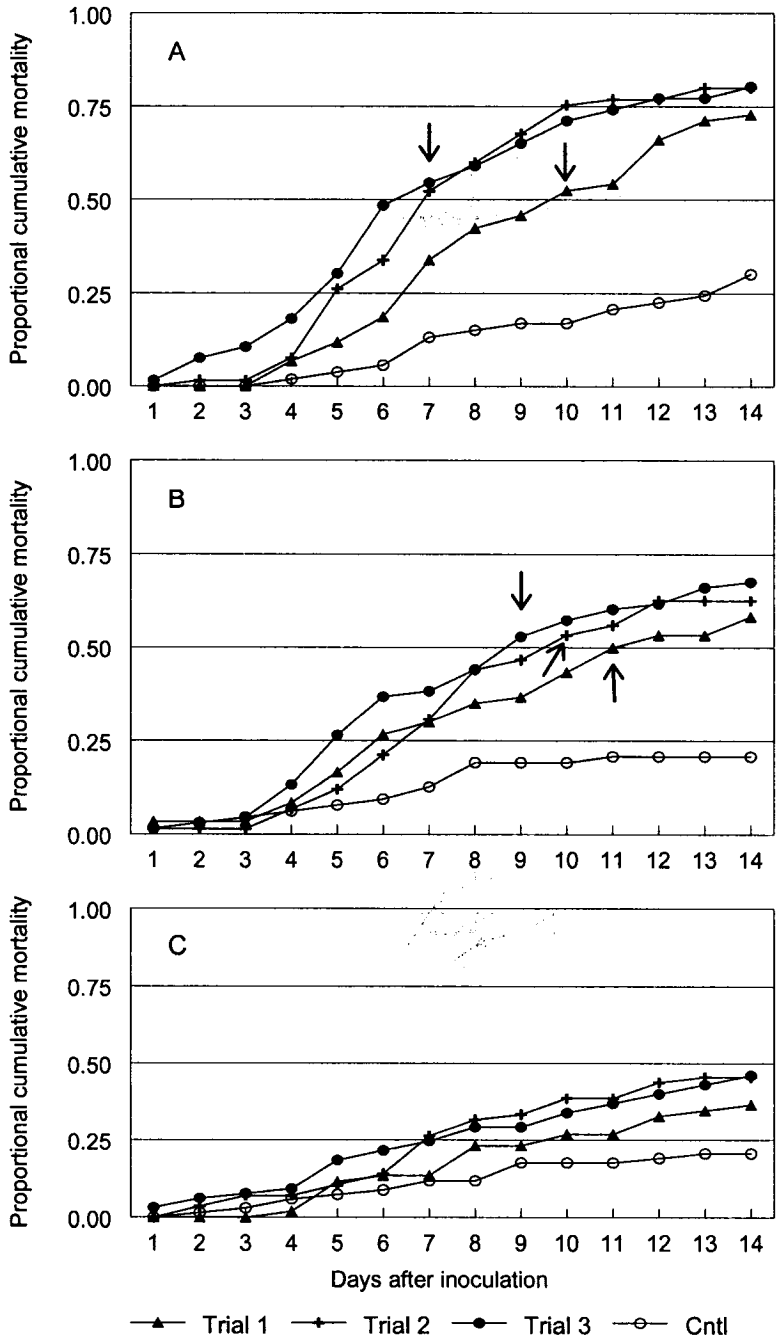

Figure 2.3. Cumulative total mortality (fungal and non-fungal) of adult *Lygus lineolaris* after spray application of one of three native isolates *Metarhizium anisopliae* (A), *Beauveria bassiana* (B), and *Verticillium lecanii* (C) at varying concentrations. Legend for bioassays: Trial 1 (solid triangle), Trial 2 (plus sign), Trial 3 (solid circle), and control (hollow circle). Arrows show LT_{50} values for *M. anisopliae* and *B. bassiana* treated insects only.

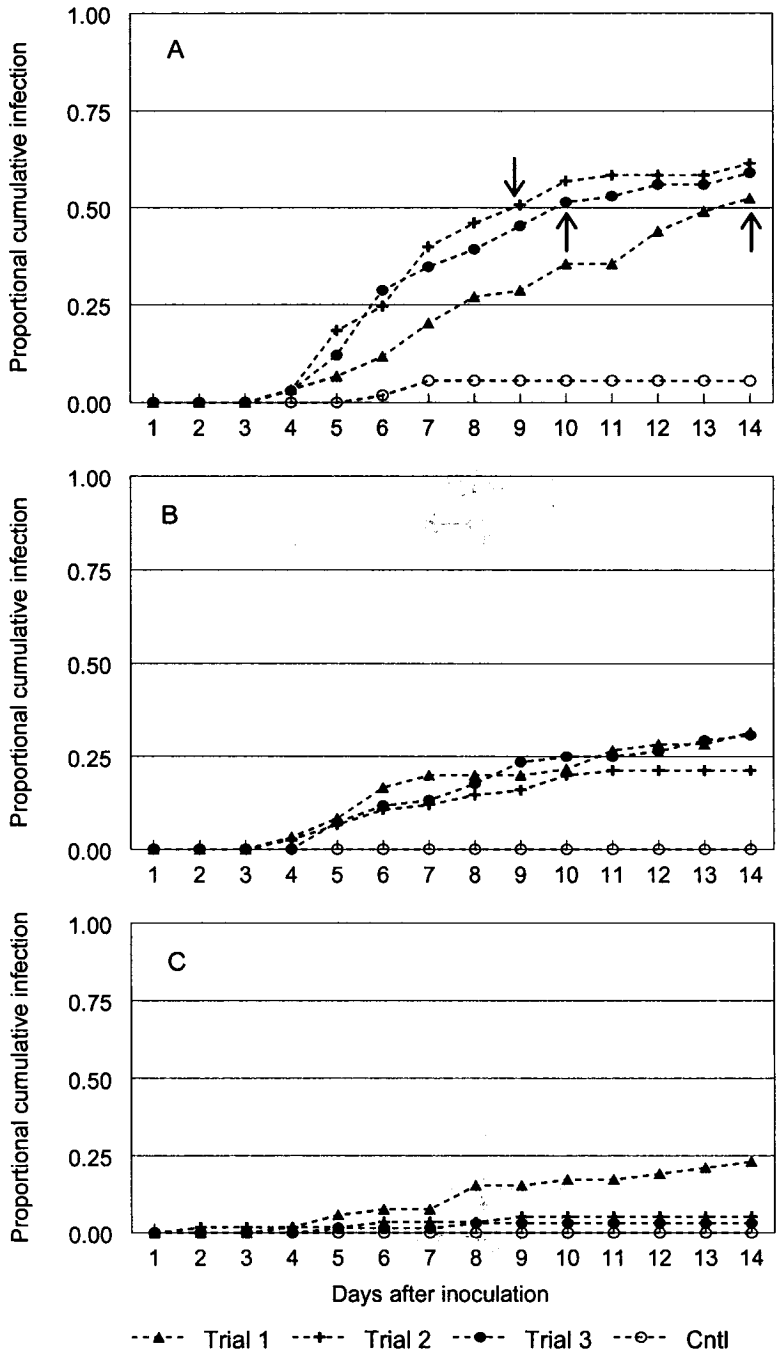

Figure 2.4. Cumulative proportion of adult *Lygus lineolaris* that died of fungal infection (infected insects/total dead insects) after spray application of each of three native isolates *Metarhizium anisopliae* (A), *Beauveria bassiana* (B), and *Verticillium lecanii* (C) at varying concentrations. Legend for bioassays: Trial 1 (solid triangle), Trial 2 (plus sign), Trial 3 (solid circle), and control (hollow circle). Arrows show LT_{50} values for *M. anisopliae* treated insects only.

Days to initial deaths were similar for all treatments, across all concentrations, whereas initial deaths of infected insects were observed on day 4 (day 2 for *V. lecanii*), as shown in Figure 2.4 B and C, versus 1 to 2 days for insects that died but were uninfected (Figure 2.3). Figure 2.3 (A-C) illustrates the effects of concentration on time of death, where higher concentrations of conidia caused earlier time of death as compared with lower concentrations for *M. anisopliae* and *B. bassiana*. With an increase in conidial concentration, the mean survival time (mean number of days from treatment to insect death ± SE) was less with *M. anisopliae* and *B. bassiana* (Table 2.6). When dead insects were removed and placed in a humid chamber, initial signs of disease development were often obvious 3 d after death and included a distended, mummified abdomen. Shortly after, mycelia were observed coming out of the cuticle, primarily through the inter-segmental membranes, anus and leg joints.

3.2 *Observations of attachment, germination, and spatial distribution of conidia*

Attachment of conidia to the insect cuticle and conidial germination were observed for all fungal isolates tested in this study (Table 2.7). Total numbers of conidia on the dorsal surface of cuticles of treated insects were much higher for insects exposed to the high concentration BotaniGard with conidia and germinating conidia counts ranging from 336 to 1,873 (n=5) and 0 to 103 (n=5), respectively (Table 2.7). Insects treated at a lower concentration of BotaniGard had conidial counts of 67 to 284 (n=6) and germinating conidial counts of 0 to 25 (n=6), similar to those achieved with the native isolate *M. anisopliae* (40 to 207 conidia and 0 to 60 germinating conidia, n=6), as

Table 2.7. Observed conidia, germinating conidial counts, and percent germination taken at varying times post-inoculation for individual insects examined using a VPSEM.

Insects sampled	Pathogen	Days after treatment	Status of insect §	Total conidia	Total germinated conidia	Percent germination
1	BotaniGard (high)	0	Live	1873	0	0
2	BotaniGard	24	Live	1072	62	5.8†
3	BotaniGard	48	Dead	758	59	7.8
4	BotaniGard	72	Dead	587	103	17.5
5	BotaniGard	96	Dead	336	3	0.9
6	BotaniGard (low)	0	Live	284	0	0
7	BotaniGard (low)	24	Dead	161	25	15.5†
8	BotaniGard (low)	48	Live	204	22	10.8
9	BotaniGard (low)	72	Dead	67	9	13.4
10	BotaniGard (low)	96	Dead	201	9	4.5
11	BotaniGard (low)	120	Dead	184	13	7.1
12	*M. anisopliae*	0	Live	104	0	0
13	*M. anisopliae*	24	Dead	207	19	9.8
14	*M. anisopliae*	48	Dead	126	60	47.6†
15	*M. anisopliae*	72	Live	201	54	26.9
16	*M. anisopliae*	96	Dead	138	9	6.5
17	*M. anisopliae*	120	Dead	40	15	37.5
18	*B. bassiana*	0	Live	60	0	0
19	*B. bassiana*	24	Dead	59	13	22
20	*B. bassiana*	48	Live	48	5	10.4
21	*B. bassiana*	72	Dead	46	7	15.2
22	*B. bassiana*	96	Dead	95	14	14.7
23	*B. bassiana*	120	Dead	87	18	20.7†
24	*V. lecanii*	0	Live	15	0	0
25	*V. lecanii*	24	Live	56	3	5.4
26	*V. lecanii*	48	Live	16	1	6.3
27	*V. lecanii*	72	Dead	34	3	8.8
28	*V. lecanii*	96	Dead	29	3	10.3

§ Status of insect when removed from bioassay and frozen for VPSEM assessment
† Individual insects where germinating conidia were recorded on every body region sampled.

shown in Table 2.7. Overall conidial counts for individual insects exposed to the additional native isolates were even lower, ranging from 46 to 95 (n=6), and 15 to 56 (n=5) for *B. bassiana* and *V. lecanii*, respectively. Germinating conidial counts per individual ranged from 0 to 18 for *B. bassiana* and 0 to 3 for *V. lecanii* (Table 2.7). In some individuals, conidial germination was observed within 24 h post inoculation for all fungal isolates tested. Areas of the insect cuticle sampled under VPSEM comprised approximately 10% of the total area on the dorsal surface available for observation (excluding the wing membrane).

Percentage germination did not exceed 50% for the 23 insects, examined 24 h to 120 h post-inoculation, and almost half (n=10) were observed with germination of less than 10% (Table 2.7). Unfortunately it proved difficult to determine an accurate assessment of percentage germination for insects treated at either concentration of BotaniGard, due to the extensive hyphal growth observed within 48 h post inoculation. In nearly every case, conidia and germinating conidia were observed on all body regions when all insects given a specific treatment were considered. Insects exposed to *V. lecanii* were the only exception where no conidia were observed on the head and germination was never observed on the head and mesoscutum. Germinating conidia were only recorded on every body region sampled for four individuals, two were from the BotaniGard treatment (Table 2.7). Patterns of conidial deposition observed on the different body regions may influence whether or not an insect subsequently dies of infection or not, but this hypothesis could not be tested due to the lack of replication.

No conidia were observed on the cuticle of untreated adults (control group) that

received only a direct application of 0.05% Tween 80 solution. No attempt was made to recover conidia that were on the sides of the glass vial that individuals had been in when sprayed. Conidia and numerous germ tubes, for all fungal pathogens tested, were observed on treated dialysis tubing 48 h post inoculation. Quantification of percentage germination was not possible due to the extensive hyphal growth on the dialysis tubing.

4. Discussion

4.1 *Pathogenicity bioassays*

4.1.1 *Pathogenicity of a commercial formulation of Beauveria bassiana, BotaniGard to adult Lygus lineolaris*

BotaniGard was highly pathogenic to adult *L. lineolaris* with a final observed mortality, after 14 days, greater than 85% at both conidial concentrations tested compared with fungal mortality in the procedural control group (< 1%) in Trial 1 (Figure 2.2). Mortality rates of this study were considerably higher than those observed by Brown et al. (1997), where a full rate (1.12 kg/ha) of Mycotrol WP (commercial formulation of *B. bassiana*, strain GHA) sprayed on adult *L. lineolaris* maintained on cotton plants in cages, resulted in 43.8% mortality seven days after treatment with only 52% of that death attributed to mycosis. This is not surprising considering that controlled laboratory trials aim to ensure that experimental insects come into contact with conidia, most often at high doses, versus insects that are inoculated through sprays in a field setting. A number of factors (e.g. ultra-violet light, temperature, humidity, etc.) further influence subsequent disease development in field and semi-field studies versus

controlled laboratory conditions (Fuxa, 1995; Leland and Behle, 2005).

Although there was mortality in the procedural controls, no infection was observed in the majority (94.3%) of insects treated. Fungal infection observed in three control insects in trial one, was probably due to cross contamination when treatments were being applied since no infection was ever detected in monitored colonies (n = 100 insects/trial) maintained in cages prior to being used in the trials. One explanation for the level of mortality observed in the controls is that adult *L. lineolaris* were field collected and of mixed age, with a proportion of the insects being first generation adults. Late season observations of *L. lineolaris* on alfalfa in Saskatchewan, suggested that all of the second generation adults, but only a proportion of first generation adults, will enter reproductive diapause and overwinter (Craig, 1983). First generation adults that are not going to overwinter would probably 'die off' at the end of the growing season.

Of interest is that the higher (3.0×10^5 versus 3.0×10^4 conidia/insect in a vial) dose of BotaniGard yielded only a modest (< 14.9%) increase in overall mortality, although the rate of kill was faster than for insects exposed to lower concentrations. Bajan and Bilewicz-Pawińska (1971) reported 100% mortality of second instar *Lygus rugulipennis* nymphs after five and 10 days exposure to high (2×10^6 conidia/ml) and low (5×10^5 conidia/ml) concentrations of *B. bassiana* suspensions, respectively. Steinkraus and Tugwell (1970), also observed high mortality in adult *L. lineolaris* five days after exposure to high concentrations versus seven days with exposure to low concentrations of *B. bassiana* conidia.

Speed of kill has been considered an important characteristic of fungal pathogens for

use as biological control agents (Roberts and St. Leger, 2004). In this study, 50% of treated insects in both the high and low concentration of BotaniGard died much sooner (4 to 6 days) than control insects (9 to 12 days). Although, the BotaniGard treatment gave high mortality and infection levels, the rate of kill may be too slow to prevent *L. lineolaris* populations approaching economic threshold. On apple, the time frame within which *L. lineolaris* attack is rather short-lived; initial dispersal onto the trees takes place shortly after the delayed-dormant bud stage and continues until fruitlets are about 12 mm in diameter (Hammer, 1939). Continued damage to the crop is the cost of delayed kill of an economic pest, especially if it takes four to six days before 50% of the target population finally succumbs to disease. However, one issue is that time of death may be much shorter in laboratory trials where temperatures are often much higher than in the field, particularly in May and June when *L. lineolaris* attack apples in Nova Scotia. Fuxa (1995), considered a three to four day knock down period post-inoculation characteristic of a 'slow' pathogen. In some cases, a slower rate of kill can be acceptable providing that insect pressure to the crop is reduced through sub-lethal impacts, such as reduced feeding or decrease in adult longevity and fecundity. However, little is known about these effects for fungi (Hajek and Goettel, 2000). Laboratory trials showed a decrease in consumption of maize leaves by second and third instar stem borer, *Chilo partellus* (Lepidoptera, Pyralidae), three and four days after exposure to *B. bassiana or M. anisopliae* suspensions, respectively (Tefera and Pringle, 2003). However, feeding by adult *L. lineolaris* after exposure to *B. bassiana* in laboratory bioassays was not reduced until late in the infection stage (Brown et al., 1997).

Another strategy is the combining of control agents to overcome the slower kill rate often observed when fungal pathogens are used alone as a treatment; this also serves in reducing chemical loads in the environment (Benz, 1971). Adult *L. lineolaris* numbers were reduced by 97.9% five days after being sprayed with a combination of Mycotrol (280 g/ha) and Imidacloprid (50 g AI/ha), in field trials on cotton. When insects were treated with only Mycotrol, mortality only reached 52.0% after five days in contrast to 67.3% for individuals treated with Imidacloprid (Steinkraus, 1996). Brown et al. (1997), also reported greater (68.7%) control after 7 days against adult *L. lineolaris* treated with a combination of Mycotrol (0.56 kg/ha) and Provado 1.6F (0.03 kg AI/ha), an insecticide containing imidacloprid, at half the recommended rates versus the full rate of Mycotrol (1.12 kg/ha) alone. In both cases, control was achieved against an insect pest after inoculation with a fungal pathogen that had been combined with a reduced rate of insecticide. One possible mechanism for the synergism is that certain insecticides interfere with grooming. In this study, lethargy and less preening was observed in daily inspection of treated insects in bioassays.

Steinkraus (1996), also suggested that modifications to application methods (e.g. spray trap crops or weedy borders) may overcome issues of rate of kill for the commercially formulated *B. bassiana*, Mycotrol, against *L. lineolaris* on cotton. Some success was also realized against larval red flour beetle, *Tribolium castaneum* (Herbst) (Coleoptera, Tenebrionidae) after exposure to wheat treated with diatomaceous earth and *B. bassiana* conidia. Improved performance of *B. bassiana* was attributed to increased conidial attachment because of damage to the insect cuticle from the

diatomaceous earth (Akbar et al., 2004). An integrated approach that combines the pathogen with reduced rates of chemical or 'organic' (e.g. diatomaceous earth) controls may offer a more practical and effective strategy. Overall, the number of insects that were still alive 14 days after inoculation in this study was low, suggesting that BotaniGard is capable of reducing populations of adult *L. lineolaris*. The implications of the slower rate of kill may be resolved if the product can be successfully used in conjunction with another control agent, since high mortality was achieved with only a small number of conidia observed on the dorsal cuticle and different application rates produced similar mortality.

4.1.2 *Pathogenicity of native isolates of Metarhizium anisopliae, Beauveria bassiana and Verticillium lecanii to adult Lygus lineolaris*

Of the three native isolates tested, *M. anisopliae* proved to be the most effective in killing *L. lineolaris*. The number of insects exposed to *M. anisopliae* that subsequently died of infection (52.5 to 61.5%) was considerably less than that observed in insects treated with BotaniGard at the high (89.5 to 96.1%) and low (73.1 to 90.3%) concentrations. Mycosis levels were even lower for native isolates of *B. bassiana* and *V. lecanii*, never exceeding 32% for either fungal pathogen. However, the concentrations used in this study were half that typically used in laboratory assays.

At the highest concentration, *M. anisopliae* resulted in 61.7% mortality, despite having been isolated from a sawfly larva (Order: Hymenoptera). Similarly, Liu et al. (2002), reported that of the 11 isolates responsible for > 90% mortality in second instar

L. lineolaris, seven had been isolated from insects not belonging to the order Hemiptera. The native isolates of *B. bassiana* and *V. lecanii* gave poor control of *L. lineolaris* in laboratory bioassays with mortality less than 68% and 47%, respectively. Liu et al. (2002), reported similar results for *V. lecanii* with < 40% mortality in adult *L. lineolaris* and > 80% average mortality in assays with eight fungal species that included *M. anisopliae* and *B. bassiana*. Variations in virulence among isolates are well known (Tanada and Kaya, 1993) so this may explain the results of this study. The lower concentrations of these fungi compared to those with BotaniGard may be an issue so comparisons were not made between these treatments. Although *M. anisopliae* was found to be more pathogenic to *L. lineolaris* than the native *B. bassiana* isolate, concentrations of the latter were always lower. For example, concentrations for *M. anisopliae* were more than double than that used for *B. bassiana* in trial three. In contrast, concentrations of *V. lecanii* were comparable to those used for *M. anisopliae* (in trial one, concentrations were actually higher), but this pathogen was the least effective against adult *L. lineolaris*. Adult western flower thrips, *Frankliniella occidentalis* Pergande (Thysanoptera, Thripidae) were also more susceptible to *M. anisopliae* than *V. lecanii* after being immersed in conidial suspensions (Vestergaard et al., 1995).

Of interest is that higher concentrations did not always result in the highest overall mortality or mycosis levels for all three fungal isolates. One possible explanation for this may have been that the range in concentrations, across the three trials for a given treatment, were not sufficiently different enough to produce detectable differences in

cumulative mortality. However, with an increase in conidial concentration, the mean time to 50% mortality (LT_{50}) was less for all native isolates tested. Observed differences in virulence among the fungal pathogens tested are difficult to explain since the infection process of entomopathogenic fungi is still not fully understood (Vestergaard et al., 1995).

Concentrations used for the native isolates *M. anisopliae* (except Trial 1) were slightly higher (Table 2.6) than those used for the lowest concentration of BotaniGard (Table 2.5). Furthermore, numbers of conidia and germinating conidia were similar for both isolates with higher numbers of germinating conidia observed for insects treated with *M. anisopliae*. Despite this, better performance (e.g. mortality and infection) was observed for BotaniGard, even at the lowest concentration tested, than *M. anisopliae*. Although, one might argue that the differences in performance between the isolates tested in this study are inherent to each fungal pathogen (e.g toxin production), it is more likely that complex interactions between the pathogen, host and environment are having the greatest impact on disease development. In addition, the performance of BotaniGard probably reflects marketable attributes (e.g. better attachment, faster times to germination, consistent performance) of a commercially formulated product.

4.2 *Observations of attachment, germination, and spatial distribution of conidia*

Conidia and germinating conidia, of all pathogens, except *V. lecanii*, were observed in each body region. With *V. lecanii*, no conidia were observed on the head and germination was never observed on the head and mesoscutum. Sosa-Gomez et al.

(1997), also observed fewer *M. anisopliae* conidia attached to smoother, well sclerotized regions (e.g. head) compared with areas that had higher numbers of setae (e.g. antennal tips) on juvenile southern green stink bug, *N. viridula* (Hemiptera, Pentatomidae). Attachment and germination are thought to be affected by topography (Boucias and Pendland, 1991), where preferential binding to structures such as setae is possibly influenced by surface chemistry (Sosa-Gomez et al., 1997). Less than half of all *L. lineolaris* examined had conidia on every body region sampled and, of these, the majority had been treated with BotaniGard. Despite the high variability observed in conidial numbers, both the high and low BotaniGard concentrations gave good overall coverage with conidia observed on all body regions on eight out of 11 insects assessed. Specific properties of fungal pathogen formulations (e.g. reduction in fungistatic compounds) can enhance fungal spore attachment and stimulate germination on the host cuticle (Butt et al., 2001). Results from this study gave no clear indication that germination was better on dead insects suggesting that fungistatic compounds, if present in the epicuticle, are not labile. Conidial counts determined for insects that had been treated with a native fungal isolate, were also highly variable among insects and pathogen treatments, even though the hydrophobic conidia of *M. anisopliae* and *B. bassiana* and the hydrophilic conidia of *V. lecanii* attach nonspecifically to the insect cuticle (Boucias and Pendland, 1991). Large numbers of conidia, of all fungal isolates attached to dialysis tubing and developed germination tubes. Although germination was extensive over the dialysis tubing surface, germ tubes were often longer than those seen on insect cuticle and no appressoria were observed. Boucias and Pendland (1991),

reported that conidia are capable of attaching to non-host substrates as well as the insect cuticle but may produce non-penetrant germ tubes on the non-host surface. Observations of fungal growth on the dialysis tubing treated with conidial suspensions suggest that there may be cues, other than thigmotropism, that are involved in fungal surface behaviour. These data suggest that formulation may improve performance in the right combination of fungus and host.

Percentage germination, for all spores on the whole insect, did not exceed 50% and almost half of the insects assessed had less than 10% germination. Germination (5-20%) was also low for conidia of *M. anisopliae* attached to the cuticle of *N. viridula* (Sosa-Gomez et al., 1997). The low correlation between number of spores versus number of penetration events on potato leafhopper, *Empoasca fabae* (Homoptera, Cicadellidae) inoculated with *Erynia radicans* (Entomophthorales, Zygomycetes), was attributed to the varying susceptibilities of individual insects (Wraight et al., 1990). In this study only two of the eight *L. lineolaris* treated with *M. anisopliae* had germinating conidia on every body region suggesting that germination may be influenced by cuticular region. Direct penetration through sclerotized or membranous cuticle was observed for larval elaterid larvae, *Limonius californicus* (Coleoptera, Elateridae), *Hypolithus bicolor* (Coleoptera, Elateridae), *Ctenicera aeripennis* (Coleoptera, Elateridae), and *Ctenicera destructor* (Coleoptera, Elateridae), after exposure to *M. anisopliae* conidia (McCauley and Zacharuk, 1968). Little is known about the relationship between conidial deposition patterns and the likelihood of infection. Steinkraus and Tugwell (1997), emphasized that the number of spores needed to kill an insect was lower than what the determined lethal

concentration might suggest, as it is based on spore concentrations in water used to inoculate the insect and not the number of spores that actually attach to the cuticle.

5. Conclusion

BotaniGard demonstrated the most potential for use as a biological control agent of adult *L. lineolaris* in Nova Scotia. At the lowest concentration (3.0×10^4 conidia/insect in a vial), exposure to this commercially formulated mycoinsecticide resulted in greater than 73.1% mycosis. Unfortunately, the native isolates did not perform as well with results showing mortality from fungus ranging from 3.1 to 61.5% mycosis. Before concluding that *M. anisopliae* cannot be considered as a potential biological control agent against *L. lineolaris*, it would be worthwhile to further examine the possible limitations to mycosis development since fungal mortality was >50% in all three trials. Despite the observed mycosis levels for insects treated with BotaniGard and the native isolate, *M. anisopliae*, overall percentage germination was low for insects exposed to all fungal treatments tested in this study. Unfortunately, the current strong push to deliver safe, environmentally friendly alternatives to existing chemical controls, often results in inconsistent successes with using fungal entomopathogens as biological control agents in field settings. Achieving high mycosis levels despite low conidial counts, and even lower germinating conidial counts suggests that factors operating at the conidia and host cuticle interface need to be more closely examined. Presuming greater coverage by simply increasing conidial concentration may not suffice if germination is the key determinant for initiation of infection and if site of infection can influence whether

successful penetration will even occur. Results from this study contribute to understanding the role of factors that influence why exposure to fungal pathogens does not always guarantee 100% mortality due to infection. Continued efforts towards investigating processes taking place at the interface between conidia and host cuticle are critical to the future success of fungal entomopathogens as the new 'convention'.

Chapter 3. Spatial deposition patterns and germination of conidia of the entomopathogen *Metarhizium anisopliae* infecting adult tarnished plant bug, *Lygus lineolaris*.

Abstract

Spatial deposition patterns and germination of conidia on the dorsal cuticle of adult *L. lineolaris* treated with conidial suspensions of *M. anisopliae* (4.8×10^4 conidia/insect in a vial) were investigated in laboratory trials. Insects that died during the bioassay were assessed for cause of death (fungal or non-fungal) and cadavers were then examined under a variable pressure scanning electron microscope, to assess the influence of body region (head, pronotum, mesoscutum, scutellum, and wings), topography (e.g. adjacent to setal socket, seam, smooth), and gender on conidial deposition, germination and appressorium formation as they related to the fate (diseased or not) of individual insects. Bioassays demonstrated that *M. anisopliae* significantly reduced *L. lineolaris* numbers with mycosis levels averaging 64% and mean days to death from fungus ranging from 5.2 ± 0.4 to 5.5 ± 0.5 for July and August trials, respectively. Relatively few conidia had attached to the cuticle, relative to the total number sprayed on the insect. Furthermore, the number of germinating conidia was approximately 15% of those that adhered, suggesting that germination may be a limitation on infection. Overall, mean numbers of conidia and germinating conidia were much higher for infected insects (28.1 ± 2.6 conidia, $n = 22$; 6.5 ± 1.3 germinating conidia, $n = 19$) versus uninfected insects (18.1 ± 3.1 conidia, $n = 19$; 1.2 ± 0.4 germinating conidia; $n = 16$). The mean percentage germination over all infected insects sampled did not exceed 25% and was less than 10% for insects that died but were not infected. Significant differences were detected between infected versus not infected insects based on germinating conidial counts in relation to body region and both conidial and germinating conidial counts for surface

topography. Conidia of *M. anisopliae* were more frequently observed in depressions adjacent to setal sockets than on inter-segmental seams, shallow depressions not adjacent to setal sockets, or smooth surfaces. Furthermore, the highest number of appressoria (51.4%) were observed for conidia in depressions adjacent to setal sockets on infected insects. Gender also significantly affected the treatment effect observed in insects that had died of infection. Higher numbers of infected males had died sooner than females that had died from fungal related causes. Results from this study contribute to a better understanding of factors operating at the spore-cuticle interface that affect mortality induced by fungal pathogens.

Introduction

Widespread use of chemicals against insect pests has become more difficult because of host resistance to these controls and heightened public safety concerns over high levels of pesticide residues on foods and in the environment. In response, interest in biological alternatives to chemical controls for use against economically important insect populations continues to increase. An effective fungal entomopathogen would be an important addition to the suite of available treatments currently used against tarnished plant bug, *Lygus lineolaris*. This insect is a major agricultural pest throughout North America, utilizing over 350 plant species as hosts for food and oviposition sites (Young, 1986). In Canada, economically important crops like apple (Bostanian and Coulombe, 1986), strawberries (Mailloux and Paradis, 1979), and alfalfa (Bidochka et al., 1993) are susceptible to injury caused by *L. lineolaris*. To date, suppression of *L. lineolaris* has been achieved mainly through the use of organophosphate and pyrethroid insecticides (Hardman et al., 2004). Although chemical controls do reduce injury to apple, Hauschild and Parker (1976) found that spraying did not prevent *L. lineolaris* from staying within treated orchards and potentially feeding after residues dissipated. Furthermore, organophosphates are slowly being de-registered due to their toxicity to mammals and pyrethroids are known to be disruptive to beneficial species resulting in outbreaks of secondary pests such as mites and aphids (Hardman et al., 2004).

One aspect that gives fungal pathogens tremendous potential in biological control is their ability to penetrate directly through the insect cuticle (Charnley, 1984). This mode of action is particularly useful for control of phloem-feeding insects like those belonging

to Orders Hemiptera and Homoptera. Historically, *Beauveria bassiana* has been the most widely used entomopathogen tested against *L. lineolaris* as a potential control agent (Bajan and Bilewicz-Pawińska, 1971; Bidochka et al., 1993; Leland and Behle, 2005; Leland et al., 2005; Liu et al., 2002; Liu et al., 2003; Noma and Strickler, 1999; Steinkraus and Tugwell, 1997). In contrast, fewer studies have examined the potential of *Metarhizium anisopliae* for use against the tarnished plant bug (Liu et al., 2002). This is despite a number of field and laboratory trials that have experimentally tested *M. anisopliae* against a wide range of Hemiptera, including stink bugs, *Nezara viridula*, *Piezodorus guildinii*, and *Euschistus heros* (Hempitera, Pentatomidae) (Sosa-Gómez et al., 1997; Sosa-Gómez and Moscardi, 1998); sugarcane froghopper, *Aeneolamia varia saccharina* (Hemiptera, Cercopidae) (Allard et al., 1990); chinch bug, *Blissus leucopterus leucopterus* (Hemiptera, Lygaeidae) (Krueger et al., 1991); and *Riptorus linearis* (Hemiptera, Coreidae) (Hu et al., 1996). In spite of the high mortality achieved in laboratory trials and, to a lesser extent, in experimental field studies, naturally-occurring infections are seldom observed in field collected *L. lineolaris* with only two reports of *B. bassiana* having been isolated from a tarnished plant bug in the wild (Broadbent, 2000; Steinkraus and Tugwell, 1997). Inconsistent results generated in trials using fungi as control agents of insect pests suggest that key challenges remain if we hope to develop entomopathogens as biological control agents (Inglis et al., 2001). Although, differences in performance between isolates tested (Chapter 1) may be inherent to each fungal pathogen (e.g. speed of kill, toxin production), better understanding of the factors that influence disease initiation and development may be

key to effective control of the target pest (Inglis et al., 2001).

A critical determinant of infection is thought to be the number of conidia that come into contact with the insect cuticle based on positive correlations between the number of spores and death from infection for most insect/pathogen combinations (Butt and Goettel, 2000). However, few researchers have explored the reasons why 100% mycosis is seldom achieved even in the laboratory. In bioassays, it is thought that infectivity is dependent on spore dosage and that a threshold exists for the number of spores needed to cause infection (Butt and Goettel, 2000). If the dose is high enough then it is assumed that an effective number of spores will adhere to the insect, germinate and cause infection. Good coverage will overcome initial barriers such as insufficient deposition on vulnerable regions of the cuticle (Goettel et al., 2000). Various cuticle characteristics can influence the likelihood of conidial attachment at specific sites including the chemical composition of the conidium wall and the epicuticle of the host (Boucias and Pendland, 1991), surface topography (Akbar et al., 2004; Boucias and Pendland, 1991) and physiochemical properties, both of which influence the formation of appressoria (Sosa-Gómez et al., 1997; St. Leger et al., 1989).

The influence of conidial spatial distribution on disease development has not been adequately addressed (Goettel et al., 2000), with surprisingly little empirical data published on the relationship between events that occur at the pathogen-insect cuticle interface, and the likelihood that the insect will die of infection. The lack of published data may be partly due to the difficulties associated with the considerable investment of time and the level of training required to process samples using specialized equipment

like scanning electron microscopes (SEM). Goettel and Inglis (1997), discuss the labour intensiveness and shortfalls (e.g. difficulties with accurately assessing spore density) of techniques such as SEM and the use of tissue-specific dyes when attempting to make direct quantification of conidia on the insect cuticle.

This study was designed to address some of these issues by evaluating the pathogenicity of a Nova Scotian isolate of *M. anisopliae* on *L. lineolaris*. In addition, the influence of body region and topography on conidial deposition and germination were related to the infection status of individual insects. This particular fungus was chosen based on preliminary data (Chapter 2) that showed it could infect *L. lineolaris* and because the characteristic shape of its conidia facilitated recognition using a variable pressure scanning electron microscope. *Metarhizium anisopliae* var. *anisopliae* conidia are cylindrical in shape and approximately 1.6 to 2 μm in width and 6 to 8 μm in length (Tulloch, 1976).

There are a number of potential explanations for the survival of some insects that are treated with infective fungal spores. One possible reason may be that certain characteristics of the insect cuticle such as region on the body (e.g. head, wings) or surface topography (e.g. setal sockets, depressions, smooth surface) may influence whether or not conidia will adhere and germinate, even when the application method gives good coverage of conidia on the cuticle. In this study, I examined how body region and surface topography of the insect cuticle may influence conidia deposition patterns and germination events, and therefore, affect the likelihood that disease will develop. Bioassays were conducted to determine mortality and mycosis levels, time of death (T_d),

and median time to death (LT_{50}) for insects treated with conidial suspensions of *M. anisopliae*. In addition, both deposition of inoculum and the spatial distribution of the conidia on the insect's body surface were related to development of disease in individual insects treated with the fungus. This was done using a variable pressure scanning electron microscope (VPSEM). Specifically I did the following: a) quantified average conidia and germinating conidia densities over different regions of the insect cuticle; b) measured and described germ tube length and appressorial development for germinating conidia; c) tested and described associations of total conidial or germinating conidial counts with body regions and topographical features; d) assessed changes in conidia densities on insect cuticle after exposure; e) assessed relationship between conidia density or germinating conidia density in different body regions and incidence of observed mycosis; and e) determined whether there was a threshold of inoculum needed to cause infection.

2. Materials and Methods

2.1 *Source of experimental pest species*

Adult *L. lineolaris* were collected in July and August 2003, from field sites located at the Atlantic Food and Horticulture Research Centre (AFHRC) in Kentville, Nova Scotia (45.063° N, 64.488° W). Composition of groundcover was predominantly broad leaf plants including dandelion, *Taraxacum officinale*; common plantain, *Plantago major*; and sheep sorrel, *Rumex acetosella*. Insects were maintained at 21°C, 70% relative humidity (RH) and 16 light:8 dark photoperiod in wooden framed isolation cages with

nylon screening (47 x 47 x 68 cm) in a temperature controlled rearing room, located at the AFHRC. Twenty-five individuals were in each isolation cage (47 x 47 x 68 cm). Cages were lined with white paper, to facilitate finding dead individuals, and three potted green bean, *Phaseolus vulgaris*, plants (Derby variety, Veseys Seeds, York, Prince Edward Island) per cage were provided as food. For additional details of maintenance and selection methods see Chapter 2.

2.2 *Source and preparation of fungus*

Trials tested a native *M. anisopliae* isolate (MET-01), chosen because of observed infection levels (52 to 62%) in *L. lineolaris* treated in bioassays comparing pathogenicity of MET-01 with a commercial formulation of *B. bassiana* and native isolates of two other pathogens against *L. lineolaris* (Chapter 2). In addition, this particular isolate had been collected locally and would be adapted to regional climatic conditions. *M. anisopliae* (MET-01) was isolated by Dr. D. B. Strongman (Saint Mary's University, Halifax, Nova Scotia) from a sawfly, *Neopareophora litura* (Klug) (Hymenoptera, Tenthredinidae) larvae collected from blueberry fields in Parrsboro and Sheffield Mills, Nova Scotia.

Fungal cultures were grown on malt extract agar (MEA) (Difco, Detroit, MI) supplemented with antibiotics (in g l^{-1}: 20 g agar, 10 g malt extract, 0.5 g Penicillin G, 0.5 g Streptomycin sulphate) in petri dishes (110 x 15 mm). incubation conditions were 25°C, 85% RH in total darkness. Conidia were harvested and stored at -80°C on ceramic beads in cryo-vials (Pro-Lab Diagnostics, Fisher Scientific, Ottawa, ON)

containing 10% glycerol. These frozen suspensions were the source of inoculum for each successive bioassay so no serial transfers were required. Conidia were harvested from 10-day old cultures using 3 mL sterile water containing 0.05% Tween 80 (Fisher Scientific, Ottawa, ON). Conidial concentrations in the inoculum suspension were determined using a Neubauer-Improved haemocytometer and adjusted to the desired final concentration with sterile distilled water. All fungal suspensions were applied to experimental insects within 24 h of preparation. Details of methods used to verify conidia viability can be seen in Chapter 2.

2.3 *Pathogenicity bioassays*

Bioassays were conducted in July and August 2003 to determine the ability of the pathogen to infect the host. A randomized complete block design (RCBD) was used to account for variation caused by block differences, where time of trial was the blocking factor (Underwood, 1997). Regimes included the treatment group (application of fungal conidia) and procedural control (application of 0.05% aqueous Tween 80). A single host in a vial (2.9 mL) was considered one replicate, with 51 to 57 replicates in the treated group and 30 to 54 replicates in the control group. Replicate vials were arbitrarily assigned to each regime. The conidial concentration (1.6×10^5 conidia/ml) used in the bioassays gave 25% to 75% mortality rates as determined in trials conducted in 2001 (Chapter 2). This mortality range was recommended by Robertson and Preisler (1992). Bioassays were conducted, following the same methods used in earlier assays detailed in Chapter 2, to determine mortality and mycosis levels of adult *L. lineolaris*. Conidia

were also applied to an inert substrate, autoclaved dialysis tubing (7 x 4 mm), to examine fungal spore germination on an insect surface. Conidia solutions were applied with a micro-atomizer at a rate of 0.3 ml of conidia suspension per insect host (4.8×10^4 conidia per insect in a vial). Insects in vials were incubated at 25°C, 70% RH in total darkness. After 24 h, individual insects were transferred to sterile petri-dishes containing a piece of green bean (approx. 15 mm) and examined every 24 hours for 14 days. Pieces of bean were replaced every three days. Individual hosts were examined for changes in behaviour (e.g. leg twitching and lethargy) and evidence of disease development, such as morbidity (slight movements when gently probed), using a binocular microscope. Daily and cumulative mortality were recorded. Dead insects were removed from the bioassay, gender was determined and recorded, and then individuals were prepared for final assessments. Once removed, each dead individual had its entire abdomen removed using sterile techniques and a small amount of tissue was dissected and cultured on both MEA and Sabouraud dextrose agar with yeast extract (SDAY) media. The remainder of the abdomen was placed on moistened filter paper (Whatman No. 1) in a petri-dish (110 x 15 mm) and incubated for three to five days to allow fungal growth. The cadaver, minus the abdomen, was frozen at -86°C for electron microscopy trials. At the end of the trial (14 days), all surviving insects were killed and processed the same way as the dead. For all trials, individuals were recorded as killed by fungus if there were signs of mycosis, as determined by the development of *M. anisopliae* in cultures or on the cadavers. Re-cultures and wet mounts were made from colonized individuals to determine that the fungus recovered was *M. anisopliae* (Barnett and

Hunter, 1972). Laboratory bioassays involving preparation and application of natural isolates of fungal pathogens followed procedures that were modified from Goettel and Inglis (1997). The entire experiment was replicated twice (Trial 1, July 2003; Trial 2, August 2003). On occasion, newly emerged adult parasitoids were observed in pathogenicity bioassays. Parasitoids were pinned and labelled for identification and are deposited in the national reference collection (DAOM), Biosystematics Research Institute, Ottawa, Ontario.

2.4 *Statistical analysis for pathogenicity bioassays*

Mean cumulative mortality and mycosis values were calculated by dividing the number of dead or infected by the total number of live and dead or infected individuals. The SAS LIFETEST procedure (SAS Institute 1990) was used to calculate non-parametric estimates of total mortality and mycosis trends over time of exposure with their corresponding estimated variances (Marcus and Eaves, 2000). Log-rank and Wilcoxon statistics were also generated for testing homogeneity of mortality and mycosis distributions between treated and control groups. Both statistics were considered as Log-Rank placed more weight on the larger survival times, whereas, the Wilcoxon is sensitive to shorter survival times. Median lethal time (LT_{50} values) leading to 50% mortality or mycosis were generated, for treated and control insects, as summary measures of the cumulative probability of mortality or mycosis. Rank tests for homogeneity indicated whether LT_{50} values generated for the different treatment groups were significantly different. Comparison of LT_{50} values were used to see if there was

significant effect of the blocking factor, time period when the experiment was conducted, and to assess the effects of survival in days after exposure on spore and germinating spore counts.

2.5 *Conidial attachment, germination, inoculum density and spatial distribution*

Observations of attachment, germination, and spatial distribution of *M. anisopliae* conidia on the cuticle of the insect host involved assessment of 41 adult *L. lineolaris* using a LEO 1450 variable pressure scanning electron microscope (VPSEM) located at Saint Mary's University, Halifax, Nova Scotia. All insects (n = 37), that had died between 24 h and 120 h post-inoculation, and insects (n = 4) that remained alive 336 h after treatment were examined. Frozen insects were mounted on stubs dorsal-side up. Only the dorsal cuticular surface was examined as it was assumed that the dorsal surface would most likely come into contact with conidia since they were applied by aerial spray in the assays. The distribution of conidia was evaluated by cuticular region, as was done in the previous chapter and illustrated in Figure 3.1. One to four observations were taken from each of seven regions on the dorsal surface of the cuticle: head, pronotum, mesoscutum, scutellum, right wing, left wing, and wing membrane (Table 3.1). Care was taken to ensure that observations were taken from approximately the same area within a body region on each insect sampled. Conidia on the dorsal surfaces of cadavers were photographed at 400x while being examined under the VPSEM. Digitized micrographs were examined at a later date to determine number, location of conidia and number of germinating conidia on the insect cuticle, at various times of death ranging

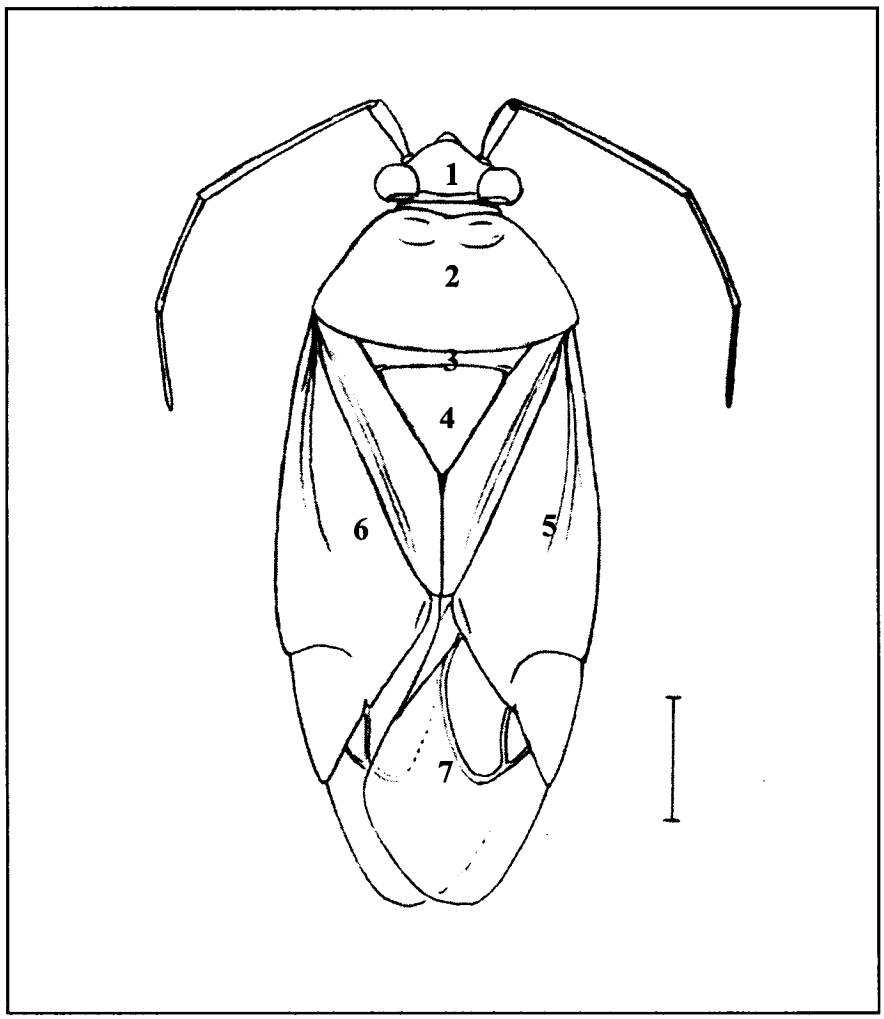

Figure 3.1. The dorsal cuticular surface of adult *L. lineolaris* was subdivided into nine regions and examined using a variable pressure scanning electron microscope at 400 x. Legend: head (1); pronotum (2); mesoscutum (3); scutellum (4); left wing (5); right wing (6); and wing membrane (7). Areas observed but not shown are the antenna and leg. Scale bar represents 1 mm.

Table 3.1. Area calculations made for different sized body regions observed on the dorsal surface of adult *Lygus lineolaris*.

Body Region	Total area of body region (mm^2)	No. of observations	Total area of observations (mm^2)	Proportion of dorsal surface[a]	Correction factor[b]
Head	0.30	2	0.12	0.011	0.407
Pronotum	2.61	4	0.24	0.021	0.069
Mesoscutum	0.45	2	0.12	0.011	0.266
Scutellum	0.55	4	0.24	0.021	0.436
Right wing	3.66	3	0.18	0.016	0.049
Left wing	3.66	3	0.18	0.016	0.049

[a] Total dorsal surface was approximately 11.23 mm^2

[b] Correction factors were calculated by dividing area observed by the total area of region

from 0 to 5 days post-inoculation both for infected insects and those killed by other causes. An acetate template (219 μm x 293 μm to scale), further sub-divided into 16 grids, was created to overlay on captured digital images of each observation made and was used to quantify conidial density, production and length of germination tubes, and appressoria formation for up to 22 observations taken per adult insect. An observation was defined as all conidia, germinating conidia, and appressoria found within one quadrat (219 μm x 293 μm to scale). Conidia were considered to have germinated if the germ tube was at least the length of the conidium. The number of ungerminated and germinated conidia, number of setae, and topography associated with the location where conidia were attached were recorded. The topography of the dorsal cuticle was described using three distinct features: (1) setal sockets, including depressed area immediately adjacent to socket, (2) shallow depressions (not adjacent to setal socket) or seams, and (3) smooth, open surfaces. Insects were held under vacuum for up to 2 h.

2.6 *Statistical analysis for scanning electron microscopy*

Mean counts were computed for conidia, germinating conidia, and appressoria for each body region observed on individual insects. The number of conidia for each observation (219 μm x 293 μm to scale) was summed and divided over the total number of observations taken for the entire region. To correct for bias between different size regions, the area of each body region was estimated using a number of geometric shapes to assume the approximate area (e.g. tetrahedron and triangles for the pronotum). The total area observed in a region was then divided by the total area of the region to

establish a correction factor for the different sized regions (Table 3.1). Correction factors are shown in the final column of Table 3.1. Mean counts for conidia and germinating conidia for each body region for individual insects were then divided by the correction factor (Table 3.1) to estimate counts of conidia and germinating conidia for the entire region. Corrected conidial counts for every body region observed were summed to compute the total conidial load on the dorsum. The number of germinating conidia with appressoria was also recorded. Examination of the mesoscutum, legs and antennae using VPSEM, provided only partial, non-uniform microscopic fields and were, therefore, not considered in the Wilcoxon Rank Sum analyses. Overall, areas sampled under VPSEM comprised approximately 10% of the total area on the dorsal surface available for observation (excluding legs, antennae and wing membrane).

Comparison of LT_{50} values were used for bioassays to assess the effects of conidia and germinating conidia counts on survival time in days after exposure. Results were analyzed using the Wilcoxon Ranks Sum option of the SAS NPAR1WAY procedure (SAS Institute 1990) to compare conidia and germinating conidia counts, and effect of body region with conidia between those insects that died of infection versus those that died of other causes or remained alive after 14 days, based on the Kruskal-Wallis test. To determine if germination differed between infected and uninfected insects, percentage germination was estimated for each body region and the entire body of each insect exposed to the pathogen. Data were then transformed to ranks using the SAS Rank procedure (SAS Institute 1990) and analyzed using the SAS ANOVA procedure (SAS Institute 1990) to obtain the Wilcoxon Ranks Sum and Kruskal-Wallis tests.

Proportions of conidia and germinating conidia observed for a specific surface feature were calculated for individual body regions and ranked using the SAS RANK procedure (SAS Institute 1990). One-way analysis of variance with type III sums of squares was used on ranked proportions to determine whether surface affected the number of conidia or germinating conidia that attached to the cuticle (SAS Institute 1990). Computed means for proportions of conidia and germinating conidia for each surface type were further separated by the Waller-Duncan k-ratio t-Test using ranked proportions. Surface types within body regions could not be compared because they were not quantified.

3. Results

3.1 *Pathogenicity Bioassays*

Mean days to death (days ± SE) for *L. lineolaris* that died after exposure to *M. anisopliae* conidia, applied at 4.8 x 10^4 conidia/vial, ranged from 4 to 5 days in the two trials (Table 3.2). Mortality data sets for the two trials could be combined because trial (Trial 1 = July, Trial 2 = August) was not shown to be a significant factor (log-rank test, $\chi^2 = 3.72$, df = 1, $P > 0.05$). Similarly, trials were combined for control insects since observed mortality did not differ significantly between trial dates (log-rank test, $\chi^2 = 2.18$, df = 1, $P = 0.140$). Mortality was significantly higher for insects treated with *M. anisopliae* versus controls in both trials (Trial 1, log-rank test, $\chi^2 = 18.63$, df = 1, $P < 0.001$; Trial 2, log-rank test, $\chi^2 = 7.76$, df = 1, $P = 0.005$), as shown in Table 3.2. Although, a high initial mortality was observed in the procedural control in Trial 2,

Table 3.2. Cumulative mortality, mycosis and associated LT_{50} values for adult *Lygus lineolaris* exposed to *Metarhizium anisopliae* (4.8 x 10^4 conidia/insect in vial) in trials conducted in July and August 2003. LT_{50} values and corresponding confidence limits were generated using the SAS LIFETEST procedure (SAS Institute 1994). Control mortality was combined because 'trial' was not shown to be significantly different, based on overlapping 95% confidence limits.

Treatment	Trial	*n*-size	Cumulative Mortality (%)†	Mean time to death (days ± SE)§	LT_{50}	95% Confidence Interval (days)	Cumulative mycosis (%)	Mean time to mycosis (days ± SE)‡
M. anisopliae	1	57	100.0	4.2 ± 0.4	4	3 - 5	64.9	5.2 ± 0.4
M. anisopliae	2	51	92.2	5.2 ± 0.4	5	4 - 5	62.8	6.1 ± 0.5
Control	1	54	68.5	8.2 ± 0.8	9	7 - 12	0	-
Control	2	30	76.7	5.6 ± 1.0	3	1 - 7	0	-
Control	1 & 2	84	71.4	7.3 ± 0.6	7	4 - 10	0	-

† Cumulative mortality represents death due to infection and unknown causes combined
§ Mean days to death included death due to infection and unknown causes combined
‡ Mean days to mycosis includes death due to infection only

cumulative mortality leveled off whereas mortality in the treated group rose steadily reaching 92.2%, eventually surpassing that recorded for the control group (Figure 3.2 A, C). Despite the high mortality observed in the control groups, 68.5% (July) and 76.7% (August), most of the death observed in inoculated *L. lineolaris* was determined to be fungal related compared with no infection in the dead examined from the control group (Figure 3.2 B, D). Adult parasitoids of the tachinid fly, *Phasia robertsonii* (Townsend) (Diptera, Tachinidae), were observed after emerging from six adult *L. lineolaris* in the control group. Parasitism was only observed in insects in the control groups and accounted for 4% ($n = 2$) and 13% ($n = 4$) of the mortality in the July and August trials, respectively. Treated insects that subsequently died of infection often showed impaired mobility (leg twitching and delayed response when gently probed) and fungal development was observed within 2-3 days of their recorded death with external sporulation occurring soon after.

When fungal and non-fungal caused deaths were analyzed separately, mean cumulative mortalities not related to infection represented only 30.3% of insects that had been treated with *M. anisopliae* versus all control insects. Most insects had died by the eleventh day after inoculation, with the exception of one individual that remained alive until the end of the trial (14 days), as shown in Figure 3.2 C. At that time, this particular individual was killed and assessed for infection and subsequently showed positive signs of mycosis. Overall, the number of treated insects that were still alive 14 days after inoculation was low, with no survivors in the July trial and four in the August trial (Fig. 3.2 A, C).

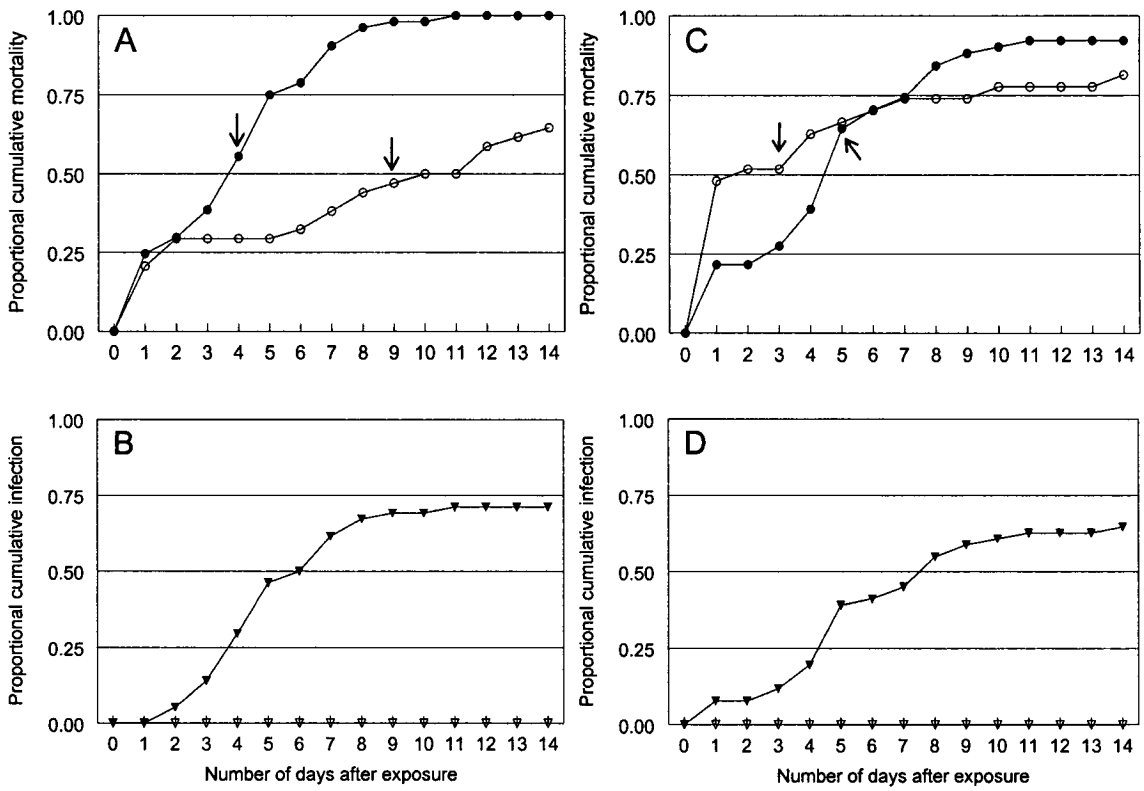

Figure 3.2. Cumulative total mortality (fungal and non-fungal) and cumulative fungal infection for adult *Lygus lineolaris* following spray application of *Metarhizium anisopliae* (4.8×10^4 conidia/insect) conducted in July (A and B) and August (C and D), 2003. Legend: ● *M. anisopliae* mortality, ▼ *M. anisopliae* infection, ○ procedural control mortality and ▽ procedural control infection; arrows denote LT_{50} values for mortality determined using SAS LIFETEST procedure.

3.2 *Separated analyses for Trials 1 and 2 to determine LT_{50}'s*

Analysis of survival data was conducted to estimate LT_{50} values for *L. lineolaris* treated with *M. anisopliae* conidia. For insects exposed to the fungal pathogen in the July trial, the LT_{50} was lower for the treated group (Fig. 3.2 A) and even the LT_{50} for uninfected (Fig. 3.2 B) insects was shorter than for those that died of other causes (Fig. 3.2 A). Significant differences in LT_{50} values were determined for treated insects that died of infection versus non-fungal causes (log-rank test; $\chi^2 = 33.06$, df = 1, $P < 0.001$). Most of the deaths among non-infected insects in the treated group were within one day, suggesting that many of the early deaths in treated and control insects were due to trauma or handling. The procedure Lifetest was unable to estimate confidence intervals for these LT_{50} values. For mortality observed in the procedural control group, for both trials combined, the calculated LT_{50} value was substantially longer at 7 days compared to 4 to 5 days for fungal treated insects. No mortality was caused by infection in the controls.

Further analysis was performed to see if gender was related to survival time for those insects that had been exposed to the fungal pathogen and also affected the observed treatment effect. When tested as a covariate, gender significantly affected the time to death of insects that had died of infection (log-rank test; $\chi^2 = 5.4$, df = 1, $P < 0.02$). Mean days to death (± SE) were 5.9 ± 0.4 and 4.6 ± 0.4 for females and males, respectively. No effect of gender was observed in insects that had been treated but died from other causes not related to infection. The proportion of females (0.76, $n = 39$) to males (0.64, $n = 30$) that died of infection or that died from other causes (0.24, $n = 12$ females versus 0.36, $n = 17$ males) were similar.

3.3 *Inoculum density, spatial distribution of conidia and the development of disease*

3.3.1 Conidia and germinating conidia counts

Variable pressure scanning electron microscopy indicated that *M. anisopliae* conidia were capable of adhering to the dorsal cuticle of *L. lineolaris*. Infection was confirmed in 22 of the 41 adults examined using VPSEM. The total number of *M. anisopliae* conidia attached to the cuticle of the 41 adult *L. lineolaris* was not found to differ significantly between individuals that died 0, 24, 48, 72, 96, 120 and 336 h post-inoculation (Kruskal-Wallis test; $\chi^2 = 7.1$, df = 5, $P = 0.214$). Counts of germinating conidia for insects that died, at the same post-inoculation times after 0 h, were also not significantly different (Kruskal-Wallis test; $\chi^2 = 6.4$, df = 5, $P = 0.273$). The lack of a significant association with time may have been due to high variability in deposition and germination among the insects sampled (Figure 3.3). Of interest is that very few conidia (< 1% of corrected counts) of the 48,000 conidia applied actually attached to the cuticle.

Conidia were observed to have attached to all body regions examined on the dorsal cuticle of insects that were dosed by spraying with the fungal pathogen, but actual mycosis was only detected in 65% and 63% of the insects in the July and August trials (Table 3.2). Overall, the mean (± SE) number of conidia and germinating conidia, based on actual counts, on the dorsal surface of cuticles of treated insects (excluding those at 0 hour) were double for infected versus uninfected insects (Figure 3.4). Observations taken on the legs were the only exception, where higher number of conidia were reported for treated but uninfected insects and mean germinating conidial counts were the same between infected and uninfected (Table 3.3). Conidia were seldom observed on the

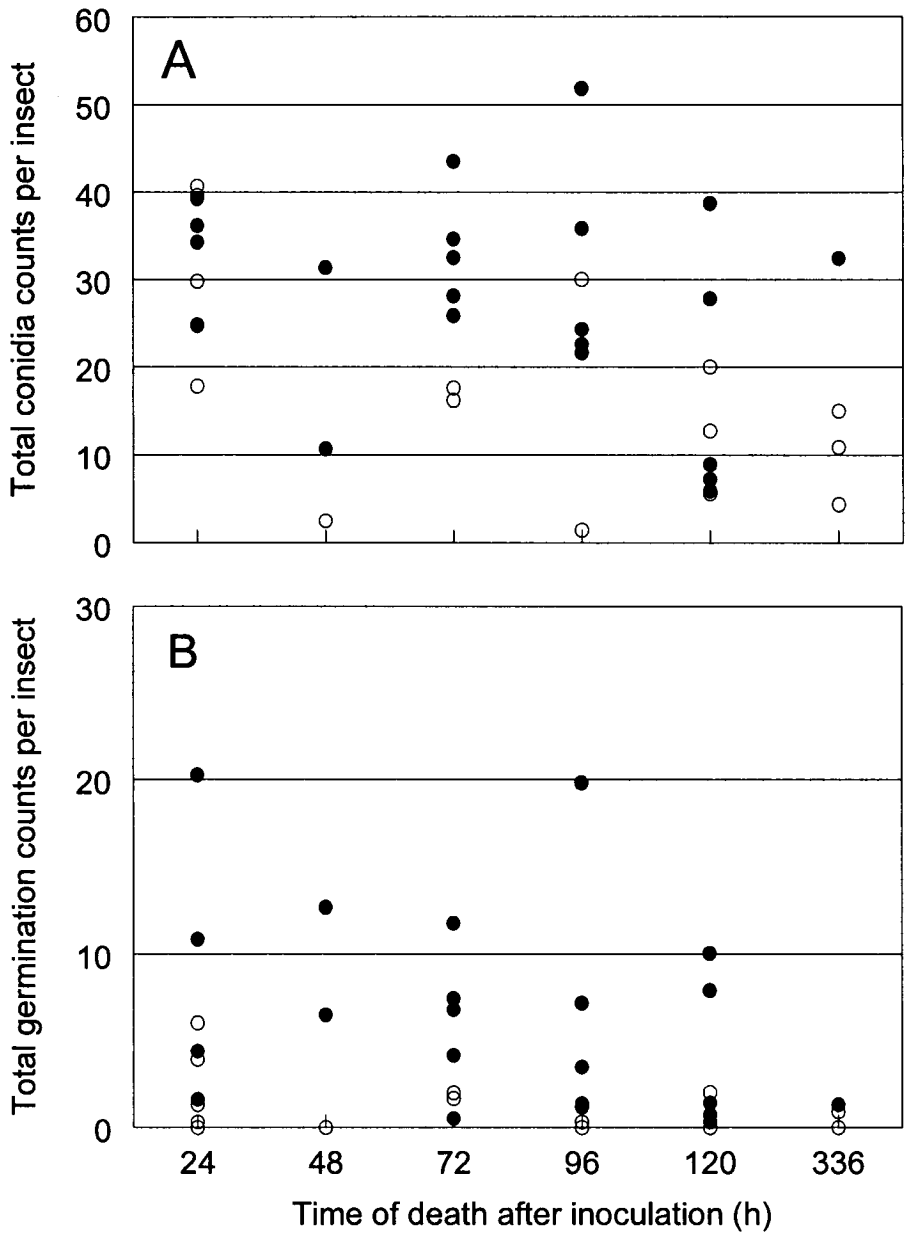

Figure 3.3. Frequency distributions showing total conidia (A) and germinating conidia (B) per *Lygus lineolaris* at different times of death after treatment with *Metarhizium anisopliae*. No difference was detected in conidia and germinating conidia at varying times of death after exposure using a Kruskal-Wallis test, $P < 0.05$. Data are pooled for the July and August trials. Legend: ● infected insects and ○ uninfected insects.

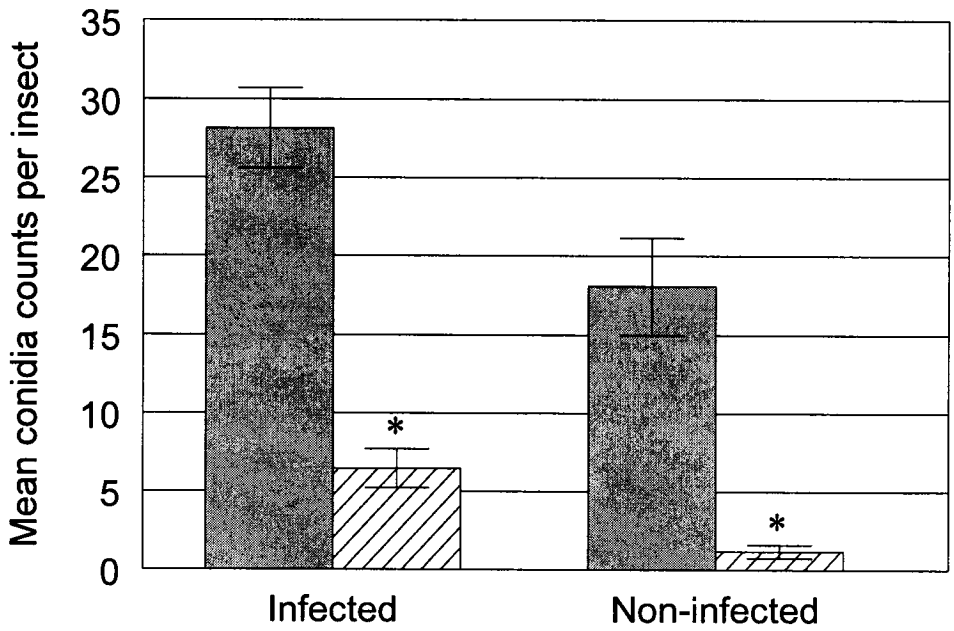

Figure 3.4. Mean number of cumulative conidia (solid bar) and germinating conidia (striped bar) observed on the dorsal surface of adult *Lygus lineolaris* after exposure to *Metarhizium anisopliae* (4.8 x 10^4 conidia/insect). Asterisks (*) denote that only germinating conidial counts observed on infected versus not infected insects were found to be significantly different using a Kruskal-Wallis test ($P < 0.05$).

Table 3.3. Descriptive statistics for conidia and geminating conidial counts in individual body regions not included in additional analyses for *Lygus lineolaris* that had died, between 24 h and 120 h, from either infection or non-fungal related causes. Data represent actual counts.

	Infected					Uninfected				
	n - size	Mean ± SE conidial counts	Range	Mean ± SE germinating conidial counts	Range	*n* - size	Mean ± SE conidial counts	Range	Mean ± SE germinating conidial counts	Range
Leg	21	3.0 ± 0.9	0 - 17	0.4 ± 0.2	0 - 3	16	4.4 ± 1.9	0 - 35	0.4 ± 0.2	0 - 3
Antennae	22	0.09 ± 0.06	0 - 1	0.0 ± 0.0	0	14	0.0 ± 0.0	0	0.0 ± 0.0	0
Wing membrane	22	0.0 ± 0.0	0	0.0 ± 0.0	0	16	0.0 ± 0.0	0	0.0 ± 0.0	0

antenna and wing membrane (Table 3.3). No significant difference (Kruskal-Wallis test; $\chi^2 = 2.63$, df = 1, $P = 0.105$) was seen in mean conidia count, when all body regions (excluding legs, antennae and wing membrane) were combined, on individuals that died from infection versus those that died from other causes. However, a significant difference (Kruskal- Wallis test; $\chi^2 = 9.68$, df = 1, $P = 0.002$) was found for the mean number of germinating conidia observed on insects that died from infection (20%, $n = 343$) or from unknown causes (6%, $n = 56$), (Figure 3.4). Appressoria ($n = 72$) were most often produced at the end of germ tubes that had developed on the cuticle of infected insects ($n = 16$). Considerably fewer appressoria ($n = 10$) were observed on insects ($n = 3$) that died but showed no infection. Although the number of germinating conidia is a small proportion of those that adhered, these results suggest that germination may be the key. No conidia were observed on the cuticle of untreated adults (control group) that received only a direct application of 0.05% Tween 80 solution.

3.3.2 Body regions

The corrected total number of conidia and germinating conidia were assessed for all body regions both for infected and uninfected individuals. Although, individual insects were exposed to 4.8×10^4 conidia/vial with insect, total conidial levels observed for individual body regions and over all regions on sampled insects were highly variable (Table 3.4). Since differences in size of individual body regions was addressed by dividing raw counts by a correction factor (final column Table 3.1) to give total counts for each region, one can presume that the observed differences were due to conidial adherence being more

Table 3.4. Descriptive statistics for conidia and geminating conidial counts in each body region for *Lygus lineolaris* that had died, between 24 h and 120 h, from either infection or non-fungal related causes. Data represent actual counts not adjusted for different sized regions. Asterisks (*) denote significant differences in germinating conidia counts per body region between infected and uninfected insects using a Wilcoxon ranks sum test ($P < 0.05$).

Body region	Infected insects					Uninfected insects				
	No. of insects	Conidial counts		Germinating conidial counts		No. of insects	Conidial counts		Germinating conidial counts	
		Mean ± SE	Range	Mean ± SE	Range		Mean ± SE	Range	Mean ± SE	Range
Head	22	1.8 ± 0.6	0.0 - 11.2	0.3 ± 0.1	0.0 - 2.1	16	1.1 ± 0.4	0.0 - 5.8	0.2 ± 0.2	0.0 - 2.5
Pronotum	22	6.7 ± 1.3	0.5 - 30.8	1.2 ± 0.2*	0.0 -3.3	16	4.5 ± 1.1	0.3 - 17.4	0.5 ± 0.2*	0.0 - 3.0
Mesoscutum	16	8.3 ± 1.5	0.0 - 16.0	2.1 ± 0.8*	0.0 -12.0	11	3.1 ± 1.0	0.0 - 9.3	0.0 - 0.0*	0.0 - 0.0
Scutellum	22	6.3 ± 1.1	0.0 - 21.1	1.1 ± 0.3*	0.0- 3.9	16	3.6 ± 0.6	0.3 - 7.7	0.04 ± 0.04*	0.0 - 0.7
Right wing	22	3.8 ± 0.7	0.0 - 12.3	0.8 ± 0.2*	0.0 - 2.3	16	3.3 ± 0.9	0.0 - 13.6	0.2 ± 0.1*	0.0 - 0.7
Left wing	22	4.3 ± 0.7	0.0 - 12.6	1.6 ± 0.4*	0.0 -7.4	16	3.6 ± 0.8	0.0 - 11.3	0.4 ± 0.17*	0.0 - 2.7

likely in some regions compared to others. Mean counts for conidia and germinating conidia for different body regions on infected and uninfected insects, after treatment are shown in Table 3.3. In all cases, mean corrected counts were higher for infected versus uninfected insects for every body region assessed (Table 3.4).

Counts of conidia varied by body regions on individual insects and also among the same body regions across all insects sampled. For example, mean counts observed on the pronotum of 22 infected insects ranged from 0 to 31 conidia (Table 3.4). Significant contrasts in the number of germinating conidia for infected and uninfected insects were observed (Table 3.4). For treated and infected insects, the highest percentage of conidia and germinating conidia were located on the mesoscutum and the fewest were on the head (Table 3.4). Whereas, uninfected insects showed the highest and lowest conidial and germinating conidial counts on the pronotum and head, respectively. No relationship was detected between conidial counts for individual body regions and whether or not infection developed (Table 3.5), even though the pronotum, mesoscutum, and scutellum of infected insects were observed having higher numbers of conidia. In contrast, when germinating conidial densities were tested for individual body regions, significant differences were found for all regions except the head (Table 3.5).

3.3.3 Percent germination

Of the 34 insects (both infected and uninfected) observed 24 to 120 hours after inoculation, almost half (n=14) were observed with germination rates (mean germination averaged over all body regions) of less than 10%, including four individuals where no

Table 3.5. Relationship between ranked mean germinating conidia counts for a given body region and infection for individual adult *L. lineolaris* exposed to *M. anisopliae* in pathogenicity bioassays. Significant effects were observed for every body region assessed (except the head) using a Wilcoxon ranks sum test ($P < 0.05$).

Body region	*n*-size	Chi-square	df	Pr > Chi-square
Head	38	2.963	1	0.085
Pronotum	38	9.438	1	0.002
Mesoscutum	27	4.009	1	0.045
Scutellum	38	14.895	1	0.0001
Right wing	38	6.684	1	0.010
Left wing	38	4.91	1	0.027

germination was observed. Percentage germination did not exceed 50%, with the exception of two individuals that died of infection 24 and 48 h after exposure, where germination reached 55.0% and 60.9%, respectively. The mean percentage germination over all infected insects (n = 22) sampled was 24.2% ± 3.4% (individual results ranged from 2.0% to 60.9%). This differed significantly (Kruskal-Wallis test; $\chi^2 = 8.63$, df = 1, P = 0.003) from treated insects that died from non-fungal related causes, where the mean percentage germination was 7.0% ± 1.9%, with individual totals ranging from 0 to 15.2% (n=16). High numbers of germinating conidia were observed on the dialysis tubing, although it was difficult to quantify because of the associated germ tube growth. This and the 80% germination in initial spore viability tests contrast with that observed on the insect cuticle. Mean germ tube lengths for germ tubes with appressoria varied among infected individuals (ranging 1.0 μm to 14.9 μm) and those uninfected (ranging 6.4 μm to 14.6 μm), as shown in Table 3.6. The highest numbers of appressoria were observed with germinated conidia on the wings (51.2%) and pronotum (23.2%) for both infected and uninfected insects (n = 14). When individual body regions were analyzed for percentage germination between the two groups sampled (infected versus uninfected), all regions but the head were significantly different (Table 3.7).

In most cases, a single germ tube emerged from a conidium and grew on the cuticle surface, with branching commonly observed (Figure 3.5). Conidia were observed to have germinated on all regions observed, but penetration sites were difficult to establish. Since infection was detected in greater than half of the individuals assessed and appressoria counts were higher for those that died of infection, one could assume that penetration had

Table 3.6. Mean lengths (± SE) for germ tubes with appressoria for germinating conidia observed on infected and uninfected adult *Lygus lineolaris* previously inoculated with *Metarhizium anisopliae*.

Body region	Infected		Uninfected	
	n^a	Germ tube length (Mean ± SE)	n^a	Germ tube length (Mean ± SE)
Pronotum	18	6.0 ± 0.9	-	-
Scutellum	8	2.4 ± 0.4	-	-
Wings	39	5.0 ± 0.9	3	14.6 ± 5.2
Mesoscutum	3	6.6 ± 2.9	-	-

n^a - Number of observed germ tubes with appressoria

Table 3.7. Percent germination (± SE) of conidia on different regions of the dorsal surface of adult *Lygus lineolaris* that died 24 h to120 h after exposure to *Metarhizium anisopliae*. Letters denote significant differences in percent germination for a given body region for infected (n = 21) versus non-infected (n = 13) insects (Kruskal-Wallis test, $P < 0.05$).

Body region	Infected (% ± SE)	Uninfected (% ± SE)
Head	12.3 ± 5.4 a	3.3 ± 3.3 a
Pronotum	23.0 ± 3.3 a	6.6 ± 2.7 b
Mesoscutum[a]	18.9 ± 6.9 a	0.0 ± 0.0 b
Scutellum	15.4 ± 3.1 a	2.0 ± 2.0 b
Right wing	20.4 ± 4.3 a	4.2 ± 1.7 b
Left wing	28.9 ± 5.2 a	7.9 ± 3.8 b

Mesoscutum[a] - Percent germination for a given body region for infected (n = 15) versus non-infected (n = 11) insects

Figure 3.5. Scanning electron micrograph showing branching in the developing germ tube observed on the scutellum of an individual that died from infection 24 h post-inoculation with *Metarhizium anisopliae*.

taken place.

3.3.4 Topography

Scanning electron micrographs showed that the body regions examined varied in their surface topography, which may affect spatial patterns of conidial deposition and subsequent germination and penetration. Characterization of the cuticle topography (depressions adjacent to setal sockets, shallow depressions not adjacent to setal sockets or epicuticular seams, and smooth/open surfaces, as shown in Figure 3.6 A-C) was related to the location of conidial deposition and revealed differences in deposition patterns between treated insects that died from infection and those that died from other causes. With the exception of a single conidium that appeared attached to the shaft of a seta, conidia were always observed in contact with the cuticle. Conidia of *M. anisopliae* were capable of attaching to most sites on the dorsal cuticle of *L. lineolaris* but were most frequently observed lodged in depressions adjacent to setal sockets (Figure 3.7 A) and less frequently on smooth surfaces (Figure 3.7 B). Topography appeared to have significantly affected conidia numbers for both infected ($F = 55.14$; df = 2; $P < 0.001$) and uninfected insects ($F = 23.54$; df = 2; $P < 0.001$) based on ranked conidial counts (Table 3.8). A relationship was also observed between surface type and the ranked number of germinating conidia (Table 3.8) observed on infected ($F = 6.69$; df = 2; $P = 0.002$) and those uninfected ($F = 3.42$; df = 2; $P = 0.042$). The highest mean proportion of conidia and germinating conidia were observed in depressions adjacent to setal sockets and the fewest observed near epicuticular seams or in shallow depressions not adjacent to

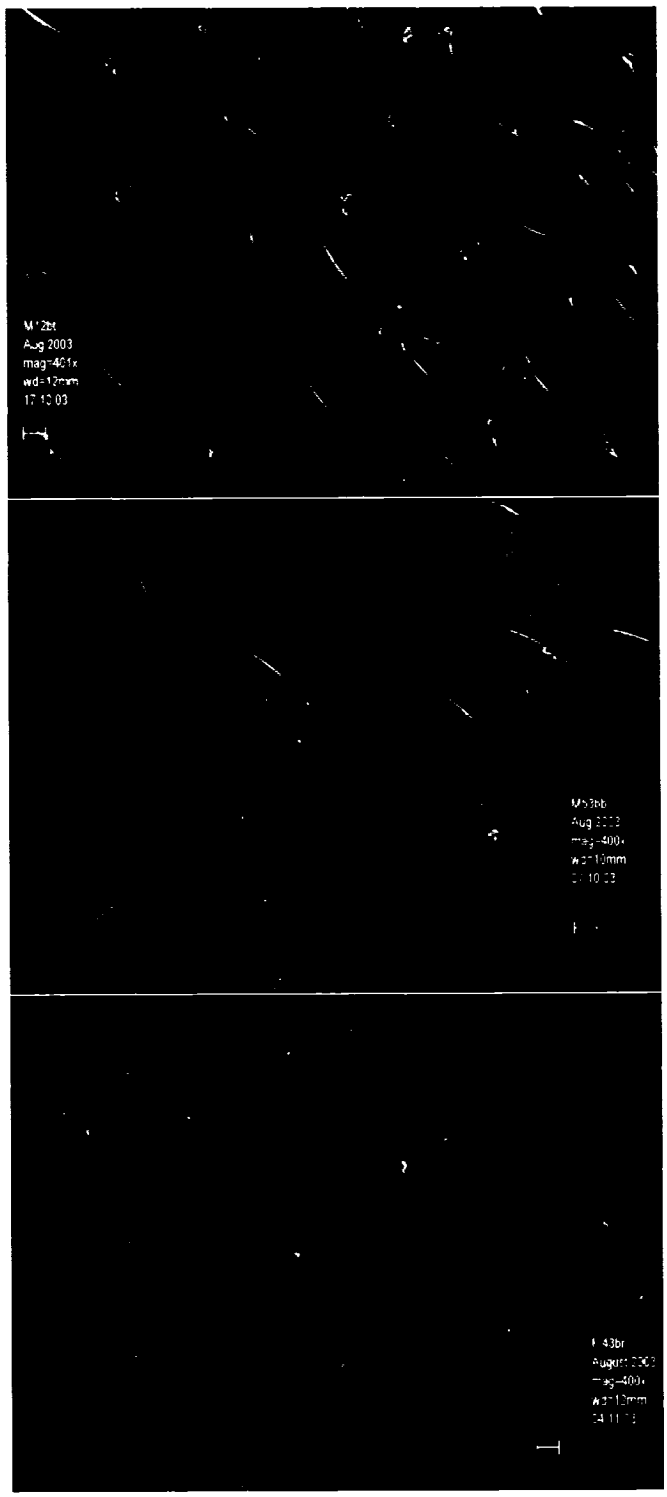

Figure 3.6. Conidia on the dorsal cuticle of adult *Lygus lineolaris* post-inoculation using *Metarhizium anisopliae*. Surface topographies include: depressions adjacent to setal sockets (A), ridges, seams or shallow depressions not adjacent to setal sockets (B), and smooth, open areas (C).

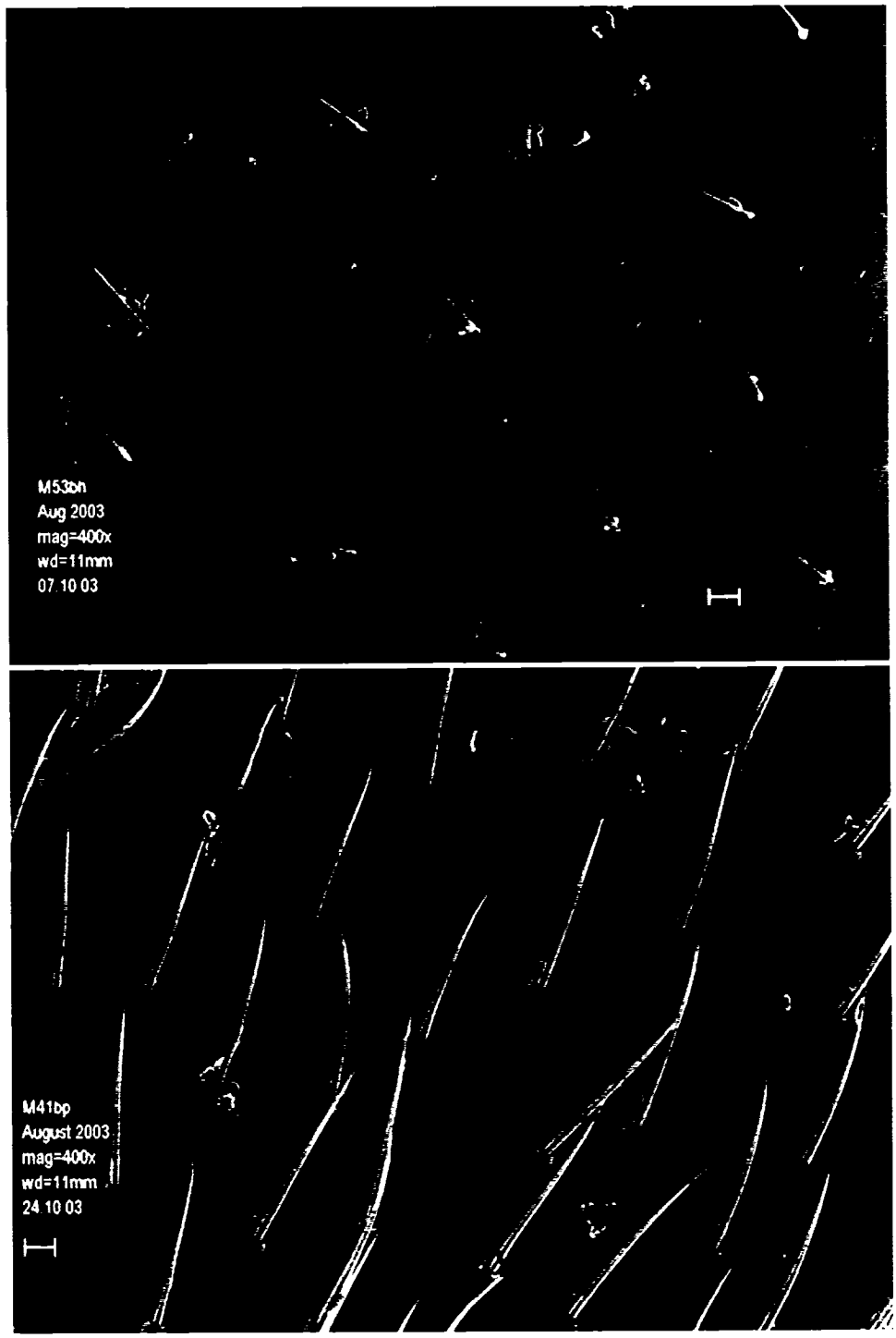

Figure 3.7. Conidial aggregations observed on the dorsal cuticle of adult *Lygus lineolaris* sprayed with *Metarhizium anisopliae*. Aggregations of conidia were observed in depressions adjacent to setal sockets on the pronotum (A) and attached to smooth surfaces on the left wing (B).

Table 3.8. Mean proportion of conidia and germinating conidia observed in relation to different topographies on the dorsal surface (body regions combined) of infected and uninfected adult *Lygus lineolaris* sprayed with *Metarhizium anisopliae* conidial suspensions. Counts on infected and uninfected insects for surface topographies with the same lowercase letter are not significantly different (Kruskal-Wallis, $P < 0.05$).

		Area adjacent to setal socket		Seams and depressions		Smooth surface	
Infection status	No. of conidia	Conidia (Mean ± SE)	Germ. conidia (Mean ± SE)	Conidia (Mean ± SE)	Germ. conidia (Mean ± SE)	Conidia (Mean ± SE)	Germ. conidia (Mean ± SE)
Infected	132	0.46 ± 0.029 a	0.25 ± 0.032 a	0.13 ± 0.020 b	0.08± 0.022 b	0.25 ± 0.023 c	0.10 ± 0.019 b
Not infected	96	0.36 ± 0.031 a	0.21 ± 0.036 a	0.11 ± 0.020 b	0.04 ± 0.014 ab	0.35 ± 0.030 a	0.12 ± 0.027 b

setal sockets (Table 3.8).

Topography also affected conidia numbers within a given body region for both infected and uninfected insects. Surface type affected conidial counts on the pronotum, scutellum, and the wings (Figure 3.8 A, B). Similar trends were observed for germinating conidia numbers where the surface characteristics of the pronotum and the wings significantly affected counts for infected and only the pronotum and wings for uninfected insects (Figure 3.8 C, D). In all comparisons, a significantly positive relationship was identified between setal sockets and total conidia or germinating conidia numbers. Areas adjacent to setal sockets on infected insects, saw the highest number of appressoria (51.4%) compared to 26.4% and 22.2% for smooth surfaces and depressions, respectively. For treated insects that died of other causes, 4 out of 5 appressoria were observed adjacent to setal sockets and one was in a depression. However, with the exception of the data for the wings, there seems to be a shift from higher numbers occurring in depressions adjacent to setal sockets (which was observed on infected insects) to more conidia on smooth surfaces and in depressions adjacent to setal sockets in treated insects that did not die from infection. Small conidial aggregations were often observed, like in Figure 3.9 (A),with multiple germ tubes observed emerging from these aggregates of conidia deposited in cuticular depressions (Figure 3.9 A-B). These were counted as a single germinating conidium as it could not be demonstrated conclusively how many spores were actually germinating since a *M. anisopliae* conidium is able to produce more than one germ tube (Figure 3.9 B). In many instances where conidia were deposited adjacent to setal sockets, germ tubes displayed strong directional growth away

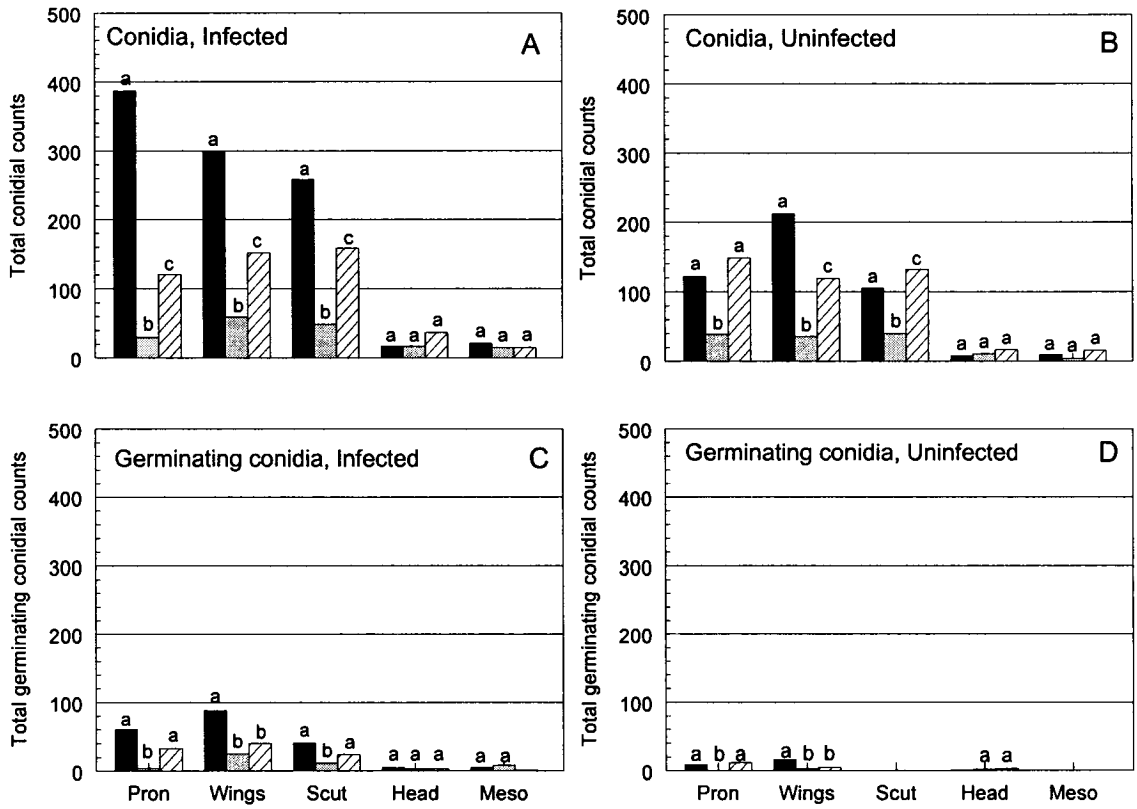

Figure 3.8. Total conidia and germinating conidial counts observed on different surface topographies for each body region on infected (A and C) and uninfected (B and D) adult *Lygus lineolaris* sprayed with *Metarhizium anisopliae*. Counts were not adjusted for different sizes of body region. Post mortem assessments determined whether death had resulted from infection. Legend for surface topographies: depression adjacent to setal socket (black), seams and ridges (grey), and smooth surfaces (striped). Counts for surface topographies within a body region with the same lowercase letter are not significantly different (Waller-Duncan k-ratio t-test).

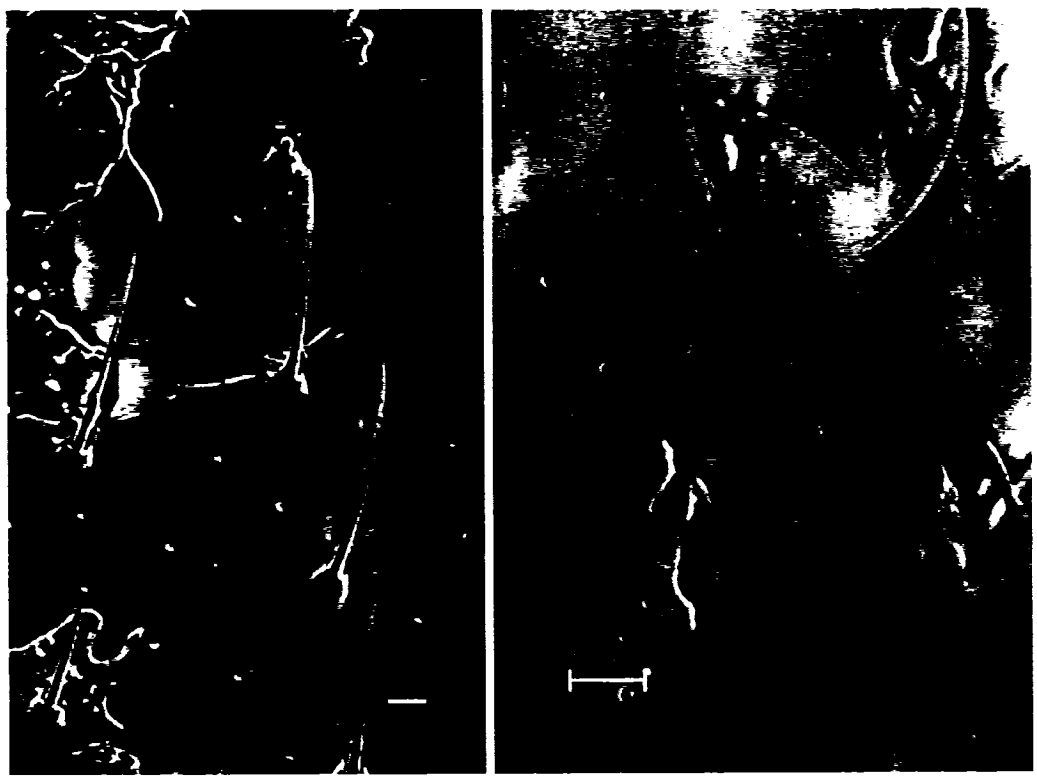

Figure 3.9. Multiple germ tubes were observed (A) and on occasion near cuticular depressions adjacent to setal sockets (B). Digitized micrographs showing growth on the wing of an adult *Lygus lineolaris* that died of infection 48 h post-inoculation (A) and the pronotum for an individual that died of infection 72 h post-inoculation (B). Scale bar represents 10 μm.

from the setal socket (Figure 3.10 A-B).

4. Discussion

This study was the first to investigate the effect of various factors at the conidium-cuticle interface as it relates to subsequent disease development in individual adult *Lygus lineolaris* exposed to *Metarhizium anisopliae*. The fate of the insect after exposure to a pathogen was found to be influenced by the percentage germination, location (specific body region where germination took place) and cuticle topography. In addition, gender also affected the treatment effect observed in infected insects only.

4.1 *Pathogenicity bioassays and LT_{50} determination*

After direct application of *M. anisopliae* on adult *L. lineolaris* mortality levels averaged 96% with mycosis responsible for 65% to 63% of the mortality. For insect death not attributed to mycosis, 50% of the mortality was observed within 24 h after inoculation. Conversely, death resulting from infection took approximately 5 days with the exception of six (8.7%) individuals that died within 24 h (n=4) and 48 h (n=2), whereas, 36.2% died after 5 days. These early deaths resulting from infection demonstrate the variation in host susceptibility or virulence in the fungus. Hence, the infected insects that died earlier than the majority were likely in a weakened state. This weakness may have facilitated infection. Attempts to maintain field-collected insects in the laboratory before treatment in bioassays can often lead to added stress on the host, brought about by less than ideal rearing conditions, often resulting in high insect mortality (Hajek and

Figure 3.10. Directional growth observed in conidia of *Metarhizium anisopliae* on the dorsal cuticle of adult *Lygus lineolaris*. Germination tubes developed from conidia observed in a depression adjacent to a setal socket on the anterior end of the left wing (A) and a germinating conidium having attached in an open area near the femur and tibia joint (B). Legend: conidia (c) and germination tubes (gt). Scale bar represents 10 μm.

Goettel, 2000). However, variation in host susceptibility or virulence in the fungus could also exist in natural populations.

Several factors may influence a insect's susceptibility to disease. Bajan and Bilewicz-Pawinska (1971) found that susceptibility was affected by the generation and geographical location of habitat of *Lygus rugulipennis* Popp. but not by life stage (adults versus nymphs). In this study, all insects received the same treatment (dose of pathogen, food, and environmental conditions) so differences observed in mortality and infection may have been related to the host's physiological age, as adults were field collected and not laboratory reared. Differences were more likely due to variation in the pathogenicity or virulence of the fungus. Observed variation possibly reflects the differences in nuclear content within the conidia produced by an isolate, so that individual conidia can express different traits (Kendrick, 2000).

A significant relationship was also found for gender in infected *L. lineolaris* , where, females took slightly longer to die. Of particular interest is that gender differences were not detected for those insects that died of causes not related to infection. One possible reason for the effect of gender may be the size difference between males and females, where females can be slightly larger (Schwartz and Foottit, 1998), thus, requiring more time for fungal invasion of tissues or possibly females have more resources to fight infection. Researchers have also reported increased susceptibility to infection in male beetles (Elateridae) that were shorter lived than females (Zacharuk and Tinline, 1968). This may be a plausible reason for the observed effect of gender in this study. In laboratory trials with fluctuating temperatures (17-30°C), longevity for female *L.*

lineolaris ranged from 31 to 68 days compared with 19 to 41 days for males (Mailloux and Bostanian, 1994). Despite some control mortality, the number of insects in the procedural control group that survived the duration of the trial was considerably higher than for insects in the treated group. Thus, exposure to *M. anisopliae* did appear to give some control against *L. lineolaris*.

4.3 *Inoculum density, spatial distribution of conidia and the development of disease*

4.3.1 Conidia counts

The data from this study suggest that conidia are able to attach, germinate and produce appressoria on *L. lineolaris*. A number of factors have been shown to affect the development of infection after exposure to infective conidia. Exposure method (Fernandez et al., 2001), concentration of spores, and temperature (Tefera and Pringle, 2003) can influence the development of infection. In this study, abdomens were removed from insects prior to examination with the VPSEM, and therefore, it remains unknown if this body area conforms to observations and events that could affect disease development. However, the assumption was that the dorsal surface of *L. lineolaris* would have received the most spray and unless the insects were flying during the application then conidia would likely hit the wings rather than the abdomen. Spatial distribution of conidia on second-instar Colorado potato beetles, *Leptinotarsa decemlineata*, varied with higher numbers of conidia observed on the dorsal abdominal cuticle for insects sprayed directly in contrast to the higher conidial counts on the ventral abdominal surface and mouthparts for insects exposed to treated foliage (Fernandez et al., 2001). Mean conidial counts were

lower (161.1 conidia/insect) for insects sprayed directly and placed on unsprayed potato foliage versus untreated insects exposed to sprayed foliage (256.1 conidia/insect) or both cases where insects were sprayed on foliage (408.3 conidia/insect). Despite the differences in conidial numbers, fewer (34%) untreated insects exposed to leaves that had been sprayed died than those sprayed directly (Fernandez et al., 2001). Leland and Behle (2005), reported higher mortality (80 times greater) for adult *L. lineolaris* sprayed directly versus exposure through contact with broccoli treated with *B. bassiana* (GHA) conidia.

The total number of conidia and germinating conidia was assessed for the dorsal cuticular surface of treated adult *L. lineolaris* that either died from infection or from causes not related to infection. Although, conidia were observed on every insect sampled, regardless of whether mycosis developed, total conidial levels were highly variable suggesting that 'effective' dose is only a fraction of that sprayed. Conidia counts were higher on infected insects but comparison of means between infected and uninfected insects did not reveal any statistical differences. This was more than likely due to the large range in total counts of conidia for each individual determined to be infected (6 to 52 conidia) and uninfected (1 to 41 conidia). Electron microscopic assessment of conidia were made for those insects that died 24, 48, 72, 96, and 120 h after exposure to *M. anisopliae*, and four additional individuals removed 30 minutes after inoculation (0 h). Total conidial counts varied for individuals that subsequently died of infection versus treated insects that died of other causes but counts were not affected by the time that lapsed between exposure and death. This is not surprising, since hydrophobic conidia are thought to adhere strongly to the host cuticle (Boucias et al., 1988). After 10 days,

sporulation was observed on adult *L. lineolaris* exposed to *B. bassiana*, suggesting that conidia may remain on the cuticle surface for a period of time before infection develops (Leland and Behle, 2005). This was also observed in this study where a single individual did not show signs of infection until the end of the trial (14 days), despite the fact that < 1% of the conidia observed on it had germinated by that time. This is not exceptional: infected, adult chinch bug, *Blissus leucopterus leucopterus* (Hemiptera, Lygaeidae), could survive for approximately 12 to 21 days after being exposed to *B. bassiana* for two weeks (Krueger et al., 1991).

Although, conidia were observed on all body regions examined, distributions of conidia on the cuticle varied from region to region and between insects for the same region. However, no significant differences in conidial counts and deposition patterns were detected. Grooming behaviour would most likely affect conidia distribution patterns on the cuticle, where regions that the insect can clean would be less likely to favour conidial attachment. Quintela and McCoy (1998), showed a reduction in conidia on the larval cuticle of *Diaprepes abbreviatus* (Coleoptera, Curculionidae), 12 h post-treatment, and suggested that the initial loss was due to the removal of non-attached conidia. Zacharuk (1970) reported that *M. anisopliae* conidia attached nonspecifically over the entire cuticular surface of three species of larval Elateridae and were more easily removed from smooth cuticular areas (e.g. sclerites) than from epicuticular folds. Although it seemed that *L. lineolaris* could groom many areas on the dorsal cuticle clean of infective conidia, it was never observed that the entire pronotum, mesoscutum or scutellum could be reached (D. Moreau, personal observation). Furthermore, preening may move conidia

from one region with a smooth surface to a region that has a high setae (e.g. wing to leg). This observed variability in conidial deposition and adherence suggests that other processes not related to adhesion may also be related to pathogenicity.

4.3.2 Germinating conidia, percent germination and body regions

Spore germination was low on all insects treated compared with checks on dialysis tubing and cultures. This may partly be a result of the conservative sampling methods used in this study, where multiple germ tubes were only counted as a single germination event if the source conidia could not be isolated from an aggregation. In situations where there was extensive hyphal growth, counts only reflected one germ tube since a single *M. anisopliae* conidium is capable of producing multiple germ tubes (Zacharuk, 1970). However, this was not observed often enough to explain the difference.

Germinating conidia were observed on all regions sampled and both counts of germinating conidia and percentage germination were significantly different for infected versus uninfected insects. The location where germination occurred was also related to whether or not disease developed, where all body regions (except the head) were significantly associated with development of disease. This leaves unresolved the question as to whether one body region is more vulnerable to infection than another (or is it merely a numbers game), since experimental conditions had been held constant for all insects. Perhaps there is a critical number of germination events required to breach the host's defenses, however, data generated in this study did not provide any clear evidence to support this idea one way or another.

For those insects that succumbed to infection, most conidia adhered to the mesoscutum, then the pronotum, scutellum, both wings, and finally the head. Vestergaard et al. (1999), reported a somewhat different pattern for conidia on adult western flower thrips, *Frankliniella occidentalis* (Thysanoptera: Thripidae) exposed to *M. anisopliae*, with the highest number of conidia reported on the wings (73%), thorax (~18%), abdomen (6%) and head (~3%). No statistical difference was detected between infected and uninfected insects based on conidia counts for different body regions. However, germinating conidial counts for the pronotum, scutellum, mesoscutum, and both wings were significantly different between treated insects (infected and uninfected). Most conidia and germinating conidia were observed attached to the wings presumably since that particular region offered the largest surface area and conidia were deposited by spraying from above. Wang and St. Leger (2005), reported 73% and 54% germination on the wings of the locust, *Schistocerca gregaria*, and the hemipteran cicada bug, *Magicicada septendecim*, respectively. Both had been directly exposed to *Metarhizium anisopliae* var. *acridum* conidia. Since this particular strain is currently being developed for use against *S. gregaria* and was isolated from a locust, *Austracris guttulosa* (Orthoptera, Acrididae) (Wang and St. Leger, 2005), this is likely the reason for the higher rate of germination on that insect. Despite having been isolated from a sawfly larva, *M. anisopliae* (MET-01) produced 63% to 65% infection in treated adult *L. lineolaris*. It is likely that infection rates would have been greater if the pathogen had originally been isolated from *Lygus*. However, Liu et al. (2002), reported that of the 11 isolates responsible for > 90% mortality in second instar *L. lineolaris*, seven had been

isolated from insects not belonging to the order Hemiptera. One of the most striking results is that germination on the cuticle was clearly suppressed compared to that observed on an inert surface (e.g. dialysis tubing) under essentially ideal conditions. This is not surprising given the number of factors operating at the cuticle interface (e.g. immune system of the host) that would not exist on dialysis tubing. Interactions such as these offer explanation for the difficulties reported in biological control attempts since contact of the pathogen with its host does not guarantee infection.

4.3.3 Topography

One plausible hypothesis as to why type of surface was a determinant for the development of infection may be related to the surface topography for a given body region. For example, the surface of the head of *L. lineolaris* is predominantly smooth compared with the pronotum, characterized by shallow, moderately to widely spaced depressions, or the shallow to moderately deep punctated wings, that also have moderately long to long, densely distributed, overlapping setae (Schwartz and Foottit, 1998). Patterns of conidia and germinating conidia counts on the different types of surfaces (sites near setal sockets, depression or seams, or smooth surfaces), differed significantly between infected versus uninfected insects. Data from this study showed that higher conidia numbers for the immediate area around the setal socket was in agreement with those of Vestergaard et al. (1999), who reported that *M. anisopliae* conidia were capable of binding to any site on the cuticle of adult western flower thrips, *F. occidentalis* (Thysanoptera, Thripidae), but were often observed trapped by setae. Likewise where the

southern green stink bug, *N. viridula* (L.) (Hemiptera, Pentatomidae), was treated there were higher densities of *M. anisopliae* conidia on body regions with higher numbers of setae (Sosa-Gomez et al., 1997). Fewer *M. anisopliae* conidia were observed from less exposed regions, such as the setal socket, on the cuticle of *Diaprepes abbreviatus* (Coleoptera, Curculionidae) larvae (Quintela and McCoy, 1998).

The force associated with spraying may lodge conidia into cuticular folds and facilitate attachment, germination, and penetration (Boucias et al., 1988; Fernandez et al., 2001). In this study with *L. lineolaris*, however, the fewest conidia were found in depressions not adjacent to setal sockets or along cuticular seams. Small aggregations of conidia were often observed in depressions adjacent to setal sockets. Rows of setae or spines, often observed adjacent to or overlapping inter-segmental seams, could have formed a protective barrier for conidia that could become lodged in the more vulnerable areas through the force of the spray or insect movement. Zacharuk (1970) reported that *M. anisopliae* conidia on cuticles of three species of larval elateridae were more easily removed from smooth cuticular areas (e.g. sclerites) than from epicuticular folds.

St. Leger et al. (1991), reported that a thigmotrophic response by conidia to surface structures can also influence initial attachment, germ tube and appressoria development. For example, the length of developing germ tubes varied between the dorsal versus ventral side of abdomens on *L. decemlineata* exposed to *B. bassiana* (Fernandez et al., 2001). Hoch et al. (1987), reported that the rust fungus, *Uromyces appendiculatus* responded (by growth orientation and appressorium formation) to surface features on both bean leaves and polystyrene replicas of the leaf surface. Spore development on the

polystyrene showed that germ tubes grew straight and perpendicular to ridges measuring 0.5 μm high similar to the height of stomatal guard cells on the leaf surface (Hoch et. al., 1987). In many instances where conidia were deposited adjacent to setal sockets on *L. lineolaris*, germ tubes displayed strong directional growth away from the structure. Wang and St. Leger (2005), observed that hyphae were frequently deflected by setal sockets and other surface structures and changed directional growth; this was observed in this study also. In contrast, germinating *M. anisopliae* conidia observed near or attached to setal sockets on *F. occidentalis*, often produced a germ tube that developed towards the setal socket (Vestergaard et al., 1999). Conversely, Wraight et al. (1990), reported that germinating *Erynia radicans* spores developed towards membranous areas on the cuticle of *Empoasca fabae* nymphs. Thus, thigmotropism may play a critical role in the infection process by enhancing the ability of developing conidia to invade at optimal penetration sites (Van Burik and Magee, 2001). However, germination of conidia on the dialysis tubing was extensive with lengthy germ tubes and no appressorial development was observed. Infected versus uninfected insects showed different germination patterns among the different body regions and for certain surface types suggesting that conidia may have responded to regional specific cues (e.g. chemical) and not exclusively a thigmotrophic response.

Wang and St. Leger (2005) suggest that regional specificity may affect whether or not appressorial development, and ultimately infection, will occur. Data from this study showed that the majority of appressoria were associated with insects that had become infected versus those that died from other causes. Since percentage germination was

highest on the wings and then the pronotum, it is not surprising that regional patterns for appressoria were similar. Germination observed on fifth instar *Manducta sexta* (Lepidoptera, Sphingidae) occurred on both smooth and convoluted surfaces but appressorial development was primarily on smooth surfaces (St. Leger et al., 1991). Vestergaard et al. (1999), observed regional influences on differentiation reporting that a single appressoria was often produced on hard, sclerotized cuticle (head, thorax and abdomen) compared with two or more appressoria formed at the end of a germ tube on intersegmental membranes on the abdomen. St. Leger et al. (1989) noted that appressorial development was only observed for *M. anisopliae* germ tubes growing on hard surfaces. In this study, conidia were sprayed on the insect cuticle in a suspension and topographical features such as depressions adjacent to setal hairs may have been a more effective 'trap' than seams or smooth surfaces. Not surprising conidia numbers were observed more often associated with depressions adjacent to setal hairs. A relationship was also identified for germinating conidia and topographical surface, with a greater proportion of conidia having germinated in depressions adjacent to setal sockets in comparison to the other surface types. Possible signals that the conidium may respond to such as different chemical cues that may be characteristic of different topographical surfaces on the cuticle of *L. lineolaris* are not known. One plausible reason for the observed differences in surface features and conidial attachment and development may simply be that certain topographies provide a more amenable micro-environment for the conidium, acting as a possible reservoir for moisture for example, or that they offer protection from grooming.

5. Conclusion

In summary, conidia of *M. anisopliae* are capable of attaching and germinating on the cuticle of adult *L. lineolaris*, resulting in mycosis. Direct observations of the host cuticle after the pathogen was applied by spray, revealed that germination varied between insects and was further affected by a variety of factors (e.g. adhesion to specific surface features). A significant association was observed between germination and location on the cuticle and topography of body regions. These associations appear to be critical determinants in relation to whether or not infection developed. This study highlights the importance of examining factors influencing the interface between conidia and host cuticle in an attempt to better understand the factors that influence the efficacy of a fungal pathogen considered for use as a biological control agent. Findings from this study suggest that *M. anisopliae* could be considered a potential candidate for biological control of *L. lineolaris*, but further work is needed to develop improved formulation and application technologies that will enhance germination rates on key regions on the insect.

Chapter 4: Survey of endemic fungal infections in host populations of *Lygus lineolaris* in managed and abandoned apple orchards and (adjacent) fields.

Abstract

A two-year survey of natural fungal infection levels in *Lygus lineolaris* (Hemiptera, Miridae) populations in six apple orchards, six adjacent weedy fields, and two fields not adjacent to orchards was conducted in 2002 and 2003 in the Annapolis Valley, Nova Scotia. *L. lineolaris* adults and nymphs (3^{rd}, 4^{th} and 5^{th} instars) were sampled weekly by sweep net from mid-May to early October. Apple orchards were categorized by grower management regime: (1) low (no spray and only mowed once), (2) moderate (fungicide spray only/routine mowing), and (3) high (insecticides, fungicides and routine mowing). Samples (n = 30) of insects from each weekly collection and from each habitat were held in isolation cages and individuals that died were removed and placed in moist chambers to detect infection by fungal pathogens. Total numbers of *L. lineolaris* (adults and nymphs combined) collected in sweep nets were 6,334 and 5,383 for 2002 and 2003, respectively. Adult insects assessed for infection totalled 2,861 in 2002 and 2,170 in 2003. No incidence of natural fungal infection was observed for *L. lineolaris* from any site sampled regardless of management regime.

1. Introduction

The tarnished plant bug, *Lygus lineolaris* (Hemiptera, Miridae), is the most widely distributed *Lygus* species in North America (Schwartz and Foottit, 1998) and is considered a principal pest of apple in Massachusetts (Prokopy et al., 1980), Quebec (Boivin and Stewart, 1982), and Nova Scotia (Hardman et al., 2004). Stings and toxic saliva injected in apple blossoms and the developing apple cause fruit to abort or produces blemishes and distortions that lead to apples at harvest being downgraded or rejected for the fresh fruit market at harvest (Boivin and Stewart, 1982; Prokopy and Hubbell, 1981). With susceptible varieties such as Jonagold, Cortland and Red Delicious (Anonymous, 2003), many apples can be downgraded unless growers apply insecticides during the 4-6 week period in May and June when their crop is at risk. Each spring, high numbers of *L. lineolaris* move into apple orchards from adjacent overwintering sites and start feeding on buds, blossoms and fruitlets on apple trees, during the period from silver tip to tight cluster. Feeding may continue up to 2 weeks after petal fall (Prokopy and Hubbell, 1981). In Nova Scotia, there are two generations of *L. lineolaris* per year (Bishop, 1993) but it is only the overwintered adult stage that poses the risk of damage to apple (Boivin and Stewart, 1982; Kelton, 1975). In response, apple growers spray their crop with insecticides such as imidacloprid, pyrethroids and organophosphates in order to control *L. lineolaris* (Anonymous, 2005). Unfortunately, some of the insecticides used to control *L. lineolaris* and other pests are known to induce outbreaks of spider mites because these chemicals are toxic to their natural enemies (Hardman et al., 1988).

Although entomopathogenic fungi are often considered widespread in their distribution (Tanada and Kaya, 1993; Zimmermann, 1993), only two published reports of fungal infected *L. lineolaris* are known, one in Arkansas in 1992 (Steinkraus, 1996) and one near Albany, New York (Broadbent, 2000). In both instances the pathogen was *Beauveria bassiana*. Strains of fungal pathogens are likely to be more effective against a specific insect if originally isolated from that same host and acclimatised to the local environment (McGuire, 2002). Hence, knowledge of indigenous fungal species can assist the development of a long-term management strategy that uses fungal pathogens as biological control agents and determination of endemic levels of indigenous pathogens is an essential component of an assessment of a biological control agent.

Assessing the impact of natural mortality agents on local pest populations in and around commercial apple orchards can facilitate the development and successful application of biological controls, and potentially reduce dependence on insecticides. Furthermore, this type of survey can provide important baseline data necessary for assessment of any biocontrol attempt. To date most studies on the potential of fungal entomopathogens for use against *Lygus* spp., have been conducted on alfalfa (Noma and Strickler, 1999) and cotton (Brown et al, 1997; Snodgrass and Elzen, 1994; Steinkraus, 1996; Steinkraus and Tugwell, 1997).

The purpose of this research was to assess the incidence of naturally occurring fungal pathogens in *L. lineolaris* populations in apple orchards and non-crop habitats in Nova Scotia. Populations of *L. lineolaris* were monitored through the growing season in 2002 and 2003 to estimate numbers and phenology in both apple orchards and non-crop fields

since *L. lineolaris* visit apple trees in an orchard block (Boivin and Stewart, 1983) and also move to ground vegetation in and around the orchard to oviposit (Boivin, 1981). Insects collected in weekly surveys were monitored in the laboratory to determine the levels of fungal pathogens throughout the growing season.

2. Materials and Methods

2.1 Study sites: Commercial apple orchards and fields

Surveys were conducted on a weekly basis from mid-May to mid October 2002 and 2003, in six commercial apple orchards with adjacent fields and two fields not bordering apple orchards, located in the Annapolis Valley, Nova Scotia, Canada (Lockhartville through Black Rock). Grower management regimes for apple orchards were categorized as (i) low (no pesticide sprays with one mowing event); (ii) moderate (fungicides only and routine mowing), and (iii) high (insecticides, fungicides and routine mowing), as shown in Table 4.1. Fields adjacent to apple orchards were mowed once in late July to mid- August, and received no pesticide treatment. The two fields not adjacent to orchards were only mowed in November after insect sampling was finished for the year. Composition of ground cover vegetation, at the sites was also assessed on three occasions during the growing season (May-June, July-August and September-October). The percentage of ground covered by an individual species was estimated for vegetation observed in ten arbitrarily placed 0.7 x 0.7 m quadrats, where two samples were taken for each of five orchard laneways and five samples were assessed for each of two diagonal transects in non-crop fields.

Table 4.1. Survey of natural fungal infection levels in *Lygus lineolaris* in commercial apple orchards with adjacent fields and two additional fields conducted in 2002 and 2003. All sites were located in the Annapolis Valley, Nova Scotia, Canada. Grower management regimes for apple orchards are explained in the text.

Survey site	Location	Habitat		Groundcover*
		Orchard	Field	
1	Black Rock (low)	√	√	Grass
2	Lockhartville (low)	√	√	Grass
3	Kentville (moderate)	√	√	Mixed vegetation**
4	Avonport (moderate)	√	√	Mixed vegetation**
5	Waterville (high)	√	√	Mixed vegetation**
6	Pereau (high)	√	√	Mixed vegetation**
7	Kentville (low†)		√	Grass
8	Sheffield Mills (low†)		√	Grass

† Mowing of the two wildflower fields not adjacent to orchards only took place after insect sampling was finished for the year.

* Predominant plant species

** Broad leaf plants (e.g. dandelion, sorel)

2.2 Evaluation of natural infection levels in Lygus lineolaris populations

Seasonal occurrence of all life stages of *L. lineolaris* for each site were based on 20 sweeps (180°C arc) with a standard 38 cm diameter insect net in each of five orchard laneways and 50 sweeps in each of two diagonal transects in adjacent non-crop fields. Sweeps were initiated at approximately the same location in the orchards and fields every week, on sunny days with ambient temperatures >15°C. Captured insects were placed in ventilated, plastic vials containing a piece of clover and kept in a styrofoam box chilled with ice packs until returned to the laboratory. Then insects were sorted by life stage (3^{rd}, 4^{th}, 5^{th} instars and adults) with species identified using keys in Schwartz and Foottit (1998). Total counts per 100 sweeps for individual life stages (3^{rd}, 4^{th}, 5^{th} instars and adults) of *L. lineolaris* were calculated for each site and each sampling date.

Sub-samples (50% of the total number of both nymphs and adults to a maximum of 30 nymphs and 30 adults) collected from each orchard and non-crop field on each collection day were reared in the lab and monitored daily for fungal infection and other causes of mortality. Adult *L. lineolaris* were maintained at 21°C, 70% RH and 16L:8D photoperiod in isolation cages in a temperature controlled rearing room, located at the Atlantic Food & Horticulture Research Centre (AFHRC), Kentville, Nova Scotia. A maximum of thirty individuals were in each isolation cage (47 x 47 x 68 cm). Cages were lined with white paper, to facilitate finding dead individuals, and were supplied with three potted green bean plants (Derby variety, Veseys Seeds, York, Prince Edward Island). Plants of the same physiological age and condition were used in all trials and were watered daily. To facilitate observations, juvenile *L. lineolaris* were monitored in

smaller cages constructed from 946 ml Ziploc® plastic storage containers with ventilated lids to prevent escape. Nymphs were given green beans as food and maintained at 21°C, 70% RH and 16L:8D photoperiod. Green beans were changed every 3 days. All cages were examined every 24 h and dead individuals were removed from the isolation cage, placed on moistened filter paper (Whatman No. 1) within a petri-dish and incubated for three to five days, at 25°C in total darkness, to promote fungal growth. In some cases, parasitoids were found to have emerged from both nymphs and adults. Larval parasitoids were collected and stored in ethanol along with the host cadaver and maintained at AFHRC, Kentville, Nova Scotia.

3. Results

3.1 Evaluation of natural infection levels in Lygus lineolaris populations

Although approximately 10,000 bugs were collected on 38 sampling dates over 2 years, no fungal infected individuals were found (Tables 4.2 and 4.3). However, low levels of parasitism were detected (n = 11 in 2002 and n = 31 in 2003), as shown in Tables 4.2 and 4.3. Two generations of *L. lineolaris* were noted in each orchard and field sampled in both years. Overall, results for 2002 (Table 4.2) showed lower levels of parasitism in both the orchard and field habitats as compared to 2003 (Table 4.3).

Intensity of management appeared to affect adult *L. lineolaris* numbers with fewer *L. lineolaris* captured in the low-management habitats in both 2002 and 2003 (Tables 4.2 and 4.3). Overwintered adults were present by mid-May (Figures 4.1 and 4.2) with first generation nymphs observed from early to late June, depending on the site (Figures 4.3

Table 4.2. Cumulative seasonal counts in 2002 for nymphs and adult *Lygus lineolaris* are shown for the six apple orchards with adjacent fields and two additional fields. All sites were located in the Annapolis Valley, Nova Scotia, and surveyed using sweep nets. Incidence of natural fungal infection and parasitism was determined in the laboratory for insects collected on each sampling date for every site surveyed. Legend:'O' and 'F' denoted counts for orchard and fields, respectively.

Apple orchards	Management regime	No. of sampling dates[b]	*L. lineolaris* life stage										Parasitism (%)		Fungal infection (%)
			3rd instar		4th instar		5th instar		Total nymphs[c]		Adults				
			O	F	O	F	O	F	O	F	O	F	O	F	
1	Low	16	14	2	51	7	83	35	148	44	125	146	0	0	0
2	Low	17	1	1	7	2	22	4	30	7	44	65	0	1.4[d]	0
3	Moderate	10	15	15	57	14	189	42	261	71	399	270	0	0	0
4	Moderate	18	5	8	58	38	87	205	150	251	309	724	1.1	0.1	0
5	High	18	4	7	31	15	141	62	176	84	694	569	0	0.2	0
6	High	16	38	14	120	21	251	70	409	105	358	360	0	0.4	0
7	Field only[a]	21	-	0	-	9	-	68	-	77	-	126	-	0	0
8	Field only[a]	19	-	39	-	17	-	45	-	101	-	231	-	0.3	0

[a] Fields were mowed once after all monitoring was completed for the year.
[b] On each sampling date, *L. lineolaris* were collected from 100 sweeps per site.
[c] Total counts represent 3rd, 4th and 5th instars combined
[d] Parasitism observed in adult host.

Table 4.3. Cumulative seasonal counts in 2003 for nymphs and adult *Lygus lineolaris* are shown for the six apple orchards with adjacent fields and two additional fields. All sites were located in the Annapolis Valley, Nova Scotia, and surveyed using sweep nets. Incidence of natural fungal infection and parasitism was determined in the laboratory for insects collected on each sampling date for every site surveyed. Legend:'O' and 'F' denoted counts for orchard and fields, respectively.

Apple orchards	Management regime	No. of sampling dates[b]	*L. lineolaris* life stage										Parasitism (%)		Fungal infection (%)
			3rd instar		4th instar		5th instar		Total nymphs[c]		Adults				
			O	F	O	F	O	F	O	F	O	F	O	F	
1	Low	15	10	15	8	89	10	175	28	279	65	130	0	0	0
2	Low	17	0	2	1	2	11	16	12	20	64	69	0	0	0
3	Moderate	15	13	12	13	17	68	47	94	76	167	378	0.8	0	0
4	Moderate	17	12	7	103	14	331	49	446	70	466	204	0.9	0.4	0
5	High	13	13	21	57	111	80	287	150	419	227	651	0.5	0.1	0
6	High	14	19	12	106	24	126	135	251	171	139	442	1.8	0.7	0
7	Field only[a]	16	-	1	-	12	-	32	-	45	-	146	-	1.6	0
8	Field only[a]	17	-	2	-	8	-	25	-	35	-	140	-	1.7	0

[a] Fields were mowed once after all monitoring was completed for the year.
[b] On each sampling date, *L. lineolaris* were collected from 100 sweeps per site.
[c] Total counts represent 3rd, 4th and 5th instars combined

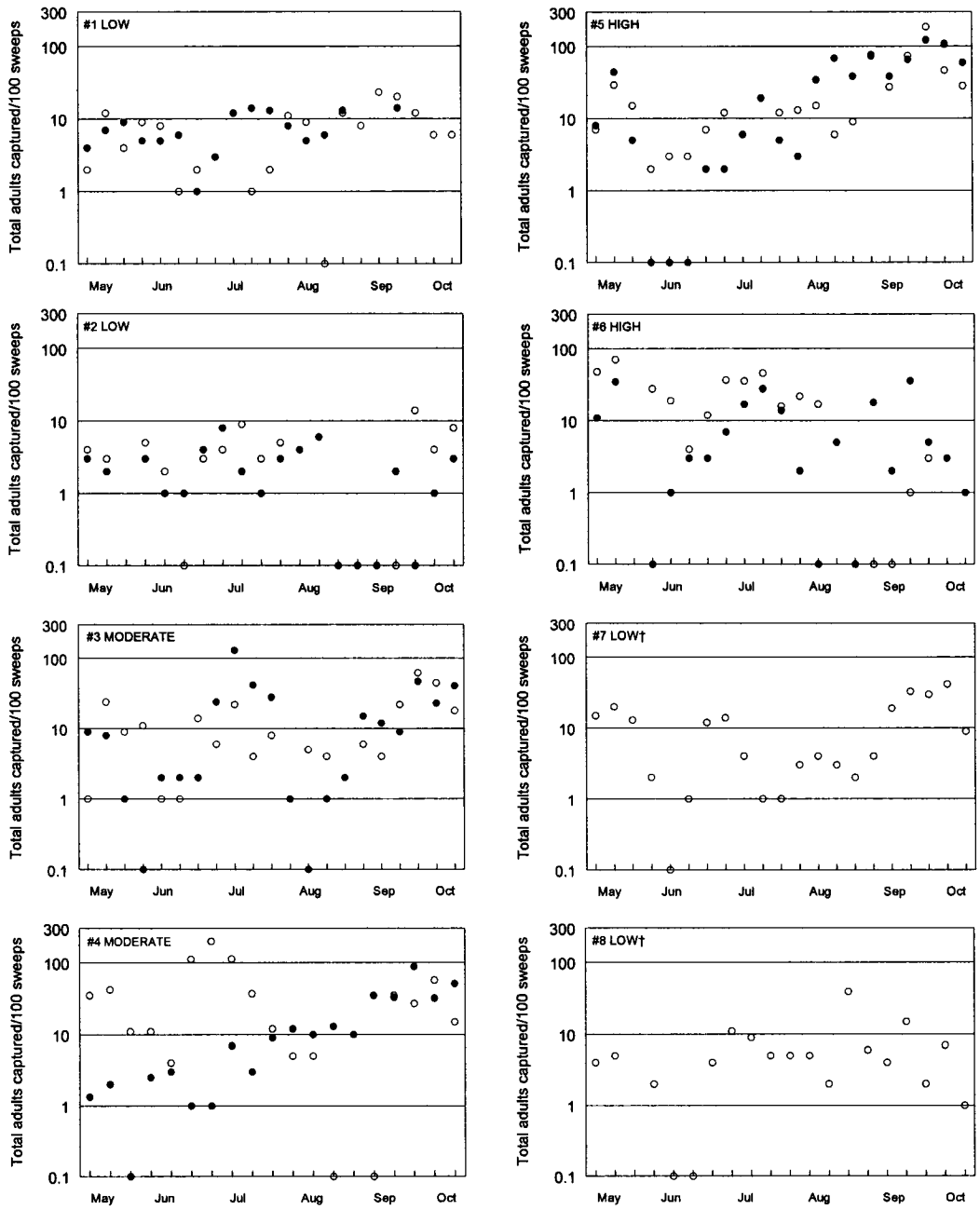

Figure 4.1. Adult *Lygus lineolaris* seasonal counts in 2002, based on weekly sampling of 100 sweeps within apple orchards and fields. Legend: solid circle represents collections taken in apple orchards and hollow circle shows counts for fields; orchard management regimes are represented by low (no spray/mowed once), moderate (fungicide/mowing), and high (insecticide/fungicide/mowing). Fields that were not sprayed and mowed only once († denotes mowing at end of survey).

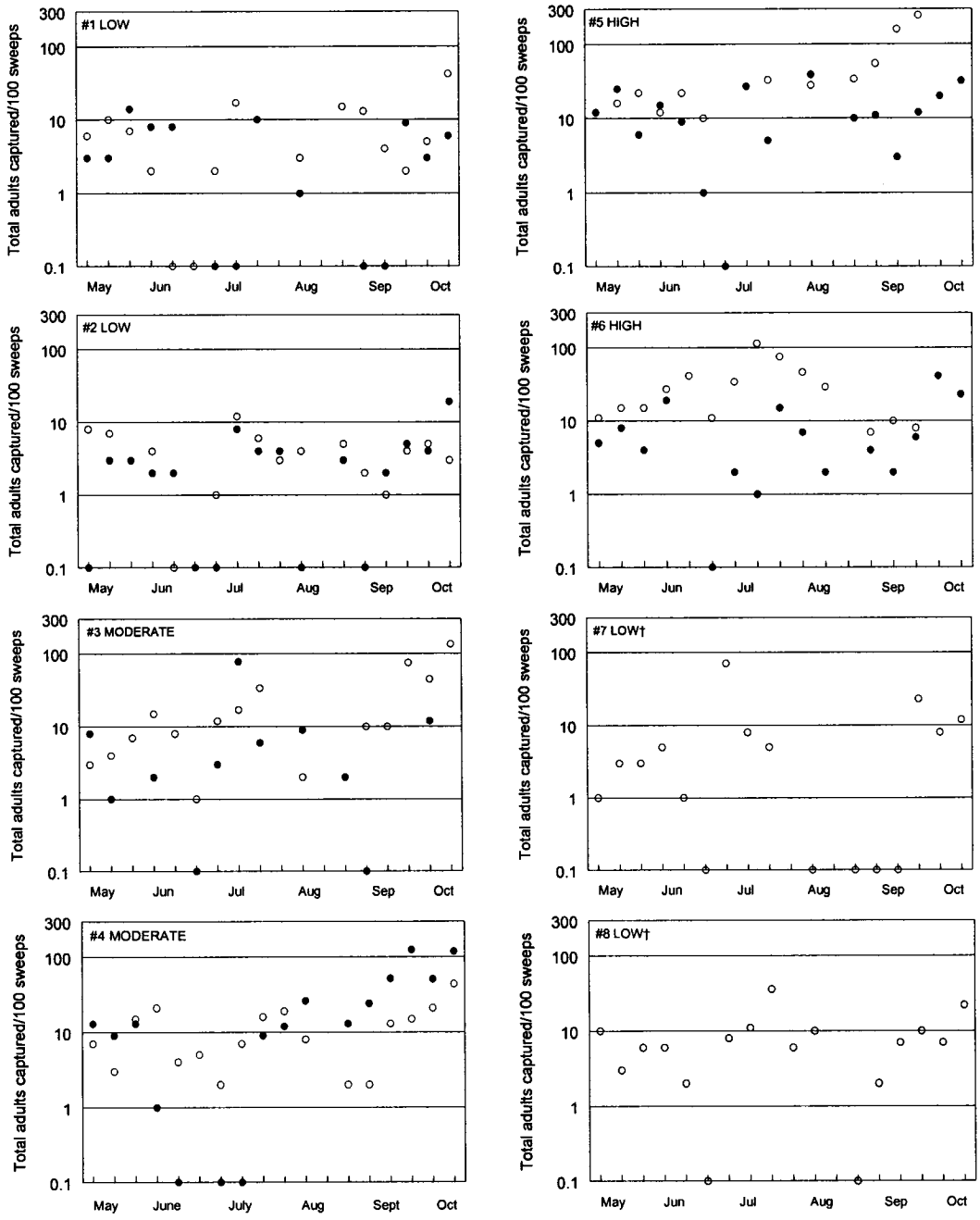

Figure 4.2. Adult *Lygus lineolaris* seasonal counts in 2003, based on weekly sampling of 100 sweeps within apple orchards and fields. Legend: solid circle represents collections taken in apple orchards and hollow circle shows counts for fields; orchard management regimes are represented by low (no spray/mowed once), moderate (fungicide/mowing), and high (insecticide/fungicide/mowing). Fields that were not sprayed and mowed only once († denotes mowing at end of survey).

and 4.4). Numbers of second generation adults peaked from mid to late July and numbers of third generation adults were high from late August to early October in both years (Figures 4.1 and 4.2). Field sites under a low management regime (69%) had mixed grasses (mostly unfavourable hosts for *L. lineolaris*) as the predominant groundcover, whereas broad-leaved species (favourable hosts) were more common in highly managed sites (38%). The relationship between groundcover and endemic fungal infection levels could not be determined because infection was not observed and overall parasitism was so low.

4. Discussion

A two-year survey of *L. lineolaris* in apple orchards and non-crop fields located in the Annapolis Valley, Nova Scotia did not detect naturally-occurring fungal infections, although several pathogenic species are known to occur in the field and can infect in laboratory inoculations (Bajan and Bilewicz-Pawińska, 1971; Bidochka et al., 1993; Brown et al., 1997; Leland and Behle, 2005; Liu et al., 2002; Steinkraus and Tugwell, 1997). Fungal infections are commonly observed in insects exposed to pathogens in laboratory trials but not in the field (Hajek et al., 1996). It is not surprising that the prevalence of infection was difficult to establish since results of similar surveys have also shown high variability between sites and generally low incidence of fungal disease (Leland and Snodgrass, 2004; McGuire 2002). *Beauveria bassiana* infection was detected in only nine out of 54 sites over a two year period in *L. lineolaris* collected on wild host plants in Mississippi. Of those sites, overall infection levels were low

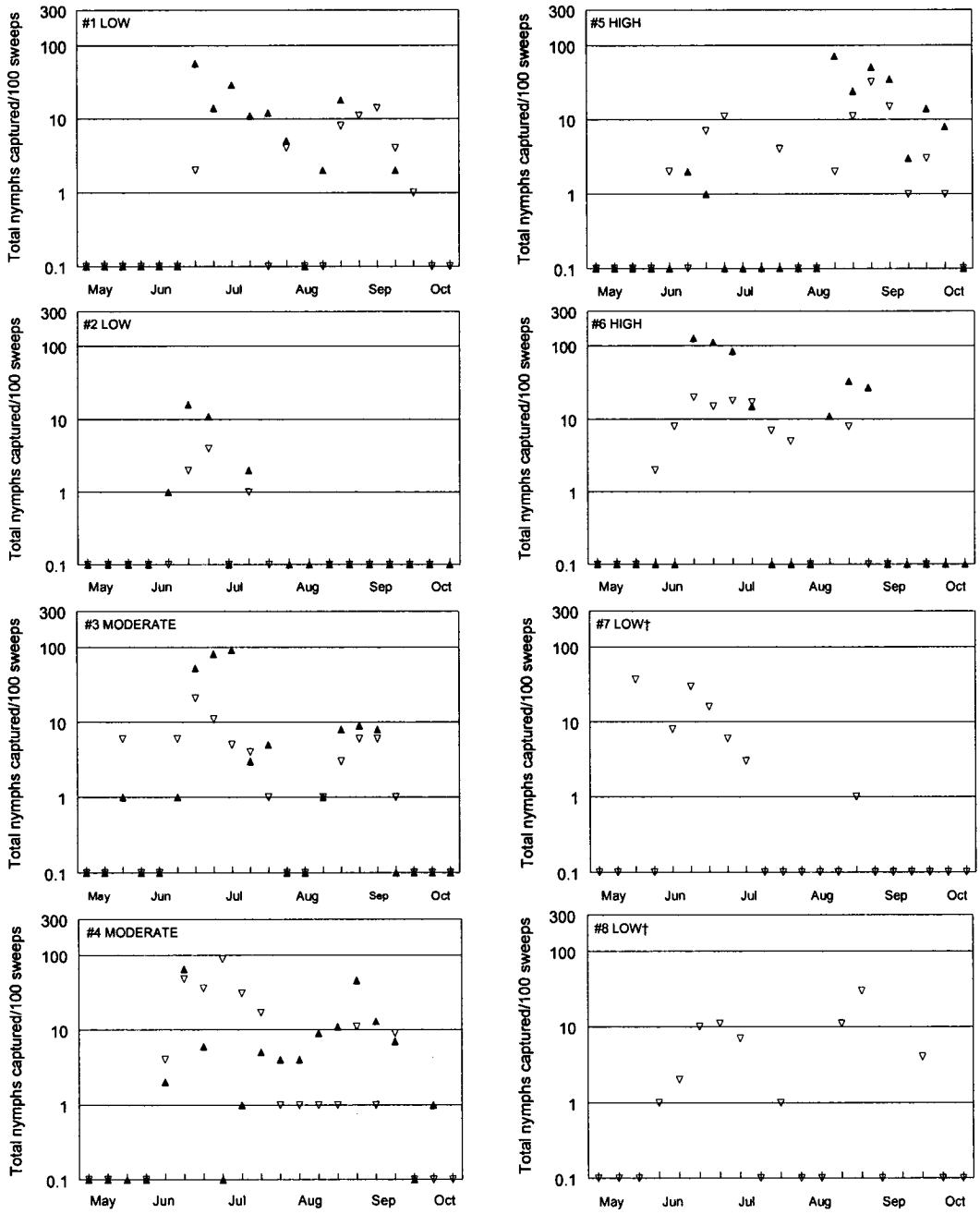

Figure 4.3. Seasonal counts in 2002 of *Lygus lineolaris* nymphs (3rd, 4th and 5th instars), based on weekly sampling of 100 sweeps within apple orchards and fields. Legend: solid triangle represents collections taken in apple orchards and hollow triangle shows counts for fields; orchard management regimes are represented by low (no spray/mowed once), moderate (fungicide/mowing), and high (insecticide/fungicide/mowing). Fields that were not sprayed and mowed only once († denotes mowing at end of survey).

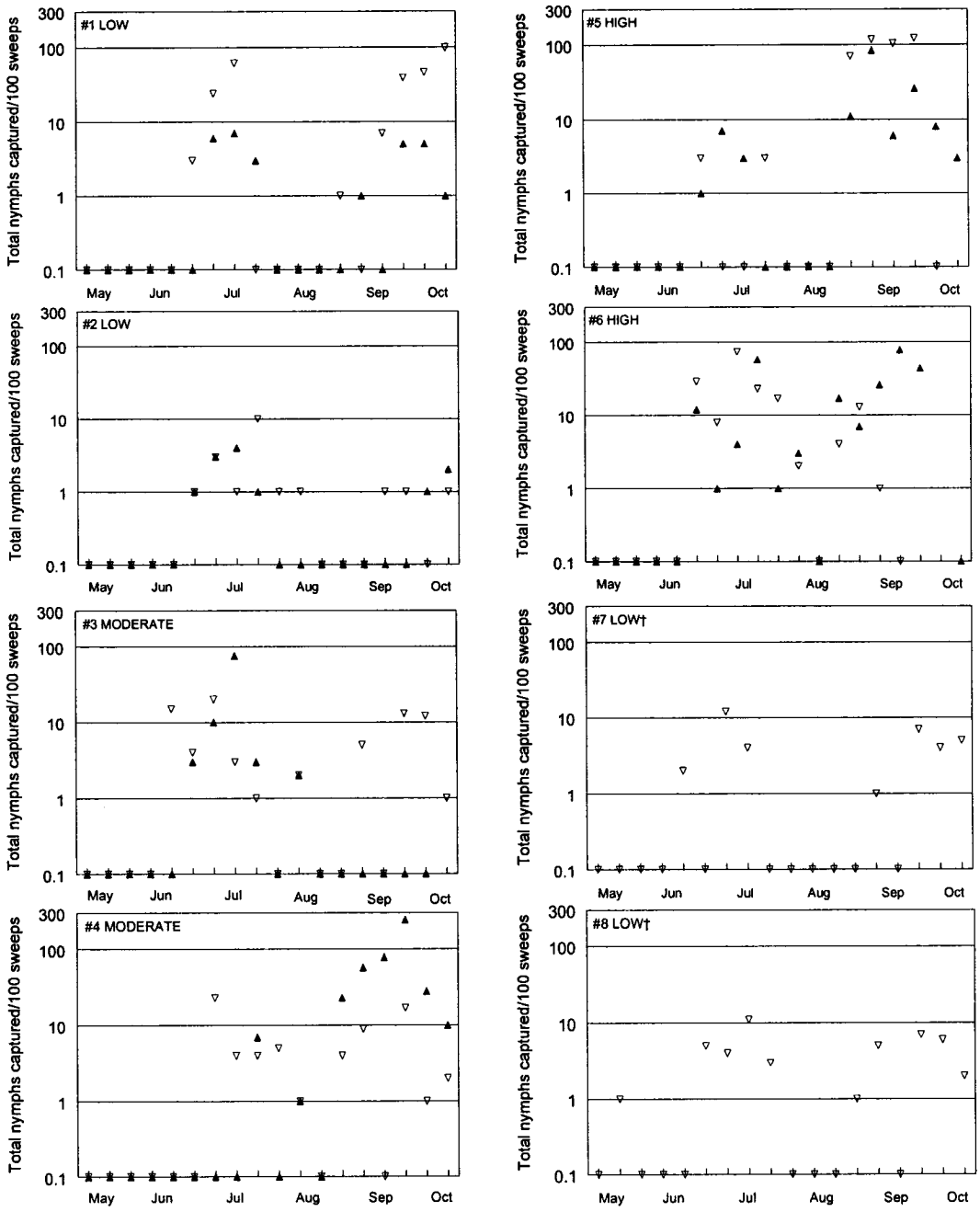

Figure 4.4. Seasonal counts in 2003 of *Lygus lineolaris* nymphs (3rd, 4th and 5th instars), based on weekly sampling of 100 sweeps within apple orchards and fields. Legend: solid triangle represents collections taken in apple orchards and hollow triangle shows counts for fields; orchard management regimes are represented by low (no spray/mowed once), moderate (fungicide/mowing), and high (insecticide/fungicide/mowing). Fields that were not sprayed and mowed only once († denotes mowing at end of survey).

averaging 0.3% ± 0.15% and ranged from 0 to 7.5% (Leland and Snodgrass, 2004). McGuire (2002) also reported varied prevalence (0% to 50%) for *B. bassiana* in *L. hesperus* populations in alfalfa or roadside weeds, located in each of six counties in California. Furthermore, this same study reported that the percentage infection for all life stages combined, averaged 2.4, 3.0, and 6.7 for spring, summer, and fall sampling times. This was consistently higher than previously discussed reports. Soil surveys conducted in pecan orchards in the southeastern United States, demonstrated that entomopathogenic fungi are widespread (collected in 16 out of 21 orchards) but highly varied in their distribution within orchards with fungi isolated from 10.4% of all sites (five sites were sampled in each orchard). In subsequent laboratory bioassays, good control was achieved with several isolates of *B. bassiana* and *M. anisopliae* against the pecan weevil, *Curculio caryae* (Coleoptera: Curculionidae) and are now being considered as potential control agents (Shapiro-Ilan et al., 2003).

It is difficult to surmise exactly why infection was not observed even at low levels in this study, especially when *L. lineolaris* numbers were high. One possibility is that the sampling methods employed in this study may have excluded dead and infected dead or moribund insects at or near the ground level or in the leaf litter. However, similar techniques have been used in other studies where infected insects were collected. Jaques and MacLellan (1965), monitored fungal development in codling moth larvae overwintering in Nova Scotia apple orchards and attributed 1.7% of the observed insect mortality to *B. bassiana*, the predominant fungal species isolated from cadavers. Diseased codling moth larvae are likely more accessible for sampling than diseased *L.*

lineolaris, which may have resulted in increased detection. The pathogen's widespread occurrence resulted in infection even at low host densities. A 2 year survey in lowbush blueberry fields in eastern Canada, showed that endemic fungal infection levels were low (4.13% ± 3.90%) for all of the mortality observed in insects (total of 5,786) collected in the field. Furthermore, infection was highly variable among sites and insects in the study (e.g. flea beetles, sawflies, Hemiptera) (Strongman et al., 1997).

Orchards are intensely managed perennial systems that provide a somewhat more stable habitat for insect pests and beneficials, unlike annual crops where the entire crop is destroyed at the end of the season (Puterka, 1999). However, apple orchards in Nova Scotia are routinely impacted by management practices such as insecticide or fungicide sprays and mowing. In this study, the impact of these practices should have been minimized in sites under low management regimes that provided a pesticide-free buffer plus infrequent or no mowing. Since *L. lineolaris* are highly mobile, feeding and reproducing on more than 350 host plants (Young, 1986), the conditions afforded by the adjacent weedy fields may have provided a greater variety of food and oviposition sites as well as protection from pesticides. These same conditions could also provide a more amenable environment for fungal pathogens underneath the canopy of broad-leaf weeds where exposure to UV is reduced and relative humidity increased. Goettel et al. (2001), reported that exposure to sunlight can reduce the half-life of a spore to minutes, however, protection of a shady environment can increase its persistence. Assuming that a particular environment supports fungal pathogen populations, the issue for control then becomes one of making contact with the insect host. This may be an even greater

challenge considering the high mobility of *L. lineolaris*.

Boivin and Stewart (1983) found that even though *L. lineolaris* adults have the potential to feed on developing apples at fruit set, they generally prefer ground vegetation. In Nova Scotia apple orchards, *L. lineolaris* utilizes a variety of host plants throughout the growing season and colonizes different host species in each generation. Weekly monitoring showed seasonal fluctuations in the number captured for both adults and nymphs which probably reflected movement patterns to and from preferred plant hosts, as well as the effects of spray regimes and ground management practices (e.g. mowing). Infection was not detected and rate of parasitism remained low despite periodic increases in *L. lineolaris* numbers in several survey sites. In contrast, McGuire (2002) found increased *B. bassiana* infection levels with higher numbers of *L. hesperus* populations in alfalfa or roadside weeds (McGuire, 2002). Similar trends were reported by Strongman et al. (1997), with higher numbers of pathogens (4-6 species) isolated from sites where insect collections exceeded 500 individuals in comparison to < 3 fungal species when host numbers were lower (100- 200). Although, high (> 5000 adult insects over 2-years) numbers of *L. lineolaris* were examined for infection over both years of this study, it is possible that an increased sampling effort could yield pathogens.

Understanding the influence of non-crop plants on natural mortality agents of *L. lineolaris*, specifically fungal pathogens (but could also include parasitoids and predators), is most relevant to sustained biological control in an apple orchard. In this study, higher numbers of *L. lineolaris* adults were observed in samples taken from the field sites in contrast to nymphs whose densities were usually greater in orchard

collections. Mortality agents, like fungal pathogens and parasitoids may also be supported by these same weed hosts, although this was not observed in this study. A survey of *L. lineolaris* in wild host plants in Mississippi, found the highest infection levels (2.4%) in populations on goldenrod. Furthermore, *B. bassiana* was isolated from all sites where insects were sampled from goldenrod (Leland and Snodgrass, 2004). Fleischer and Gaylor (1987), found evidence of much higher populations of *L. lineolaris* on certain weed species and suggested that management of specific growth stages (e.g. to maintain a continued presence of the weed host) could reduce the number of *L. lineolaris* attacking the cultivated crop. Although, several native parasitoids are known to attack *Lygus* insects at much higher levels in certain weeds than in crops (Lim and Stewart, 1976; Ruberson, 1998), parasitism levels observed in this study were not sufficient to exert any appreciable effect on host populations at such low densities.

5. Conclusion

The surveys conducted in this study are a necessary prelude to conservation, testing, and introduction of fungal entomopathogens for biological control of *L. lineolaris* both inside and on the margins of orchards. Evaluation of control efficacy should be made under field conditions because virulence and persistence of entomopathogenic fungi may be limited by abiotic and biotic constraints that exist in the field but not in the laboratory or semi-field plots or cages. The extensive use by *L. lineolaris* of most host plants stresses the necessity of considering the numerous plant species of groundcover and hedge rows as an integral part of the orchard ecosystem (Boivin and Stewart, 1983).

Despite, not having observed natural infection levels in field populations of *L. lineolaris*, Harper (1987), emphasized the potential of introducing a pathogen if the habitat including the host population is proved suitable for the pathogen. In a biological control program for apple, it may prove efficacious to introduce a fungal entomopathogen early in the spring on non-crop or weed hosts in order to maintain *L. lineolaris* populations below the economic threshold level when the stage of fruit development is most vulnerable to attack. Treatment of orchard edges in the spring before overwintering adults have migrated into the orchard may favour infection because the weather is cool and moist, the rank vegetation is damp, and overwintered adult *L. lineolaris* may be more vulnerable (e.g. hungry and weak). Although, fruit is not vulnerable to attack in the fall treatments at this time could potentially reduce the overwintering generation that will eventually emerge in the spring and then feed on apple. Higher incidence of fungal infection could reduce *L. lineolaris* damage to apples and lessen growers' dependence on conventional, broad-spectrum insecticides and help to reduce overall pesticide load on the environment.

Chapter 5: General Discussion

Control efforts against *Lygus lineolaris* after application of the fungus *Beauveria bassiana* and, to a lesser extent, *Metarhizium anisopliae* have had varying results (Bidochka et al., 1993; Leland and Behle, 2005; Brown et al., 1997; Kovach, 1995; Leland and Behle, 2005; Leland et al., 2005; Liu et al., 2002; Liu et al., 2003; Snodgrass and Elzen, 1994; Steinkraus, 1996; Steinkraus and Tugwell, 1997). Although the results from the objectives of this study (Figure 5.1) add to the growing support for continued research in the assessment of fungal entomopathogens as biological control agents, their use against *L. lineolaris* on economically important crops in Nova Scotia may be limited using the conventional means of application, such as aerial sprays. In summary, varying levels of mortality and mycosis were realized for a commercial formulation of *B. bassiana*, Botanigard, and three native fungal isolates, *M. anisopliae*, *B. bassiana*, and *V. lecanii* assessed for their potential as control agents against adult *L. lineolaris*. Although, microscopic examination revealed that conidia were capable of attaching and germinating on the cuticle of adult *L. lineolaris*, germination of *M. anisopliae* conidia may be the most critical determinant in whether or not infection developed. Disease development was positively correlated with number of germination events as further influenced by location on the cuticle and integumentary surface features. The conclusion that can be drawn from these patterns is that the number of conidia that actually attach to insects and germinate is a potential limiting factor to efficacy.

The lack of commercial success of fungal pathogens for use in pest management may be found in the assumptions and expectations currently held with regards to application methods and the evaluation of efficacy of microbial-based insecticides in the field. The

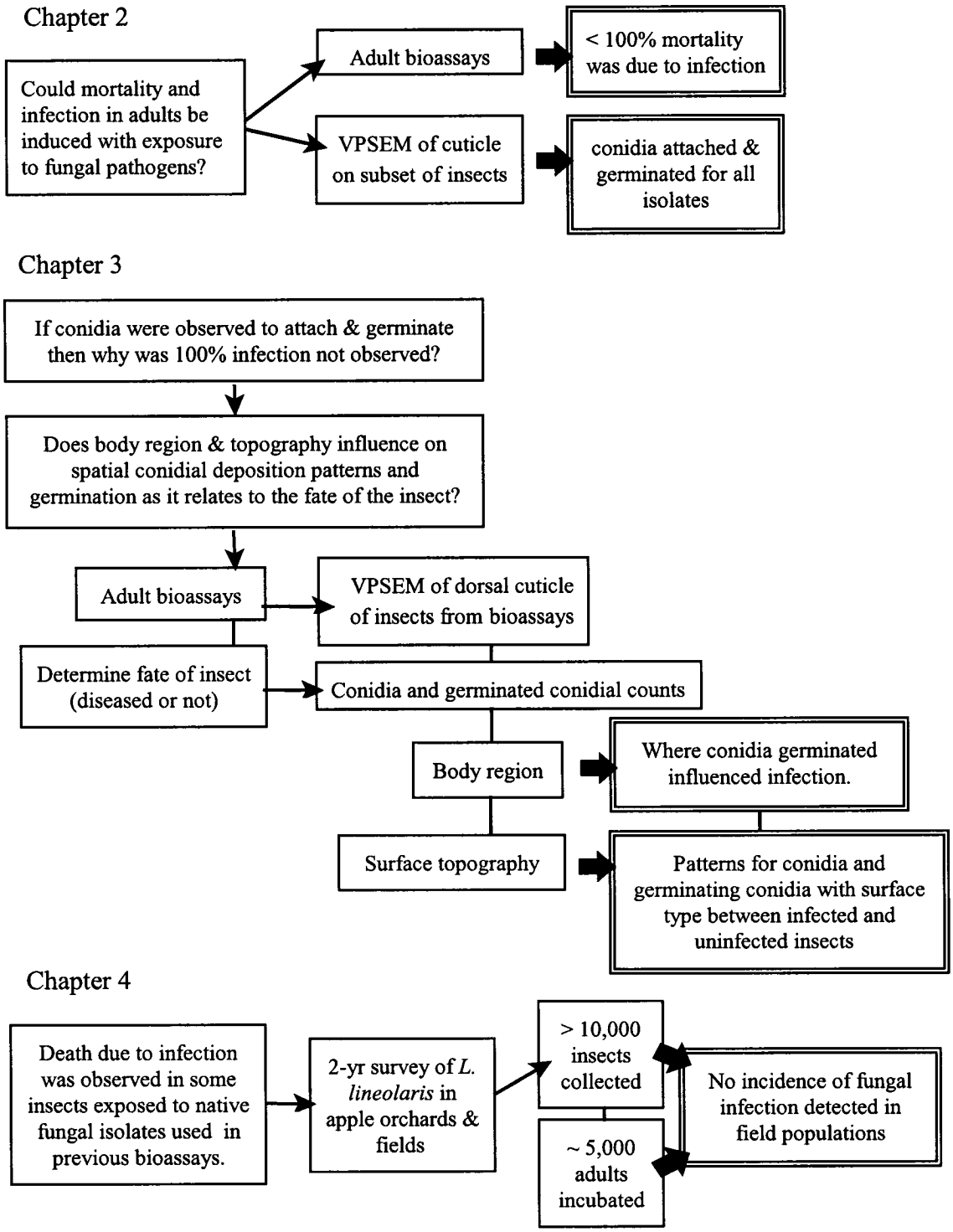

Figure 5.1. An overview of the development of objectives investigated in thesis chapters two, three and four.

accepted paradigm used for commercializing microbial insecticides is based on a pesticide model where products are inexpensive, stable and easy-to-use and provide quick kill, low persistence requiring repeated application (Waage, 1997). Fungal pathogens as biological control agents fit this chemical model poorly (Gaugler, 1997; Inglis et al., 2001; Wraight and Carruthers, 1999). The assumption that fungal pathogens should act like insecticides may explain in part the inconsistent results obtained in efficacy trials. Directly spraying individual insects resulted in > 90% mortality with approximately 60% resulting from infection (Chapter 3). Microscopic examination revealed that differences in the deposition patterns for germinating conidial on the cuticle was related to the development of disease (Chapter 3). Findings in this thesis suggest that conventional approaches (e.g. spraying) to using fungal pathogens for control of insect pests may simply be outdated. Perhaps the problem is twofold; researchers have not yet been able to put the deeper insights generated by detailed laboratory studies to practical use in the field and the predictive power of laboratory results falls short because trials have not been oriented to the complexities of the field.

Laboratory procedures ensure that experimental insects come into contact with conidia, most often at high doses, whereas, control in field settings is assumed through optimum coverage of the target area. Under controlled experimental conditions, infection levels never reached 100% in bioassays where every insect died after being sprayed directly with conidial suspensions, despite conidia viability determined to be > 80% and despite every insect being treated similarly in terms of exposure method, dose, temperature and relative humidity (Chapter 2 and 3). This same pattern was observed

for both the commercial formulation of *B. bassiana*, Botanigard and the native fungal isolates, *M. anisopliae*, *B. bassiana*, and *Verticillium lecanii* (Chapter 2). Attempts to understand the mechanism for why infection may or may not develop between pathogen and host are not clearly or easily understood. Close examination of the conidia-cuticle interface, revealed that the number of germinating conidia attached to the cuticle was a more critical determinant of whether an insect died of mycosis (Chapter 3), and that there is little correlation between concentration and conidial deposition/adhesion, germination and pathogenesis.

Another symptom of the chemical mindset is the use of very high dosages of pathogens in an attempt to achieve high levels of mortality. With conventional insecticides, it is customary to apply a sufficient spray volume to ensure good coverage of the crop, even if this involves pesticide drift or seepage into ground water. The objective is to get full coverage of a target area, thereby, leaving no unaffected refuge for the pest. There is also some allowance for dissipation of residues: sufficient material is applied so that the killing power of the application remains high for a period of several days to several weeks depending on operational requirements. This same model has been the convention for field based studies using fungal pathogens, since conidia also need to come into direct contact with the host. However, one major problem exists when attempting to extrapolate an effective dose that was determined in laboratory trials to that needed in field settings, since the amount that insects will be exposed to in the field is not known. This is particularly problematic when assessing the impact of large-scale field applications on non-target hosts (Hajek and Goettel, 2000). Historically,

application of fungal pathogens at high dose did not always guarantee effective control against the target pest. Yet, a single infective unit, in this case a condium, may be all that is needed to infect an insect. This study showed that relatively few conidia adhered to the dorsal cuticle and fewer still germinated, yet, infection did develop among a high proportion of the adult *L. lineolaris* treated with both Botanigard and *M. anisopliae* (Chapters 2 and 3). Achieving high mycosis levels despite low conidial counts, and even lower germinating conidial counts suggests that the next priority for research would be to examine ways to achieve these necessary numbers or conversely what factors cause the numbers to be so low even when so many spores are sprayed into the vial. Presuming greater coverage by simply increasing conidial concentration may not suffice if germination is the key determinant for initiation of infection and site of infection can influence whether successful penetration will even occur. Certain surface features (e.g. depressions adjacent to setal sockets) may act as conidia traps during application resulting in higher numbers of conidia deposited to a certain area. Regional features on the insect cuticle are known to influence conidial attachment and subsequent development (Wang and St. Leger, 2005). Assuming that we can identify the most vulnerable regions of the cuticle, the goal will now be to research and develop technologies to achieve better deposition, better adherence and better germination on these sites.

Results attained in controlled laboratory studies often indicate that high levels of mortality are possible, however, this is seldom achieved in field trials. Although the determination of pathogenicity of fungal isolates against its insect host is important in

studies evaluating pathogens for their potential as control agents, current evidence (Chapter 2 and 3) suggests that survivorship bioassays, on their own, are not sufficient to make effective assessment of performance in a field setting. Indeed, even in controlled laboratory trials complex processes are at work (e.g. influence of pathogen-cuticle interface on infection) that often are difficult to incorporate into the design of field studies established to evaluate the efficacy of promising fungal candidates to be developed as microbial-based insecticides. Accurate assessment of the long-term impact of a pathogen application (e.g. persistence of fungus in the environment) are also difficult, since endemic levels of indigenous pathogens can remain in the environment at very low levels and not be easily detected, if at all (Goettel et. al., 2001). A 2-year survey of endemic fungal infection levels on *L. lineolaris* in six apple orchards, six adjacent fields, and two additional fields in the Annapolis Valley, Nova Scotia did not detect any diseased insects (Chapter 4), despite having sampled 6,334 and 5,383 insects in 2002 and 2003, respectively. Although, 50 to 65% mortality in adult *L. lineolaris* was caused by the native fungal isolate, *M. anisopliae*, when tested in laboratory assays (Chapter 2 and 3), it is unlikely that these results could be repeated in field trials. Concentrations of fungal suspensions would be considerably higher ($> 10^6$) for use in large scale applications versus laboratory assays where individual insects were exposed to doses $\leq 3.0 \times 10^5$ conidia (Chapter 2 and 3), however, individual insects will likely come in contact with fewer conidia in the field. It is promising that the commercial formulation of *B. bassiana*, BotaniGard, and *M. anisopliae* (at doses $> 3.0 \times 10^4$ conidia) produced > 60% mycosis, however, efficacy as a biological control agent in a

field setting may need to rely on novel application methods.

Unlike their chemical counterparts, there are a number of additional factors that influence the probability that the conidia will remain viable in the environment until coming into contact with the host: pathogen virulence and persistence, host condition and behaviour, sunlight, desiccation, use of fungicides, temperature and humidity (Fuxa, 1995; Goettel et al., 2001; Lacey and Goettel, 1995; Van Burik and Magee, 2001). Waage (1997), argues that opportunities to utilize the inherent strengths of the pathogen (e.g. secondary inoculum production and host to host transmission) are missed when the development of microbial agents is done under the existing chemical model. Better results may be obtained by using improved formulations or more effective means of application to optimize existing characteristics of the fungal pathogen, rather than attempting to redesign such intrinsic qualities as speed of kill. Knowledge of the biological characteristics of both pathogen and host need to be extended to methods of application and delivery to the host (Evans, 1999). In trials where honey bees, *Apis mellifera* Linnaeus (Hymenoptera, Apoidea), carried *M. anisopliae* conidia to developing flowers of oilseed rape there was high mortality in the target host, adult pollen beetles, *Meligethes aeneus* Fabricius (Coleoptera, Nitidulidae) even though infection rates varied from 57-98% on spring rape and 31-41% on winter rape (Butt et al., 1998). Ensuring contact with the fungal pathogen becomes especially difficult when dealing with insects whose larval stages live within protected habitats (e.g. in the soil or within galleries in tree trunks). Commercially available traps baited with a floral lure and further modified to hold *M. anisopliae* have been used successfully to attract and

infect adult Japanese beetles, *Popillia japonica* Newman (Coleoptera, Scarabaeidae). Once the beetles came in contact with the fungus, they became infected and dispersed, suggesting the potential for dissemination and horizontal transmission of conidia to the habitats of their larvae (Klein and Lacey, 1999). When assessing the efficacy of a combination of *B. bassiana* and diatomaceous earth against the red flour beetle, *Tribolium castaneum* (Herbst) (Coleoptera, Tenebrionidae), researchers found that diatomaceous earth caused significant increase in conidial attachment on larvae even though there was no impact on adults (Akbar et al., 2004). Thus, imaginative approaches such as these can lead to successful use of fungal pathogens. Additional work on formulation, application, and choices of how and when to use these products need to be evaluated based on the realities of biological systems. These examples illustrate the benefits of a paradigm shift from the 'chemical model' where pathogen formulation and means of delivery take advantage of the characteristics of the pathogen and the biology of the target pest. The paradigm must be altered if we are going to be successful in developing useful fungal insecticides. The objective of all these approaches is to ensure that an adequate number of spores will germinate on vulnerable sites on the cuticle to allow infection to occur. Incorporating knowledge of optimum conidial dose, target cuticle regions, and insect biology into an application technology can only further serve to enhance the efficacy of fungal pathogens for use as control agents.

In laboratory studies, insect mortality can most often be attributed to a single predator or parasitoid, whereas pest densities in the field are more likely regulated by a

number of control agents including fungal pathogens. A much needed paradigm shift would be to acknowledge the synergy between natural control agents, permitting IPM programs to adapt to the new opportunities which microbial insecticides provide (Waage, 1997). Conventional single approaches using biological control measures to replace pesticides may not work because outbreaks of some pests or pest complexes may require a blended management approach (Hill et al., 1999). In a survey of 78 phytophagous insects from four orders (Lepidoptera, Diptera, Coleoptera and Hymenoptera), predators, parasitoids and pathogens all acted as mortality agents, with parasitoids causing the highest levels of mortality (Hawkins et al., 1997). In IPM programs, inoculations of potential control agents like naturally occurring pathogens or commercial fungal preparations could be combined with other control measures (Ekbom and Pickering, 1990; Fuxa, 1995; Puterka, 1999), including predators or parasitoids or be used in combination with modified pesticide spray programs. Begon et al., (1999), suggested that a pathogen and parasitoid combination is a good strategy for biological control providing that both control agents show clumped attack patterns, therefore preventing excessive competition but maintaining some degree of overlap in attacks, and both are effective exploiters of healthy hosts.

Unlike conventional insecticides, fungal pathogens are living organisms that interact with the environment and successful integration of microbial controls into an IPM system will require a broad understanding of biological and ecological factors and their interactions (Tatchel, 1997). Gaugler (1997), emphasizes the merits of longer term approaches such as conservation and inoculative biological control that are based on

fundamental ecological processes between pathogens and their hosts that are often ignored in the chemical paradigm. More specifically, future evaluations of pathogens in the field require quantitative assessments of longer term efficacy in order to realize the opportunities and overcome the constraints within an IPM system (Evans, 1999). One example of this approach is with the redheaded pasture cockchafer larvae, *Adoryphorus couloni* (Burmeister) (Coleoptera, Scarabaeidae). Populations were reduced over four years after a single application of *M. anisopliae*, equivalent to $5.1 \pm 0.7 \times 10^4$ spores g^{-1} soil equivalent, to the top 10 cm of soil. The long term control observed in larvae was dependent on the maintenance of the fungal pathogen in the soil and not on the insect host (Rath et al., 1995). Although, some control was realized against *L. lineolaris* when exposed to a native isolate of *M. anisopliae*, laboratory results did not suggest that this particular pathogenic strain could exert effective control over field populations on its own. Several authors have discussed the benefits of combined control measures, as practiced in and integrated pest management program (Puterka, 1999).

Another strategy is the combining of control agents to overcome the slower kill rate often observed when fungal pathogens are used alone as a treatment; this also serves to reduce chemical loads in the environment (Benz, 1971). Adult *L. lineolaris* numbers were reduced by 97.9% five days after being sprayed with a combination of Mycotrol (280 g/ha), a commercial formulation of *B. bassiana*, and imidacloprid (50 g AI/ha), in field trials on cotton. When insects were treated with only Mycotrol (280 g/ha), mortality only reached 52.0% after five days in contrast to 67.3% for individuals treated with imidacloprid (Steinkraus, 1996). Brown et al. (1997), also reported greater (68.7%

) control after 7 days against adult *L. lineolaris* treated with a combination of Mycotrol (0.56 kg/ha) and Provado 1.6F (0.027 kg AI/ha), an insecticide containing imidacloprid, at half the recommended rates versus the full rate of Mycotrol (1.12 kg/ha) alone. In both cases, control was achieved against an insect pest after inoculation with a fungal pathogen that had been combined with a reduced rate of insecticide. One possible mechanism for the synergism may be that certain insecticides interfere with grooming. In this study, lethargy and less preening was observed in daily inspection of treated insects in bioassays.

Overall, the number of insects that were still alive 14 days after inoculation in this study was low, suggesting that BotaniGard is capable of reducing populations of adult *L. lineolaris* (Chapter 2). The implications of the slower rate of kill may be resolved if the product can be successfully used in conjunction with another control agent. Researchers face many challenges in their attempts to change or modify the pathogen in ways that will guarantee consistent and long term pest control. For example, the ability of the pathogen to replicate within its host results in slower times to death but produces secondary inoculum (Evans, 1999). The real advantage over chemical controls is that the 'recycling' of the pest through subsequent production of additional infective units increases the probability of exposure by other individuals to the local patches of high conidia densities. Leaves treated topically with *B. bassiana* and systemically with a sublethal dose of imidacloprid showed increased movement and higher mortality of peach potato aphids, *Myzus persicae* Sulzer (Homoptera, Aphididae). Since there was no synergistic effect of *B. bassiana* and imidacloprid, higher mortality was probably

indicative of the increased exposure to conidia with greater movement over treated leaves (Roditakis et al., 2000). Consequently, when choosing recommended doses/concentrations of pathogens, one might want to consider the potential for secondary spore production. Another consideration is time of application of the fungal inoculum: sprays could be timed to take advantage of the cooler temperatures at dusk or dawn, thereby, reducing insect movement and providing more amenable microclimatic conditions (e.g. reduced UV) favouring the pathogen.

Conclusions

For fungal pathogens to work, one must ensure that a sufficient number of spores germinate on vulnerable body surfaces always assuming that the pathogen is sufficiently virulent. The possibility remains that with the improvement of application methods and techniques, the opportunity for disease development will likely improve. Higher incidence of fungal infection combined with other control measures in an integrated management program could reduce *L. lineolaris* damage to apples and lessen growers' dependence on conventional, broad-spectrum insecticides and help to reduce overall pesticide load on the environment.

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