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Study of conidia production and transmission of *Beauverza bassiana* (balsamo) vuill. in Colorado potato beetle (*leptinotarsa decemlineata*)

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STUDY OF CONIDIA PRODUCTION AND TRANSMISSION OF *BEAUVERIA BASSIANA* (BALSAMO) VUILL. IN COLORADO POTATO BEETLE *(LEPTINOTARSA DECEMLINEATA)*

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Thesis Advisor: Dr. Eleanor Groden

An Abstract of the Thesis Presented
in Partial Fulfillment of the Requirements for the
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This work is an investigation of: (1) mortality of the Colorado potato beetle (CPB), *Leptinotarsa decemlineata* (Say), under different modes of exposure to the pathogen *Beauveria bassiana* (Bals.) Vuill., *(2)* host and environmental factors affecting sporulation, production, and viability of *B. bassiana* conidia from infected cadavers of CPB larvae, and (3) transmission of the disease in CPB populations. The research leads to an improved understanding of the process of transmission of *B. bassiana* in CPB populations. This may enhance its use as a biological control agent for this pest insect.

The highest rate of mortality of CPB larvae (77 percent) was achieved through direct spray of the insects with conidial suspensions of *B. bassiana* together with exposure to inoculated foliage. Direct spraying alone resulted in 76 percent mortality and exposure to inoculated foliage resulted in 34 percent.

B. bassiana killed larvae sporulated within 2 days after death at 100 percent relative humidity and 25°C, and produced 1.75×10^4 to 8.7×10^8 conidia/insect. Cadaver

size had the largest impact on the amount of conidia produced. Larvae treated as third and fourth instars produce significantly lower densities of conidia than larvae treated as first and seconds instars. Lower dosages of *B. bassiana* increased conidia production.

Cadavers did not produce conidia at humidity levels lower than 95 percent. Exposure to intermediate levels of humidity resulted in a longer time to sporulation when at a later date the cadaver were transferred to optimal conditions for sporulation. Thus lower relative humidities are detrimental to the fungus. An optimum range of temperature for maximum production of conidia was observed to be between 15 and 30°C. Cadavers exposed to soil produced on average more conidia than cadavers incubated without soil for the same period of time.

In the field, the most favorable environmental conditions for sporulation, production and viability of *B. bassiana* conidia occurred under a potato canopy. High relative humidity (>95%) and soil moisture benefit sporulation and conidia production. Cadavers can persist in the field for more than 30 days and viability of conidia decreased over time but recovered after a rain event.

Transmission of the disease in the potato canopy was too low to detect. Transmission was more likely to occur at the soil level where pre-pupae may encounter fallen sporulated cadavers while searching for a site to burrow for pupation.

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LITERATURE REVIEW

Introduction

Biological control of insect pests is a widely considered alternative to chemical use in modern agriculture systems. Organisms used as agents range from microorganisms to macroinvertebrates and vertebrates such as birds. In order to take advantage of the full potential of these organisms, a description of the patterns and processes, and an understanding of the mechanisms involved in pest and biological agent dynamics must be achieved.

Beauveria bassiana (Bals.) Vuill. is an entornopathogenic fungus, one of many natural enemies of the Colorado potato beetle (CPB), *Leptinotarsa decemlineata* (Say) (Groden, 1989). Most of the research conducted on the pathogen's potential as a control agent has focused on the effects of environmental conditions on the development and survival of the fungus, and manipulations to increase its efficacy as a mycoinsecticide (Carruthers et al., 1985; Gaugler et al., 1989; Hywel-Jones and Gillespie, 1990). However, as a naturally occurring disease causing agent in CPB populations, ***B.*** *bassiana* has to replicate, disperse and transmit infection to suitable host individuals. Very little is known about the mechanisms of production of conidia and transmission of the disease or the rate of spread in a beetle population. My dissertation will attempt to identify factors affecting secondary production of conidia, and mechanisms of horizontal transmission, where and when it is more likely to occur, and to what degree. The major objective of this study will be to understand sporulation and factors affecting sporulation and conidia production, as well as factors and mechanisms affecting the transmission of a fungal disease, *B. bassiana,* in CPB populations.

In agroecosystems, disease phenomena are influenced by four-way interactions that include the host and pathogen populations, the environment, and humans as managers of the ecosystem (Carruthers and Soper, 1987; Zadoks and Schein, 1979). Research that

examines the cause and effect relationships among these components is essential for full exploitation of these organisms for biological control of insect pests.

Colorado Potato Beetle, *Leptinotarsa decemlineata* (Say)

History

Colorado potato beetle (CPB), *Leptinotarsa decemlineata* (Say), was first collected in the United States in 1811 near the Iowa-Nebraska border feeding on *Solanum rostratum* Dunal (Casagrande, 1987). By the late 1850s, a population of beetles had changed its eating preference to potatoes, and acquired the status of a pest. The first insecticide to be applied to control CPB was the arsenical insecticide, Paris green, (Broson and Anderson, 1952). Currently, CPB is considered a major defoliator of several economically important crops from the family *Solanaceae* which includes potatoes, tomatoes, and eggplants in the United States and Europe (Biever and Chauvin, 1992). Traditionally CPB has been controlled with the intensive use of chemicals, but with several generations per year (2-3 in Massachusetts and New York, and 1-2 in Maine) populations of this insect have become resistant to these pesticides quite rapidly (Ferro, 1985).

Life Cycle

One to two generations of the beetle are produced each year in Maine (Groden personal communication). In late summer adults burrow down 7.6 to 12.7 cm. into the soil (Lashomb et al., 1984) and overwinter as diapausing adults. Ninety percent of cumulative overwintering adults emergence occurs with 130-150 (based 10°C) degree days of soil temperature (Lashomb, et al., 1984). After eclosion, adults emerge from the soil and start feeding on the potato foliage as they begin to sprout. Females become reproductively active in 5 to 10 days and start laying eggs in masses (20-60 eggs/mass) on the underside of leaves located close to the ground (Hare, 1990). After eclosion, adults emerge from the soil and feed on potato foliage. Fecundity per female can exceed 4,000 eggs in laboratory-

reared colonies (Brown et al., 1980). Eggs from the same mass generally hatch simultaneously (Hare, 1990). As larvae feed on the host plant foliage they develop through four larval stadia. Mature fourth instars stop feeding, climb off the plant and burrow into the soil to pupate (Hare, 1990). Depending on diapause induction cues, they will either mate and oviposit or return to the soil to overwinter (either within the field or along the field borders). Diapause is induced by photoperiod, although temperature and food quality may also serve as cues (Wilde et al., 1959; Wilde, 1965). Adults resulting from larvae exposed to short photoperiods burrow down into the soil to over-winter. Critical photoperiod changes with latitude (Hare, 1990). CPB development rate is correlated with temperature. The number of degree-days (base 10°C)required for the development of eggs is 72. For the four instars it is 36, 32, 37 and 69, respectively, and for the pupa it is 176 (Logan et al., 1985).

Consumption of foliage varies among instars. First instars are responsible for 3% of the total leaf consumption; seconds are responsible for about 5%; thirds for 15% and fourths for up to 77%. Hence, fourth instars are responsible for the majority of crop losses (Ferro et al. 1985; Logan et al., 1985).

***Beauveria bassiana* (Bals.) Vuill.**

Beauveria bassiana is named after the Italian scientist Agostino Bassi who established the germ theory after his study of this fungus ten years prior to the work of Luis Pasteur (Steinhaus, 1975). *B. bassiana* is also called the white muscardine fungus, because of its white powdery appearance which resembles a French sugary candy (Steinhaus, 1975). *B. bassiana* is a soil borne fungus observed to be pathogenic to many insects worldwide, including the CPB (Groden, 1989) and it has been reported to be the most common fungus isolated from dead and moribund insects in nature (Macleod, 1954) *B. bassiana* is considered harmless to humans although some cases of fungal keratitis have been reported (Kisla et al., 2000).

Beauveria bassiana is classified under the subdivision Deuteromycotinia and genus *Beauveria*. The history of the genus *Beauveria* dates back to 900 AD, when it was observed in silkworms in Japan (Steinhaus, 1975), although fossil records of this insect disease go back some millions of years. Poinar and Thomas (1984) discovered a worker ant covered with a fungus of similar characteristics to *B. bassiana* that was embedded in amber 25 million years ago.

Description

B. bassiana is usually found growing densely through the exoskeleton of insect cadavers killed by the fungus. The conidiogenous cells are usually clustered, colorless, with a globose base and a denticulate apical extension (Humber, 1997). Conidia are **2-6** μm in diameter and are borne out of zigzag phialides or apical extensions *(rachis)* (Poinar and Thomas, 1984; Humber, 1997; Boucias and Pendland, 1998). *B. bassiana* has also been reported to be endophytic (Bing and Lewis, 1992). Wagner and Lewis (2000) observed penetration of developing hyphae on the leaf surface of *Zea mays* L. that reached the xylem and provided insecticidal protection against damage by the European corn borer, *Ostrinia nubilalis* (Hiibner) (Bing and Lewis, 1991).

Life Cycle

As all entomopathogenic fungi from the subclass Deuteromycotina, *B. bassiana* infects its insect host with conidia. *B. bassiana* conidia have rodlets that prevent them from desiccation and aid in the attachment to the host by hydrophobicity of the rodlets and the insect cuticle (Boucias et al., 1988). The time required for successful attachment to the host is about 5 minutes (Boucias et al., 1988). Some substances such as detergent, solvents and high molecular weight proteins are known to neutralize the hydrophobicity and reduce conidial binding (Boucias et al., 1988).

Germination of conidia depends largely on environmental conditions including temperature, light and especially relative humidity. Most fungal entomopathogens require relative humidity above 97% for germination (Gillespie and Crowford, 1986) and temperatures between 25-30°C (Hall, 1981). For entomopathogenic Deuteromycotina species, a relative humidity above 90% is needed for conidial germination *in vitro*. *In vitro*, *B. bassiana* conidia germinate at a range of temperatures between 8" and 35°C, with an optimum between 25" and 30°C (Hallsworth and Magan, 1999; Fargues et al., 1997). Hywel-Jones and Gillespie (1990) observed faster conidial germination at 25°C than at higher temperatures. Rapid germination is desired in field situations to avoid the effects of ultraviolet light which is highly detrimental for germination and survival of the fungus (Ignoffo, 1992; Moore et al., 1996; Inglis et al., 1997; Edington et al., 2000; Joergensen, 2000). Ferron (1977) found, however, that insects can be infected with *B. bassiana* at ambient relative humidities less than the 92% required for germination and mycelial growth *in vitro*. He suggests that the initial infective phase (germination on the cuticle of the insect) may be less dependent on ambient humidity, because the microclimate of the insect cuticle is similar to that of their host plants. *In vivo*, the ranges of temperature and humidity for germination are broader. Temperature required for germination ranges from 0 to **40°C** with an optimum at 20-30°C (Schaerffenberg, 1964; Fargues, 1972; Benz, 1987); and germination has been recorded at humidities less than 50% (Ramoska, 1984; Walstad et al., 1970; Fargues, 1972; Studdert and Kaya, 1990).

Nutritional factors are also important for germination. Many entomopathogenic fungi conidia will survive in the environment until they contact a nutritional source that will trigger germination (e. g., Smith and Grula, 1981; Hunt et al., 1984). ***B.*** *bassiana* germination depends on sources of carbon such as glucose, glucosamine, chitin and starch. Nitrogen is also necessary for hyphal growth (Tanada and Kaya, 1993).

As previously stated, entomopathogenic Deuteromycotinia fungi infect their host via conidia which produce hyphae that grow directly through insect integument. In the case of

B. *bassiana,* the most common route of infection is through the cuticle (Vey and Fargues, 1977; Ferron, 1978; Pekrul and Grula, 1979). The fungus produces several classes of cuticle degrading enzymes such as proteases, chitinases and lipases upon germination (Boucias and Pendland, 1998). The lack of production of these enzymes in some strains of ***B.*** *bassiana* may delay the infection process in certain insect species (Bidochka and Khachatourians, 1990). It has been reported that germination of ***B.*** *bassiana* on CPB larvae involves the formation of an appressorium (Vey and Fargues, 1977), but this is not necessarily the case for all insects (Boucias and Pendland, 1998).

Other infection sites include natural body openings such as the mouth cavity where environmental conditions, particularly moisture, aid germination (Tanada and Kaya, 1993). On the other hand, it has been suggested that digestive fluids from the alimentary track may destroy germinating hyphae (Tanada and Kaya, 1993). Allee et al. (1990) found penetration of conidia of ***B.*** *bassiana* through the gut in starved CPB larvae suggesting that food passage through the gut may prevent conidial infection. ***B.*** *bassiana* has been reported to infect several mosquito species through the posterior siphon and through the respiratory system (Clark et al., 1968); invade imported fire ants through the mouth parts (Siebeneicher, 1992); attack termites through the alimentary track (Bao and Yendol, 1971); and penetrate *Heliothis zea* (Boddie) through the spiracles (Pekrul and Grula, 1979). In the alfalfa weevil, *Hypera postica* (Gyllenhal), conidia of ***B.*** *bassiana* penetrate its host through the spiracles and the tracheal system, but do not penetrate through the external cuticle (Hedlund and Pass, 1968). Infection of the conidia through the integument depends primarily on the nature of the cuticle, its thickness, sclerotization, and the presence of antifungal and nutritional substances (Charnley, 1984).

Hyphae penetrate the cuticle through a series of mechanical and enzymatic processes (Ferron, 1981). The insect cuticular layer is distorted by the penetrating hyphal pressure (Tanada and Kaya, 1993). Enzymes such as proteases, lipases and chitinases are detected on germ tubes (Smith and Grula, 1983; Leopold and Samsinakova, 1970).

Proteases are considered the major cuticular degrading enzyme, and their activity appears to precede the action of chitinases (Smith et al., **1981;** St. Leger et al., **1989).** Lipases and proteases could aid growth of *B. bassiana* by solubilizing tissue such as fat body or any host tissue that is fatty or proteinaceous (Pekrul and Grula, **1979).**

Successful infection depends on the percentage of spore germination, germination rate (dependent on temperature and humidity), aggressiveness of the fungus and host specificity (Samson et al., **1988).** Other factors influencing host susceptibility to fungal infections are the age and stage of the insect at the time of infection (Watanabe, **1987),** host nutrition (Watanabe, **1987),** and exposure to chemical insecticides (Furlong and Groden, **2001).** The amount of *B. bassiana* inoculum needs to be increased with the older instars of CPB to achieve the same level of mortality (Fargues, **1972).** This is also true for other entomopathogenic Deuteromycotinia-host interactions. Sieglaff et al. **(1998)** observed less susceptibility to *Metarhizium flavoviridae* (Gams and Rozsypa) of the sixth instar *Schistocerca americana* (Drury) than of the fourth instar. Feng et al. **(1985)** found first instar *Ostrinia nubilalis* (Hubner) to be more susceptible to *B. bassiana* than later instars. **A** decrease in susceptibility to entomopathogenic Deuteromycotinia of younger instars has also been observed in *Costelytra zealandica* (White) when infected with *Metarhizium anisopliae* (Metschnikov) (Glare, **1994).** However, Vandenberg et al., **(1998)** found Diamond back moth early stages to be less susceptible to *B. bassiana.* Rizzo **(1977)** found no effect of adult age at challenge time to *B. bassiana* or *M. anisopliae* on the susceptibility in three fly species.

Food quality can also influence the susceptibility of the host. Larvae of the codling moth, *Cydia pomonella* L., reared on an artificial diet containing different amounts of ascorbic acid, showed different *B. bassiana* susceptibility. Larvae that fed on less than 0.6-0.8% ascorbic acid became highly susceptible to *B. bassiana* (Pristavko and Dovzhenok, **1974).** Costa and Gaugler **(1989a)** found that host plants with significantly different concentrations of glycoalkaloids do not influence CPB susceptibility to *B. bassiana*

infection when the fungus is applied topically. However, in another study Costa and Gaugler (1989b) reported that the glycoalkaloid tomatine inhibits colony formation and growth of *B. bassiana* more than solanine. They suggest that ingestion of ***B.*** *bassiana* conidia along with foliage containing high levels of tomatine can inhibit spore germination and hyphal growth.

After penetration hyphae reach the homeocoel and produce hyphal bodies (blastospores) that circulate through the hemolymph (Tanada and Kaya, 1993) and multiply by budding. Budding continues for a period of 3 to **7** days before the fungus reverts to a hyphal form, which infects other tissues and organs. Hedlund and Pass (1968) observed that the first tissue to be penetrated by the hyphae of ***B.*** *bassiana* after infection of the alfalfa weevil is the fat body. The same sequence of hyphal invasion is observed for infected corn borer larvae (Lefebvre, 1934). Muscle and glandular tissues are attacked next, followed by nerve tissue (Hedlund and Pass, 1968).

Development of hyphal bodies in the hemolymph of *B.* bassiana-infected *Spodotera* ***exigua*** (Hubner) are known to disrupt the cellular defense response of hemocytes (Hung and Boucias, 1992; Hung et al., 1993). Pendland et al. (1993) concluded that the lack of structural components (e.g., chitin) of the hyphal bodies in the hemolymph of S. ***exigua*** larvae is an important factor for evasion of host cellular defense mechanisms. Entomopathogenic Deuteromycotinia also produce cyclic peptides that are found to inhibit phagocytic activity of insect plasmocytes in a dose-dependent manner (Vilcinskas et al., 1997). In order to overcome insect defenses, the fungus can also produce other mycotoxins (Tanada and Kaya, 1993). Some of these toxins are proteases that damage the principal functions of the hemolymph or produce toxic by-products in the insect (Kucera and Samsinakova, 1968). Other toxins are low molecular weight compounds such as beauvericin, enniatins and bassianolide that have been demonstrated to be insecticidal (Castlebury et al., 1999; Gupta et al., 1995; Zizka and Weiser, 1993). These toxins also function as antimicrobials that prevent infected silkworms from subsequently acquiring

bacterial infections (Kodaira, 1961). In one strain of the fungus, the antibiotic oosperin was produced in submerged culture containing glutamic acid at equivalent nitrogen concentrations (Vining et al., 1962). Mazet et al. (1994) also found high molecular weight metabolites produced in the hemolymph of *B. bussiunu* infected *S. exigua* larvae. These metabolites have the ability to disrupt metamorphosis. The fungus causes insect mortality by nutritional depletion, invasion and destruction of tissue, and the release of toxins (Tanada and Kaya, 1993).

Time to death is dependent on the time required for attachment, germination and growth of the fungus before the death of the insect (Stimac et al., 1993). Stimac et al. (1993) observed that laboratory colonies of the ant *Solenopsis invicta* (Buren) treated with ***B.*** *bussiuna* have an exponential increase in mortality 3 days after inoculation, reaching highest mortality on days 4 and 5 post-treatment. Time to death also depends significantly on the insect stadium at which infection takes place (Glare, 1994), and the inoculum dose that the insect receives. Entomopathogenic Deuteromycotinia kill in a dose dependent manner. The higher the dose the higher the proportion mortality and the shorter the time to death (Mulock and Chandler, 2000; Smith et al., 2000; Poprawski et al., 1999; Jeffs et al., 1997; Ignoffo et al., 1983). Environmental conditions also affect mortality of entomopathogenic Deuteromycotinia infected insects. Clark (1980) investigated the time to death of second and fourth instar CPB larvae topically inoculated with *B. bassiunu* and held at different temperatures. She found that time to death decreased as the temperature rose.

Parasitic development of the fungus ends with the death of the insect. Saprophytic growth of the mycelial mass depletes the substrate (Lackey et al., 1992) and reproductive spores are produced from conidiophores that emerge from the cadaver if the environmental conditions are favorable. This results in the insect cadaver being covered with a characteristic "white mold" consisting of hyphae and conidiophores with conidia.

Sporulation or conidiation involves the conversion of captured resources into new fungal structures and does not require competition for additional resources (Lackey et al.,

1992). The saprophytic phase of the fungal cycle does not depend on environmental humidity, but is influenced by temperature (Ferron, 1977). However, the process of conidiation is mainly dependent on humidity levels higher than 95% (Luz and Fargues, 1998; Marcandier and Khachatourians, 1987), and to a certain extent on temperature. Survival of conidia is detrimentally affected by solar radiation (Ignoffo, 1992; Edington et al., 2000; Jorgensen, 2000). Considerable research on formulation of conidia for insecticidal purposes has been focused on developing ultra violet protectants (Pereira and Roberts, 1991; Inglis et al., 1995; Edington et al., 2000).

Little research has focused on the release and dispersion of *B.* bassiana in the environment. Unlike Entomophthoralean entomopathogenic fungi, entomopathogenic Deuteromycotinia lack active dispersal. *B.* bassiana depends on physical disruptions such as wind or rain to distribute conidia in the environment. Doberski and Tribe (1980) observed that rainwater percolating between the bark and wood of elm trees disperses conidia from mummified larvae of ***B.*** bassiana-infected elm bark beetle thus initiating epizootics.

Epizootics and Horizontal Transmission

Sinnecker (1976) defines epizootiology as the science of causes and forms of the mass phenomena of disease at all levels of intensity in a host population. Fuxa and Tanada (1987) state that the major theoretical objective of insect epizootiology is explanation of patterns (or lack of them) in host populations. Pattern descriptions may lead to an understanding of disease dynamics. The knowledge of these dynamics may be used for manipulation of the system for human benefit.

Diseases are recognized to exist in two stages: enzootic and epizootic. Diseases that are low in prevalence but always present and in balance with the host population (Van der Plank, 1975) are in the enzootic stage (Fuxa and Tanada, 1987). Enzootic diseases exhibit

little, if any, spread (Van der Plank, 1975), low virulence (Anderson and May, 1979), and a ratio of daughter to parent infection approximately equal to one (Van der Plank, 1975).

Diseases in the epizootic stage are characterized by imbalance over a short period of time, with rapid changes in prevalence and the ratio of daughter to parent infection that exceeds one (Van der Plank, 1975). Sinnecker (1976) identifies massive production of causal organisms reinforced by the environmental conditions and host factors in such diseases. For the epizootic phase to develop, the host must be susceptible to the causal agent (Fuxa and Tanada, 1987), and the infectious organism must be efficient in transmission (Lilienfield, 1976). Pathogens with high chances of undergoing an epizootic stage in an insect population may be preferable for use as biological control agents.

Anderson and May (1982) defines transmission as the process by which a pathogen is passed from a source of infection to a new uninfected host. In contrast to human populations, where diseases can frequently be transmitted by direct contact of infected to healthy individuals, insect pathogens are more likely to infect new individuals through the environment. In the case of fungal diseases, infective spores are released in the environment where healthy individuals encounter them. There are two categories of transmission (Canning, 1982). Transmission is said to be horizontal when the pathogen passes from one individual to another within the same generation. Vertical transmission occurs when an infected host transfers the disease to its progeny, from one generation to the next.

High host densities are generally needed for the transmission of the disease and the development of epizootics because high densities will increase the contact between infected and healthy individuals, and between host and pathogen (Watanabe, 1987; Long et al., 2000). Transmission of the pathogen occurs in a density-dependent fashion (Hicks and Watt, 2000). But, epizootics can also develop at low host densities if the pathogen is widely distributed in the host environment (Bird and Elgee, 1957; Clark and Thompson, 1954). The more inoculum that is present the higher is the probability of disease progression.

There are several quantitative measures that can be used to describe epizootics as specified in "Epizootiology of Insects Diseases" by Fuxa and Tanada **(1987).** Traditionally, horizontal infection has been approached as the proportion of diseased organisms resulting from the interaction between the densities of infected populations and susceptible populations (Brown and Nordin, **1982).** Infection rate, which is proportionally constant for the number of encounters between susceptible and infected hosts (Anderson and May, **1980;** May and Anderson, **1979)** can also be used as a more general approach to horizontal transmission. However, since the resulting coefficient is constant it does not take into account the dynamics of the pathogens in terms of limitations in virulence due to biotic and abiotic factors (Groden and Drummond, **1994).**

An alternative to the general approach of the density dependent coefficient of transmission is a mechanistic approach that takes into account the pathogen population (Andreadis, **1987).** Many studies relate the pathogen infective unit's dynamics (in the case of ***B.*** *bassiana* conidia) of survival, dispersal and dose-infection relationship, to model epizootics in field situations (Carruthers et al., **1988;** Onstand and Maddox, **1989)** where spore production, survival and dispersal are treated independently (Groden and Drummond, personal communication).

Drummond and Groden (personal communication) propose to use a combination of both approaches (general and mechanistic) to understand the dynamics of horizontal transmission of ***B.*** *bassiana* in CPB populations. Horizontal transmission is modeled with the following equation:

$$dI_h / dt = b_f \; I, S^a - -vI_h$$

where $I_{,,}$ = density of infected insects

t = time

b_f = transmission function, f (biotic and abiotic factors such as age, relative humidity, dosage, etc.)

$I,$ = Sporulated cadaver density

S = Susceptible host population

a = nonlinear density effect (activity coefficient)

v = virulence or mean time to death of horizontally infected CPB

In this approach, infection is based on the density of infective cadavers and susceptible hosts, but b_f is considered a dynamic transmission function whose components would be sporulation, spore survival, spore dispersal and spore germination. Other important components would also be the rate and quantity of spores produced by infected insects and the probability of healthy insects encountering those spores in relation to the spatial distribution of infective units.

Although there is an abundance of information on sporulation requirements of ***B. bassiana*** in artificial media (Schaerfenberg, 1964; Samsinakova, 1966; Walstad et al., 1969), little information is available on the quantity of conidia produced per infected host. Environmental conditions that affect sporulation can also affect the quantity of conidia produced per sporulated cadaver. Relative humidity is especially important for the mycosis of entomopathogenic Deuteromycotinia, and therefore it has been considered **a** limiting factor for fungal epizootics (Benz, 1987). Vandenberg (1992) quantified spore production of the chalkbrood fungus ***Ascusphaera aggregata*** Skou on the alfalfa leafcutting bee ***Magachile rotundata*** and found it to be dependent on dose and age. Although environmental and host factors have been investigated relative to their impact on ***B. bassiana*** induced mortality of CPB (Clark, 1980; Ignoffo et al., 1983; Groden, unpublished), previous studies have not examined the impact of these factors on conidia production.

In order for transmission of a disease to occur in a population, conidia from sporulated cadavers must be able to disperse in the environment. Meredith (1973) defines spore release as the process that places the spore in motion toward the ultimate infection site. Dispersion in a group of entornopathogenic fungi, Entomophthorales, has been extensively studied (Akoi, 1981; Mullens and Rodriguez, 1985; Newman and Carner, 1974). Fungi in this group actively disperse their spores by discharging primary conidia

from sporangia at maturity (Tanada and Kaya, 1993). This discharge is influenced by environmental temperature and humidity (Steinkraus and Slaymaker, 1994). Primary spore discharge is also influenced by diurnal pattern. Akoi (1981) and Carruthers (1982) report periods of conidial discharge at night coinciding with high relative humidity in the *Entomophthora grylli* infected *Mamestra brassicae* (L) and *Entomophthora muscae* (Cohn) infected onion flies, *Delia antiqua* (Meigen), respectively.

Entomophtora species may also exhibit secondary and tertiary conidia production (King and Humber, 1981). Primary and secondary conidia (capilliconidia) production, germination and viability of Entomophthorales fungi is also dependent on environmental conditions. Conidia production by *Pandora neoaphidis* (Remaudière & Hennebert) infected tobacco aphid *Myzus nicotianae* (Blackman) was greater at temperatures between 10-20°C and humidities higher than 98% RH or in free water (Yu et al., 1995). Similar optimum ranges of temperature and humidity are observed in the germination and sporulation of secondary conidia of *Erynia conica* infected adult black flies, *Simulium rostratum* (Nadeau et al., 1995) and of *Neozygites floridana* infected cassava green mite, *Mononychellus tanajoa* (Oduor et al., 1996a, 1996b). Secondary conidia produced by primary conidia may also discharge if the latter do not contact an insect host increasing the possibility for the pathogen to transmit itself and persist. Entomophthorales are also known for altering their host behavior by sending them to the top of the canopy right before death (Samson et al., 1979) enhancing the probability of the fungus encountering a new host.

Entornopathogenic Deuteromycotinia species are passive in terms of dispersion. Spores of this group of species do not actively discharge or alter the behavior of their host. They depend on wind, running water, or rain to disperse. Stepanov (1935) observed the effect of wind on spore release of several plant pathogenic fungi. He finds that the wind speed required for detachment of spores varies from fungus to fungus, and that turbulence releases more spores than constant wind. Gottwald and Tedders (1982) determined that the release of conidia by ***B. bassiana*** and *M. anisopliae* (another entomopathogenic

Deuteromycotinia) is stimulated by low relative humidity (less than 50%), darkness, and vibration.

Canning **(1982)** stated that pathogens that rely on horizontal transmission for their survival must either be maintained within a living host or cadaver throughout the year or have a resistant free-living stage that enables them to survive during periods when the host is not susceptible. Abundant information is available on spore survival. Clerk **(1969)** found that adding silk to distilled water aids in the germination of ***B.*** *bassiana,* and concluded that the presence of an insect in the soil might influence the behavior of fungal conidia that otherwise would lie dormant in the soil due to soil fungistasis. Some fungal conidiophores can also survive long periods of desiccation at low temperatures (Benz, **1987).** It is also known that ***B.*** *bassiana* survives two and a half years at 4°C, but no longer than **12** weeks at **23°C** under desiccation conditions (Benz, **1987).**

At the foliar level, it is determined that sunlight is one of the most detrimental factors in the survival of ***B.*** *bassiana.* Conidia that are exposed to sunlight for **24** hours to a few days on foliage lose between 50 and **100%** of their viability and virulence (Gardner et al., **1977).**

Soil conditions such as high relative humidity, low temperature and protection from solar radiation make an optimal environment for the survival and persistence of ***B.*** *bassiana* for several years (Gaugler et al., **1989;** Roberts and Campbell, **1977).** The survival of ***B.*** *bassiana* in soil is reduced with increasing temperature and water content in soil (Lingg and Donaldson, **1981;** Krueger et al., **1991).** Increasing moisture content decreases O_2 and increases CO_2 concentrations which are detrimental to fungi (Griffin, **1963).** Additions of carbon and nitrogen stimulate the soil microflora, which also reduce the survival of ***B.*** *bassiana* (Lingg and Donaldson, **1981**).

My dissertation consists of five parts. It has as its objective the investigation of production and transmission of ***B.*** *bassiana* spores in CPB populations following different modes of exposure and under different environmental conditions. In the first manuscript, I

evaluate the acquisition of infective conidia and resulting mortality rates in CPB following different modes of exposure to the fungal pathogen. The second manuscript contains a description of the production of *B. bassiana* conidia by cadavers of infected CPB larvae, and examines the influence of cadaver size, pathogen dosage, and stage at infection on conidia production. Environmental factors impacting conidia production by CPB cadavers are a focus of the third manuscript, and the fourth manuscript investigates sporulation, production and viability of *B. bassiana* conidia on CPB cadavers under field conditions. The fifth and final manuscript describes research addressing the question of whether significant transmission of *B. bassiana* conidia to CPB larvae takes place on potato foliage.

It is expected that the understanding of the process of transmission of *B. bassiana* in CPB populations provided in this study may enable the use of the fungal pathogen as an effective biological control agent for the pest insect, by taking advantage of the fungus' potential for secondary cycling and inducing additional mortality of the pest through horizontal transmission.

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MANUSCRIPT 1

THE EFFECT OF MODE OF EXPOSURE TO *BEAUVERZA BASSZANA* (BALS.) VUILL. ON CONIDIA ACQUISITION AND HOST MORTALITY OF COLORADO POTATO BEETLE, *LEPTZNOTARSA DECEMLINEATA* (SAY)

Introduction

Colorado potato beetle (CPB), *Leptinotarsa decemlineata*, is a major defoliator of the genus *Solanum* in the United States, Canada and Europe. Traditionally CPB has been controlled with extensive use of chemicals but populations of this insect have become resistant to pesticides quite rapidly (Ferro, 1985). An alternative to chemical control is the use of *Beauveria bassiana*, a naturally occurring fungal pathogen in CPB populations. One of the advantages of using this fungus for pest control is that a suspension of its conidia can be applied with conventional insecticide spray equipment. Foliar applications usually result in the sprayed conidia landing directly on the insect and/or the insect acquiring the conidia through feeding and movement on contaminated foliage. However, it is not know which type of contact results in higher mortality. The distribution of conidia on the insect is likely to differ with the mode of exposure, and different sites of conidial attachment on the insect cuticle may result in different routes of successful penetration and infection. With direct application to the insect cuticle, conidia rapidly attach and germinate (Boucias *et al.*, 1988). If the insect is exposed to contaminated foliage, there is still potential for infection, however, that process depends on the capability of the fungus to survive until contacting a host.

B. bassiana conidia are hyaline and are sensitive to sunlight (Inglis *et al.*, 1993, 1995). Inglis *et al.* (1993) found that plant architecture and leaf position within the canopy of alfalfa had an effect on conidial survival of ***B. bassiana***. Conidia survived significantly longer within the canopy than at the top. Potato canopy has an architecture quite different from alfalfa, but because of its broader leaves, survival within the potato

canopy may be high enough to cause infection some time after a spray. In the future survival of conidia on the foliage may be enhanced with sunscreens added to the formulations (Inglis *et al.*, 1995).

CPB egg masses are laid on the underside of the lower leaves in potato plants. Once the eggs hatch, the first instars start feeding and migrate to higher parts of the canopy. Field applications of *B.* bassiana to control CPB are targeted against first and second instars. The duration of these stages depends mainly on temperature but can last from **3-8** days in northern Maine. Exposure of larvae to contaminated foliage over this time period may have a significant effect on infection and consequently on control.

The objectives of this study were: **(1)** to determine which mode of exposure of CPB larvae to ***B. bassiana,*** direct spray or exposure to *B.* bassiana-sprayed foliage results in higher mortality; and (2) to examine differences in distribution and germination rates of conidia on the insect cuticle following treatment.

Materials and Methods

Fungal culture

The fungus used for both bioassays and microscopy experiments was ***B.*** *bassiana* strain GHA (supplied as technical powder by Mycotech Corporation, 630 Utah Av. Box 4109, Butte MT 59702). Conidia were aseptically transferred to Sabouraud's dextrose agar (SDA) and incubated at 25°C under a 16:8 hr light:dark cycle. After 10 days plates were transferred to 4°C until used in bioassays. The conidia used in the experiments were harvested from the first or second subculture from technical powders and were less than 1 month old. Viability of the conidia was determined on SDA prior to every experiment and was always ≥ 99%.

Insect culture

Second instar CPB were obtained from our laboratory colony previously established from field collected adults. Colony adults were kept at a ratio of 4 females to 2 males in 300 cc. paper cups and maintained in an environmental chamber at 25°C, 68-70% RH and a 16:8 hr light:dark cycle. Adults were fed daily fresh greenhouse grown potato foliage and eggs were collected and stored for up to 8 days at 12°C. For assays, egg masses were transferred to 25°C, and upon eclosion, larvae were fed fresh potato foliage daily until reaching the second stadium. Eggs from field-collected adults were added to the colony annually.

Bioassays

The effect of direct or indirect exposure to ***B.*** *bassiana* conidia on second instar CPB mortality and sporulation was assessed with the following four treatments: 1) insect exposure, larvae were sprayed with a suspension of ***B.*** *bassiana* conidia and were then fed on unsprayed potato foliage; 2) foliar exposure, unsprayed larvae fed on ***B.*** *bassiana*-sprayed foliage; 3) insect exposure plus foliar exposure, larvae sprayed with a suspension

of *B.* bassiana conidia and fed on *B.* bassiana-sprayed potato foliage; and 4) control, unsprayed larvae exposed to unsprayed foliage.

B. bassiana dry conidia were harvested from SDA plates and suspended in 0.1% Tween-208 (Sigma Chemical Company, P.O. Box 14508, St. Louis MO, 63178) by vortexing. Conidia concentrations were adjusted following estimation on a hemacytometer. Leaf discs (20 mm in diameter) were carefully placed face up in 9 cm diameter petri dishes containing 2% water agar. Second instar CPB were placed in 9 cm diameter petri dishes with moist filter paper. Dishes containing leaf discs and insects were sprayed with the conidial suspension using a Burkard computer controlled spraying apparatus set at 8 psi (Burkard Manufacturing Co. Ltd., Rickmansworth Hertfordshire, England). Two water agar plates per spray run were observed at 400X to determine the density of conidia per mm^2 achieved. Unsprayed or *B.* bassiana-treated larvae were placed individually in 5 cm diameter petri dishes with moist filter paper and either a *B.* bassiana-inoculated leaf disc or an unsprayed disc. Insects were exposed to these discs for 24 hours at 25°C and a 16:8 h light:dark cycle. After this the remains of the leaf disc were discarded and fresh untreated potato foliage was fed to all insects. Insects were maintained at 25°C and a 16:8 h light:dark cycle with fresh foliage and monitored daily for mortality. Upon death, foliage was removed and dishes with cadavers were placed in 100% humidity chambers (belljars with water) at 25°C and a 16:8 h light:dark cycle to induce sporulation. Sporulation of cadavers was monitored daily.

Four bioassay, with variations, were carried out in a randomized block design at four different times. In bioassay 1 and 2, conducted in February 1997 and February 1998, respectively, three dosages of *B.* bassiana were applied to insects and leaf discs. Fifty newly molted second instars per treatment-dose combination were exposed directly or indirectly to 3.2, 6.4, and 24 conidia/mm^2 in bioassay 1 and 35.46, 97.3, and 321.34 conidia/mm^2 in bioassay 2. In bioassays 3 and 4, conducted in November and December 1998, respectively, two *B.* bassiana dosages were tested and 50 second instars treated

within eighteen hours of eclosion were used per treatment-dose combination. Treating within 18 hours of eclosion assured that no larvae molted within the 24 hours period post exposure. Dosages were 30.2 and 45.3 conidia/mm^2 in bioassay 3, and 12.5 and 26.0 conidia/mm^2 in bioassay **4.**

Treatment mortality was adjusted for control mortality using Abbot's formula: $P_T = (((P_O - P_C)/(100 - P_C))x100)$ where P_T = corrected mortality; P_O = observed mortality; and P, = control mortality, all expressed as percentages (Busvune, 1971). Logistic regressions (JMP, SAS Institute Inc., **SAS** Campus Drive, Cary, NC 27513) were used to test treatment and dose effects on mortality. Where significant treatment effects were detected, comparisons of individual treatments were tested by re-running logistic regressions with pair-wise comparisons of treatments.

Fluorescence Microscopy

Ten second instars were dissected and their cuticle flattened on a microscope slide in order to measure the total surface area of each body part examined in this study. Insects were observed at 100X and a grid micrometer was used for measurements. The dorsal surface of the abdomen included the area between but not including the spiracles, from the antecostal suture of the metathorax to the posterior tip of the abdomen. The ventral surface of the abdomen included the area between the beginning of the posterior legs at the anterior edge of the abdomen to the posterior tip of the abdomen, and laterally between the pleuro-ventral lines. The legs were considered two dimensional, and only one side of each leg was measured and multiplied by two to get an approximation of the total surface area of the legs. The ventral view of the head included the area which extended anteriorly from the cervix and included the ventral view of the mouth parts with postgena and gena, and antenna. Another 10 second instar CPB were dissected and the entire surface area of each insect was similarly measured using the grid micrometer.

The effect of direct and indirect exposure to ***B.*** *bassiana* conidia on the number of conidia and proportion of germinated conidia on different body parts of second instar CPB was assessed over time for the following three treatments: **1)** insect exposure, **2)** foliar exposure, and **3)** insect exposure plus foliar exposure. Protocols for exposure were the same as described above for the bioassays, but higher conidia concentrations of **1,264** conidia/mm^2 were used. Two experiments were conducted at different times with **10** and **20** insects per treatment, respectively. Insects were fixed in **4%** formaldehyde at **24** and **36** hours after initial exposure and stored at room temperature for up to 5 days before viewing.

Prior to microscopic examination, fixed cadavers were rinsed, blotted *dry* and individually weighed. Cadavers were mounted dorsal or ventral side up with a drop of 0.25% (w/v) calcofluor white (Fluorescencent brightener **28,** Sigma Chemical Co.) on microscope slides with concave depression of 0.8 cm. Slides were covered with a glass cover slip for observation. Examinations were conducted with an Olympus HBS Photomicroscope with an epifluorescence using a UV dichroic filter set. The combination of exciter filter and dichroic mirror was **405-435** and **455** nm, respectively. The total number of conidia and germinated conidia on the insect cuticle were counted on four parts of each insect. Conidia were counted in two microscope fields **(0.64**mm^2**)** of the dorsal surface of the abdomen, two microscope fields of the ventral surface of the abdomen, on one surface of three legs (which was aproximately equivalent to the surface area observed in three microscope fields of the abdominal section) and on the entire ventral view of the head which consisted mostly of the ventral view of the mouth parts. Cover slips were removed and insects were carefully turned when necessary to continue observations.

Separate analyses of variance (ANOVAs) were conducted (SuperANOVA, Abacus Concepts Inc., Berkeley, CA, **1989)** for each of the four body parts to test for differences in the total density of conidia per mm^2 of integument and the proportion of

germinated conidia following the three exposure treatments. The two repetitions of the experiment were treated as blocks in the analyses. The data were transformed to the logarithm of conidia density +0.01, and arcsin square root of the proportion conidia germinated.

An additional experiment was conducted to determine if exposure to conidial suspensions via spraying insects or foliage versus dipping insects or foliage affected the rate of attachment and conidial germination within the first 24 hours of exposure. Second instars were exposed to one of four treatments: 1) direct spray of ***B. bassiana*** conidia (and fed untreated foliage); 2) sprayed foliage (untreated insects); 3) dipped in ***B. bassiana*** suspension (and fed untreated foliage); and 4) dipped foliage (untreated insects). Insects were held individually in petri dishes with treated or untreated foliage for 0, 4, 8, 12, 16, 20, and 24 hours after exposure, and fixed as described above. Microscopic examinations were conducted on four insects per exposure treatment and time. Conidia were observed in one field of observation (0.32 mm^2) on each of the ventral and dorsal views of the abdomen, one leg, and the entire ventral view of the head. This experiment was replicated twice with conidial suspensions of 6.2 and 6.3 x 10^7 conidia/ml for both methods of application (sprayed and dipped). These solutions resulted in sprayed densities of 1,200 and 1,700 conidia/mm^2 for experiment 1 and **2,** respectively.

ANOVAs were conducted to test for differences in total conidia/mm^2 and proportion of germinated conidia over time and differences in proportion germinated conidia following the four exposure methods for each body part. All body parts were analyzed together as an independent factor in the ANOVA in order to detect differences in exposure mode on the proportion of germinated conidia. The data were transformed to the logarithm of conidia/mm^2 +0.01, and arcsin square root of the proportion conidia germinated.

Results

Bioassays

Control mortality was 2% in bioassays 1 and 4, and 12 and 6% in bioassays 2, and 3. Significant interactions were detected between bioassay and dose for the two dependent variables, mortality ($p < 0.0001$; $df = 3, 1499$; $\chi^2 = 82.72$) and sporulation of host ($p < 0.0001$; $df = 3, 1514$; $\chi^2 = 75.91$). Positive dose responses were observed for mortality and sporulation in all bioassays, however, the slope of the dose responses differed between bioassays.

Exposure mode significantly impacted mortality ($p < 0.0001$; $df = 2, 1481$; $\chi^2 = 54.26$) and sporulation ($p = 0.0001$; $df = 2, 1496$; $\chi^2 = 17.67$) (Fig. 1.1). In all bioassays, insects exposed to sprayed foliage experienced less mortality than those sprayed directly and exposed to sprayed foliage. However, no significant differences in mortality were observed between sprayed larvae and larvae sprayed and exposed to inoculated foliage. **A** significant interaction between bioassay and mode of exposure was detected for mortality ($p < 0.0001$; $df = 6, 1499$; $\chi^2 = 30.99$), and for sporulation ($p < 0.0001$; $df = 6, 1514$; $\chi^2 = 30.19$). For bioassays 1, 2 and 4, larvae exposed to sprayed foliage alone resulted in significantly lower mortality than the other exposure treatments. However in bioassay 3, no significant differences were observed between insect exposure alone.

The proportion of cadavers sporulated with ***B. bassiana*** was less consistent between bioassays. In bioassay 1 and 2, exposure to only sprayed foliage resulted in a significantly lower proportion sporulation when compared to treatments in which insects were directly sprayed or sprayed and exposed to sprayed foliage (Fig. 1.2). However, for bioassay 3 and 4, no significant differences were detected in sporulation between the three exposure treatments (Fig. 1.2).

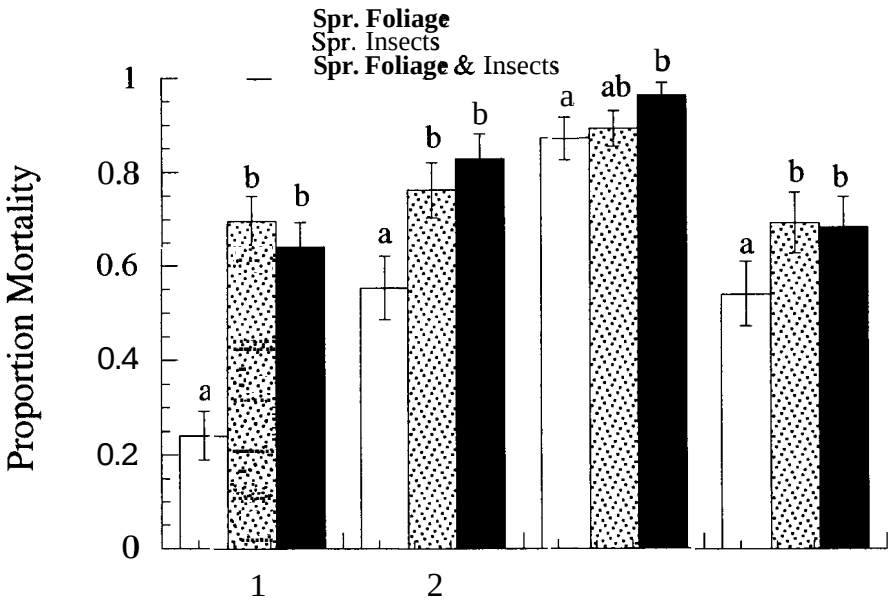


Figure 1.1. Mortality of second instar Colorado potato beetle following different modes of exposure to ***B. bassiana*** conidia. Proportion mortality adjusted for control mortality using Abbott's formula. Spr. Insect = insect sprayed directly with conidial suspensions; spr. foliage = insect exposed to foliage sprayed with conidial suspension; and spr. insect & foliage = insects both sprayed directly and exposed to sprayed foliage. Letters correspond to results of all possible pairwise comparisons using nominal logistic regression within a bioassay (significance level $p < 0.05$).

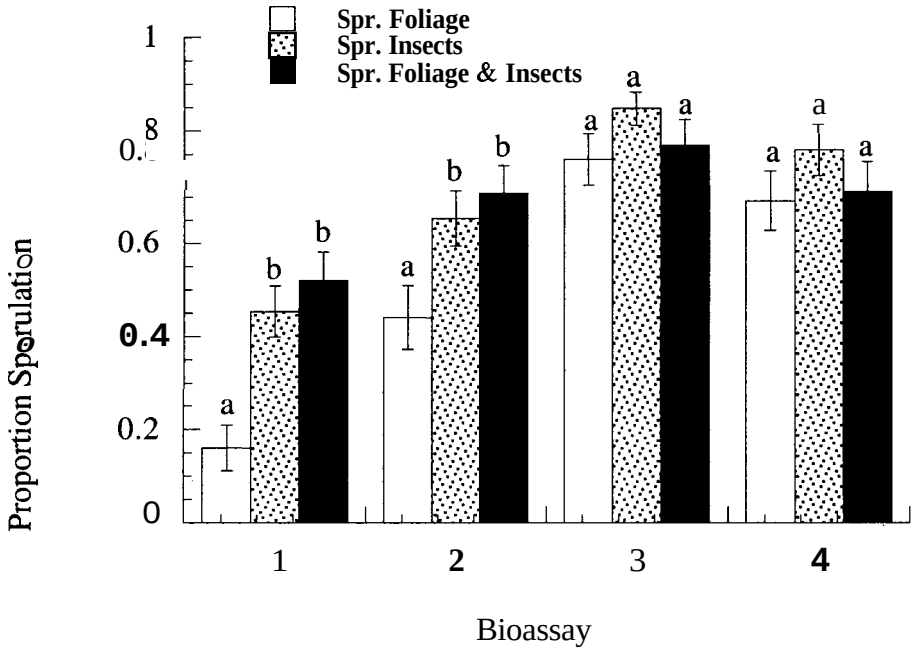


Figure 1.2 Proportion of cadavers sporulating of second instar Colorado potato beetle following different modes of exposure to ***B. bassiana*** conidia. Spr. Insect = insect sprayed directly with conidial suspensions; spr. foliage = insect exposed to foliage sprayed with conidial suspension; and **spr.** insect & foliage = insects both sprayed directly and exposed to sprayed foliage. Letters correspond to results of all possible pairwise comparisons using nominal logistic regression within a bioassay (significance level $p < 0.05$).

Fluorescence Microscopy

The total measured surface area of the second instar larva was $10.90 \pm 0.38\ mm^2$. The body regions observed microscopically in this study constituted ca. 70% of the total surface area of the insect body. Of the observed body regions the legs represented the greatest proportion of total surface area (Table 1.1).

Larvae sprayed directly with conidia of ***B.*** bassiana accumulated significantly more conidia on the dorsal surface of the abdomen than larvae exposed to treated foliage ($F = 171.30$; $df = 2, 172$; $p < 0.001$) (Fig. 1.3a). There was a significant interaction between mode of exposure and exposure time, with a higher density of conidia observed at 36 hours than at 24 hours for those insects sprayed directly ($F = 5.32$; $df = 2, 172$; $p = 0.006$). No such increase over time was observed for insects exposed to sprayed foliage alone. There was also a significant interaction between block (replicate of the experiment) and mode of exposure on the density of conidia on the dorsal view of the abdomen ($F = 5.62$; $df = 2, 167$; $p = 0.004$). In the first of these experiments there was no difference between the density of conidia on the dorsal surface of the insects which were sprayed and exposed to inoculated foliage and those insects which were only sprayed. In the second, there were significantly more conidia on insects which experienced both modes of exposure compared with those only sprayed directly.

On the ventral surface of the abdomen and head, and on the legs, the densities of conidia were higher for treatments in which insects were exposed to ***B.*** bassiana-treated foliage compared with those which were only sprayed directly (ventral surface of abdomen: $F = 85.80$; $df = 2, 173$; $p < 0.001$; head: $F = 45.71$; $df = 2, 173$; $p < 0.001$; and legs: $F = 55.38$; $df = 2, 173$; $p < 0.001$) (Fig. 1.3b-d). However, the density of conidia did not differ significantly between those insects only exposed to treated foliage and those sprayed directly and exposed to treated foliage ($p>0.05$; Tukey's multiple range test). **A** significant interaction between exposure treatment and exposure time was detected on the ventral surface of the abdomen ($F = 8.82$; $df = 2, 173$; $p < 0.001$) with

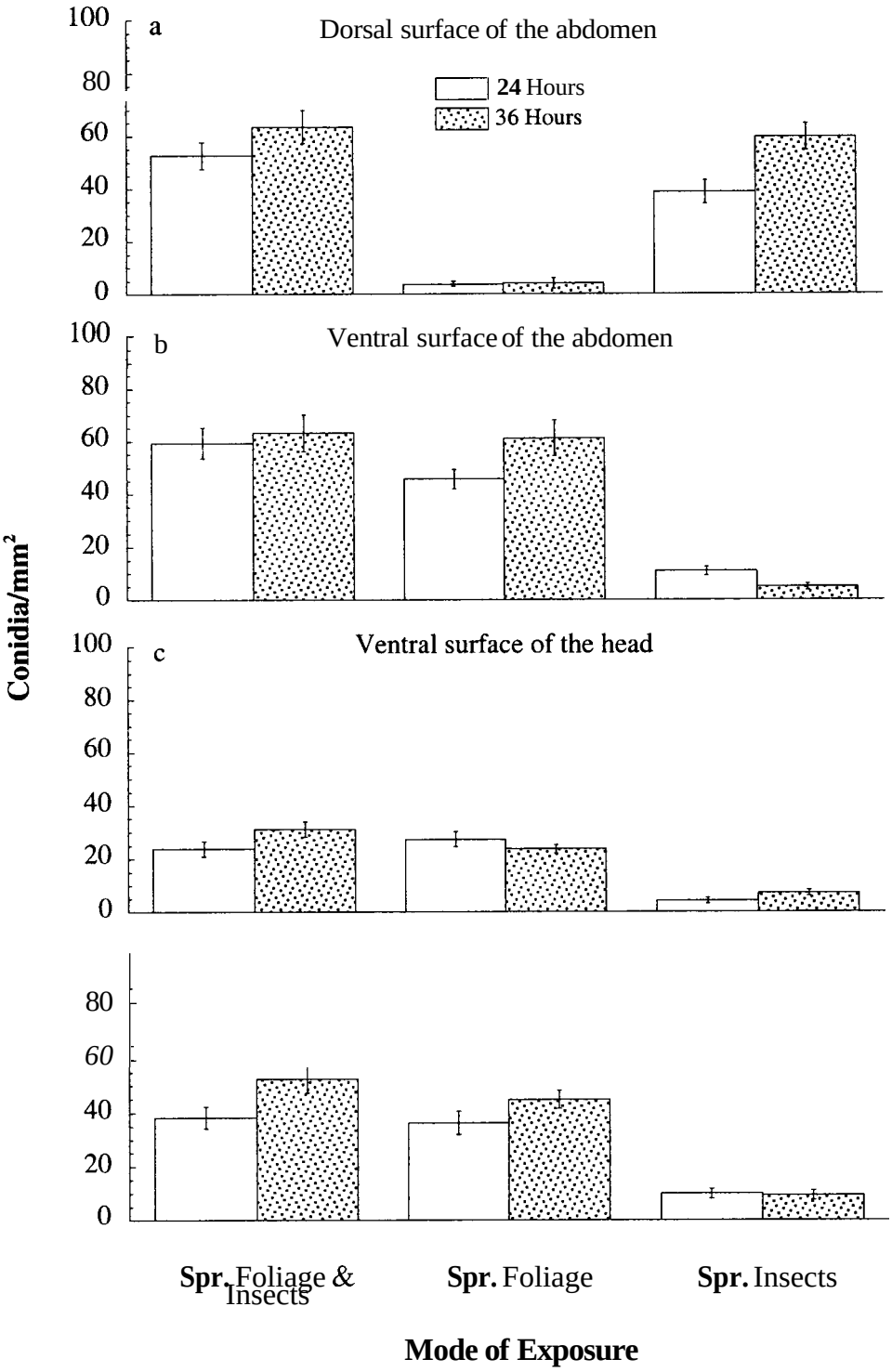
TABLE 1.1

The surface area and number of conidia on different body regions of second instar Colorado potato beetle following different modes of exposure to ***Beauveria bassiana.***

	Mean Surface area	Exposure Treatment Number of conidia per body part"		
Body part	mm^2	Sprayed insect	Sprayed foliage	Sprayed insect & foliage
Dorsal side of the abdomen	**2.24 ± 0.14**	**109.94 ± 80.6**	**8.78 ± 2.42**	**130.03 ± 9.09**
Ventral side of the abdomen	**1.49 ± 0.16**	**11.81 ± 6.76**	**79.95 ± 5.87**	**91.57 ± 6.76**
Ventral side of the head	**0.47 ± 0.03**	**2.71 ± 0.37**	**12.10 ± 0.77**	**12.97 ± 0.97**
Legs (6)	**3.49 ± 0.38**	**36.67 ± 4.86**	**155.24 ± 10.95**	**173.73 ± 12.71**
Total Insect[6]	**10.90 ± 0.38**			
Estimated total density of conidia for all body parts		**161.14 ± 19.55**	**256.07 ± 20.03**	**408.30 ± 29.53**

[a] Number of conidia (mean ± SE) on total surface of body part estimated by (surface area x mean conidia/mm^2) observed at **24** and **36** hours post exposure.
[b] Total surface of a second instar Colorado potato beetle.

Figure **1.3.** The density of ***B. bassiana*** conidia on: a) dorsal surface of the abdomen; b) ventral surface of the abdomen; c) ventral surface of the head; and d) legs of second instar Colorado potato beetle at **24** and **36** hours following different modes of conidial exposure. Spr. Insect = insect sprayed directly with conidial suspensions; spr. foliage = insect exposed to foliage sprayed with conidial suspension; and spr. insect & foliage = insects both sprayed directly and exposed to sprayed foliage. Bars correspond to the mean ± **SE** of two experiments, using 10 and 20 insects, respectively.

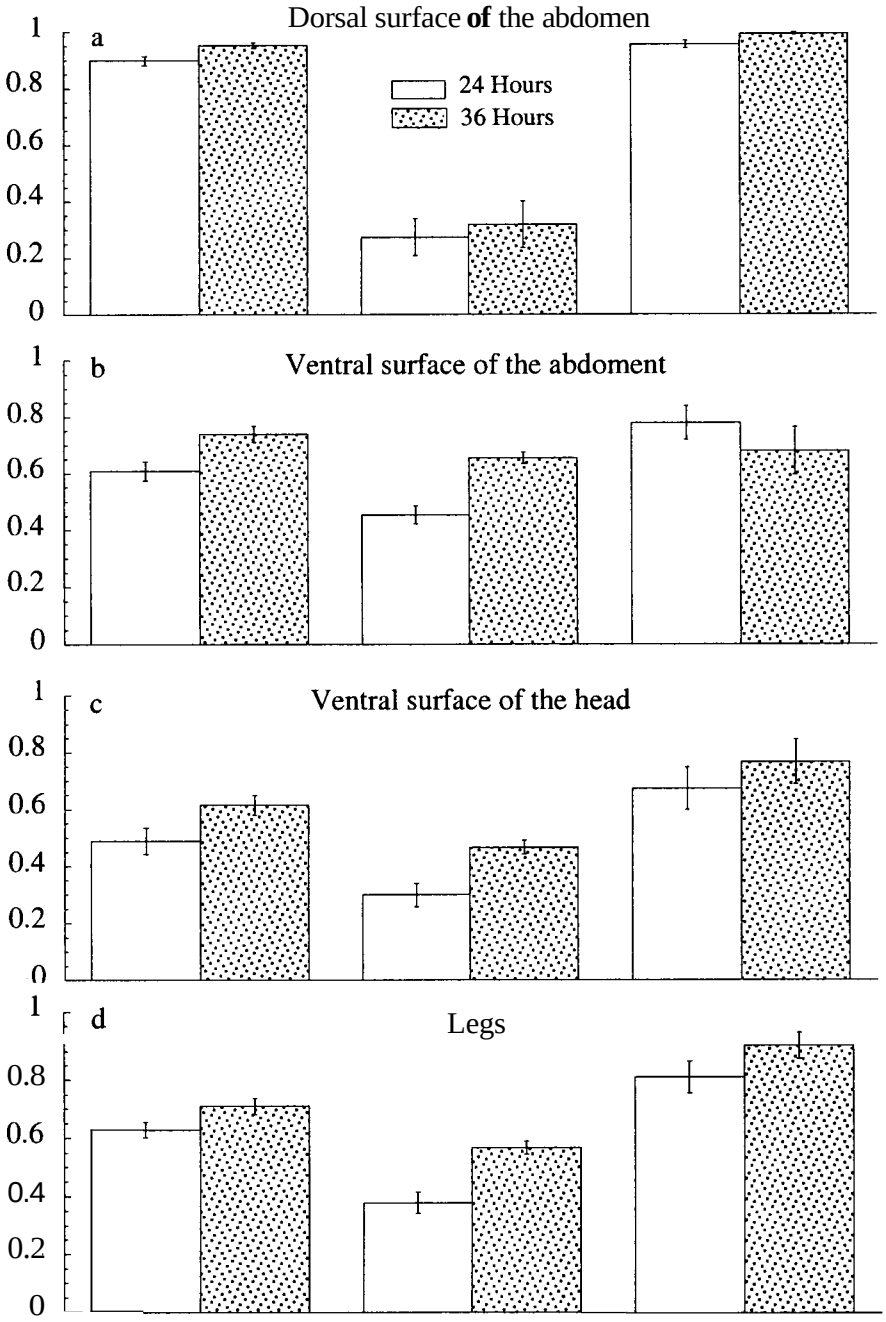

a
Dorsal surface of the abdomen
24 Hours
36 Hours
b
Ventral surface of the abdomen
c
Ventral surface of the head
100
80
60
40
20
0
Conidia/mm²
Spr. Foliage & Insects
Spr. Foliage
Spr. Insects
Mode of Exposure

conidia density increasing over time in treatments exposed to treated foliage, but decreasing in the sprayed only treatment. Conidia density did not change over time and there was no interaction between treatment and time for conidia density on the ventral surface of the head ($p = 0.11$; interaction: $p = 0.48$). Conidia densities in the legs did not differ significantly at the $p < 0.05$ level, however a trend of increased density over time was observed on the legs (time effect: $p = 0.09$; interaction: $p = 0.61$) (Fig. 1.3d).

The surface of the dorsal side of the abdomen of the observed CPB larva was morphologically different from the surface of the ventral side of the abdomen. The surface of the ventral side contained a larger density of seta than the dorsal side. Many ***B. bassiana*** conidia were observed attached to setal sockets. Conidia are also observed trapped in the intersegmental membranes of legs, mouth parts and abdomen.

For all of the observed body parts, the proportion of germinated conidia was significantly greater on insects which were sprayed directly compared with those only exposed to inoculated foliage (dorsal abdomen: $F = 144.04$; $df = 2, 172$; $p < 0.001$; ventral abdomen: $F = 5.92$; $df = 2, 173$; $p = 0.003$; head: $F = 15.73$; $df = 2, 173$; $p = 0.0001$; and legs: $F = 49.59$; $df = 2, 173$; $p < 0.001$) (Fig. 1.4a-d). On the ventral and dorsal surfaces of the abdomen, the proportion of germinated conidia did not differ between those directly sprayed only and those directly sprayed and exposed to inoculated foliage ($p < 0.05$ for a Tukey's multiple range test). On the legs and head a higher proportion of the observed conidia had germinated on insects which were only directly sprayed compared with those which were sprayed and exposed to inoculated foliage ($p < 0.05$ for a Tukey's multiple range test). The proportion of germinated conidia increased from 24 to 36 hours on the head ($F = 7.49$; $df = 1, 173$; $p = 0.007$) and legs ($F = 16.35$; $df = 1, 173$; $p < 0.001$) of insects receiving all types of exposure, and on the ventral surface of the abdomen for those insects exposed to inoculated foliage treatments ($F = 4.00$; $df = 2, 173$; $p = 0.02$). There was no increase in germination over time observed on the

Figure **1.4.** The proportion of germinated ***B. bassiana*** conidia on: a) dorsal surface of the abdomen; b) ventral surface of the abdomen; c) ventral surface of the head; and d) legs of second instar Colorado potato beetle at **24** and **36** hours following different modes of conidial exposure. Spr. Insect = insect sprayed directly with conidial suspensions; spr. foliage = insect exposed to foliage sprayed with conidial suspension; and spr. insect & foliage = insects bothsprayed directly and exposed to sprayed foliage. Bars correspond to the mean ± **SE** of two experiments, using 10 and **20** insects, respectively.


a
Dorsal surface of the abdomen
24 Hours
36 Hours
1
0.8
0.6
0.4
0.2
0
b
Ventral surface of the abdoment
c
Ventral surface of the head
d
Legs

ventral surface of the abdomen of insects only sprayed directly, and for all exposure modes on the dorsal surface of the abdomen ($p = 0.11$).

The appearance of germinated conidia differed between body regions. Conidia on the dorsal side of the abdomen produced relatively long hyphae before penetrating the cuticle, whereas those on the ventral body regions produced relatively short germ tubes over the same incubation time.

When comparing the impact of exposure modes on the density of conidia acquired over a 24 hour period following exposure, sprayed foliage resulted in a significantly higher density of conidia compared to the other exposure modes ($F = 2.66$; $df = 3,785$; $p = 0.005$). However, there was an interaction between mode of exposure and body part ($F = 22.25$; $df = 9,785$; $p < 0.001$). On the dorsal surface of the abdomen, a higher density of conidia was observed on sprayed and dipped insects, whereas on the ventral surface of the abdomen, head and legs, a higher density of conidia was detected on insects exposed to sprayed or dipped foliage (Fig. 1.5a-d). Over all, the highest density of conidia was observed on the ventral surface of the abdomen ($F = 14.28$; $df = 3,785$; $p < 0.001$) (Fig. 1.5b). A significant interaction between mode of exposure and time was detected ($F = 2.81$; $df = 18,785$; $p < 0.001$). As in the previous experiment, the density of conidia increased significantly over time on the ventral surface of the abdomen for those insects exposed to treated foliage, but not for insects sprayed directly or dipped in the conidial suspension. This same increase in conidial density over time was observed on the legs of insects exposed to sprayed foliage.

Germination of conidia was faster in sprayed insects compared to other modes of exposure ($F = 30.89$; $df = 3,785$; $p < 0.001$) (Fig. 1.6a-d). Germination after direct spray exposure was more than twice that observed in the other treatments at 8 and 12 hours ($F = 3.90$; $df = 18,785$; $p < 0.001$). As expected, proportion germination increased significantly with time ($F = 100.36$; $df = 6,785$; $p < 0.001$). However, a significant interaction between exposure method and exposure time was detected. The proportion

Figure 1.5. The density of ***B. bassiana*** conidia on: a) dorsal surface of the abdomen; b) ventral surface of the abdomen; c) ventral surface of the head; and d) legs of second instar Colorado potato beetle at 0, 4, 8, 12, **16, 20,** and **24** hours following different modes of conidial exposure. Spr. Insect = insect sprayed directly with conidial suspensions; spr. foliage = insect exposed to foliage sprayed with conidial suspension; sipped insect = insect dipped in a conidial suspension; and dipped foliage = insect exposed to foliage dipped in a conidial suspension. Data points correspond to the mean ± **SE** of two experiments, using **4** insects per treatment/time/experiment.

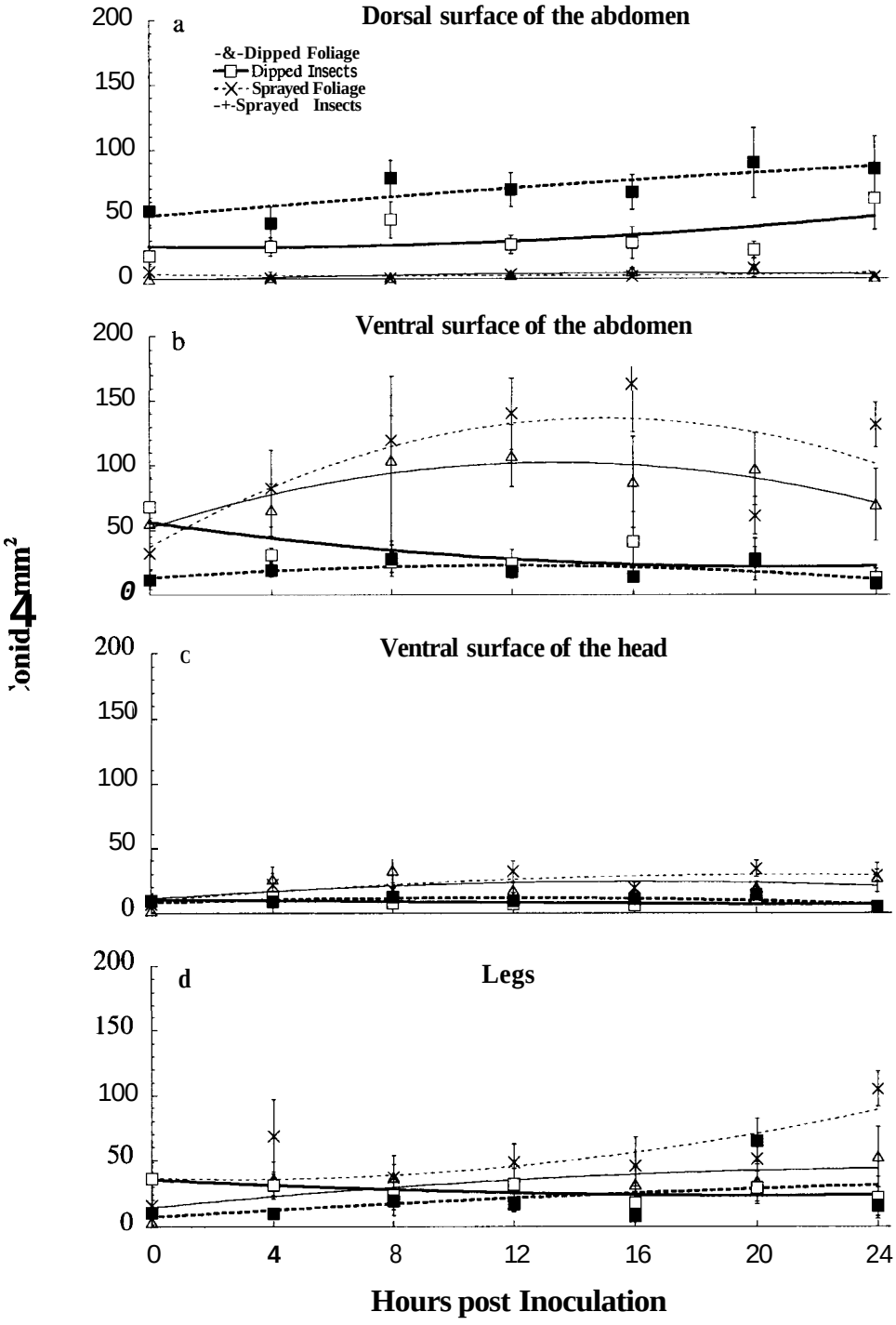
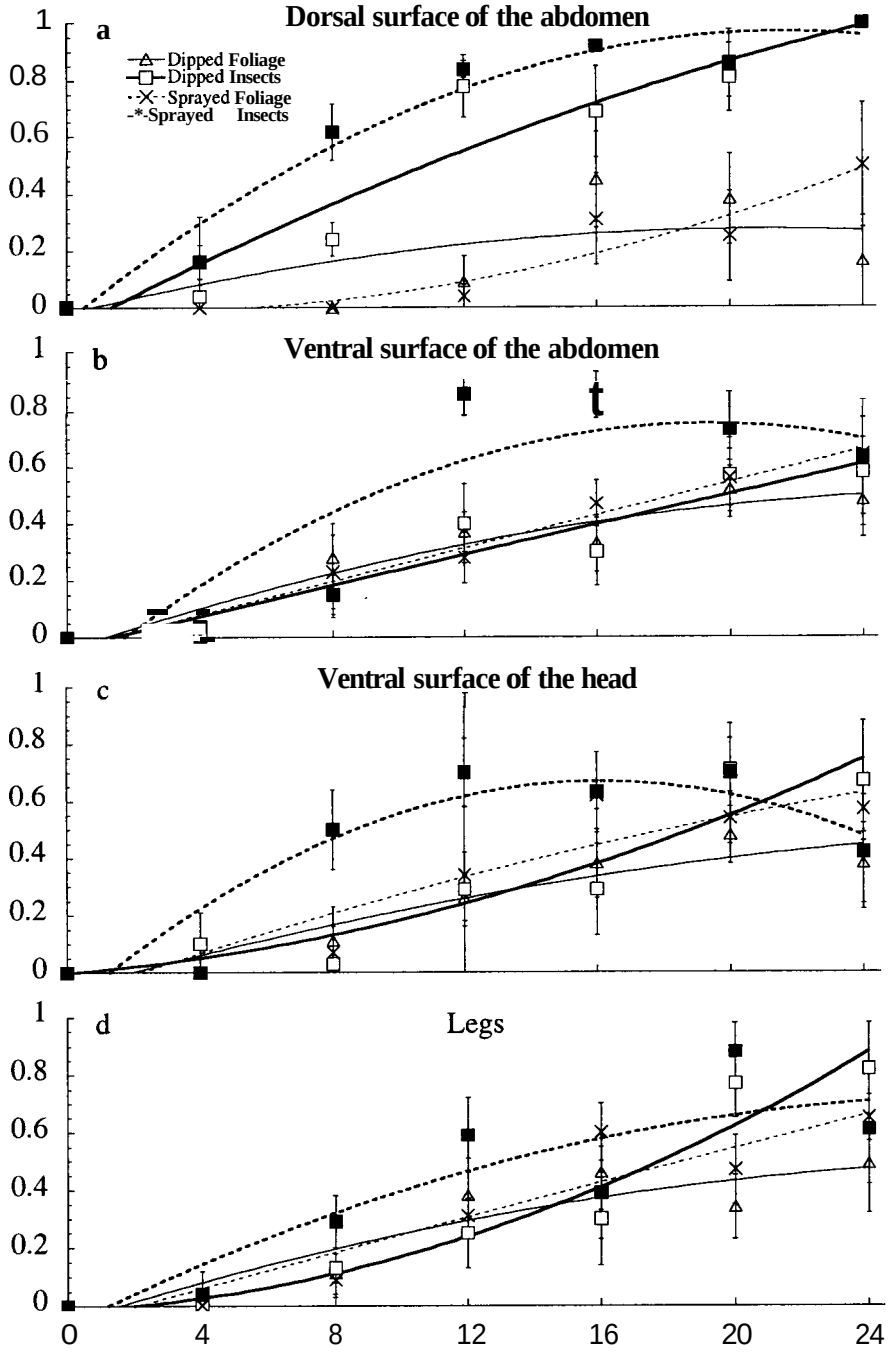

a
Dorsal surface of the abdomen
-&-Dipped Foliage
Dipped Insects
Sprayed Foliage
Sprayed Insects
b
Ventral surface of the abdomen
c
Ventral surface of the head
d
Legs
200
150
100
50
0
0
4
8
12
16
20
24
Hours post Inoculation
conid4mm2

Figure **1.6.** The proportion of germinated ***B.*** *bassiana* conidia on: a) dorsal surface of the abdomen; b) ventral surface of the abdomen; c) ventral surface of the head; and d) legs of second instar Colorado potato beetle at **0, 4,** 8, **12, 16, 20,** and **24** hours following different modes of conidial exposure. Spr. Insect = insect sprayed directly with conidial suspensions; spr. foliage = insect exposed to foliage sprayed with conidial suspension; sipped insect = insect dipped in a conidial suspension; and dipped foliage = insect exposed to foliage dipped in a conidial suspension. Data points correspond to the mean ± **SE** of two experiments, using **4** insects per treatment/time/experiment.


a
Dorsal surface of the abdomen
Dipped Foliage
Dipped Insects
Sprayed Foliage
Sprayed Insects
b
Ventral surface of the abdomen
c
Ventral surface of the head
d
Legs
1
0.8
0.6
0.4
0.2
0
0
4
8
12
16
20
24

germination was higher in sprayed insects for all exposures times lower than 20 hours, after which dipped insects showed as equal or higher proportion of germinated conidia ($F = 3.90$; $df = 18,785$ $p < 0.001$). The foliar treatments, as observed in previous experiments, resulted in a lower proportion of germinated conidia at all times than the treated insects. On the ventral body regions of larvae exposed to treated foliage (weather sprayed or dipped), ungerminated conidia as well as relatively short germ tubes were observed whereas conidia on the dorsal side of the abdomen of dipped or sprayed insects produced relatively long hypha over the same incubation time.

Although there were no significant differences among body parts in the overall proportion of conidia germinated ($F = 1.19$; $df = 3$; $p = 0.31$), consistent with the findings of the previous experiment, there was a significant interaction between body part and mode of exposure with regard to germination ($F = 6.49$; $df = 9$; $p < 0.001$). **A** higher proportion of germinated conidia occurred on the dorsal surface of the abdomen for sprayed and dipped insects compared to insects exposed to treated foliage regardless of the treatment method, sprayed or dipped foliage.

The estimated total density of conidia was greater on insects exposed both by direct spray and contact with treated foliage (Table 1.1). There was approximately 40% more conidia on insects exposed to treated foliage than sprayed directly, and approximately 40% more conidia on insects both sprayed and fed treated foliage than those fed on treated foliage alone.

Discussion

Beauveria bassiana conidia sprayed directly on Colorado potato beetle larvae are more significant determinants of mortality than are conidia acquired by larvae moving about on conidia-treated foliage. Although the actual dose of conidia per insect is higher when the insects move across contaminated leaf surfaces (Table **2.1**), the sites on the ventral surface of the insect appear not to be as conducive to conidial infection as those exposed to direct spray.

On the dorsal surface of the abdomen of CPB larvae, clusters of conidia were found trapped in the intersegmental folds where the microenvironment may be more suitable for penetration. Inglis ***et al.*** **(1995)** observed conidia on grasshoppers apparently trapped in the intersegmental folds during oviposition when the abdomen was extended. Schreiter ***et al.*** **(1994)** also found colonies of ***Verticillium lecanii*** in the intersegmental regions between the head and the thorax, and between the thorax and the abdomen of western flower thrips.

Although high concentration of conidia were found attached to the dorsal surface of the abdomen of insects following direct spray, considerably more conidia were picked up on the legs and ventral surface of the abdomen and head with exposure to sprayed foliage. Based on the surface areas of the different body regions and the observed densities of conidia within these regions, the total conidia attached to the insect after **24** hours of exposure to sprayed foliage was 37% more than on directly sprayed insects (Table **2.1**). In particular, the surface of the legs adds considerably to the area available for attachment of conidia. With the average density of conidia of **20.47 ± 0.98** conidia/mm^2 following exposure to treated foliage, the legs make the most significant contribution to the total density of conidia attached per insect. However, these sites, being heavily sclerotized, may not be suitable for penetration and infection. Conidia attached to them, do not contribute to mortality.

The differences in the character of the observed body parts may also have contributed to differences in infection. The ventral surface of the abdomen contained a higher density of seta with ***B.*** *bassiana* conidia than the dorsal surface. Schreiter *et al.* **(1994)** also reported these sockets as sites for attachment of conidia of *V. lecanii* on western flower thrips. Setal sockets are considered a definite area of weakness in the insect cuticle (David, **1967).** However, Wigglesworth **(1990)** describes the cuticle of the larvae of *Rhodnius* as composed by two types of cuticle: a soft extensive one that allows expansion of the abdomen after a meal of blood, and another composed by small plaques of hard exocuticle, each surrounding a seta. It is possible that even if in these areas of exocuticle where we have observed great numbers of conidia attached, the penetration of these may be inhibited by the cuticle hardness. The conidia attached to ventral abdomen and legs could suffer abrasion against the surface of the leaves, specially while extending the abdomen while feeding. This may negatively affect the viability of conidia and hence prevent infection. Abdominal cuticles of *Rhodnius* after a blood meal, suffer abrasion by being exposed to emery cloth or filter paper (Wigglesworth, **1990).**

Although insects feeding in treated foliage consume ***B.*** *bassiana* conidia, mortality probably occurs as a consequence of infection through the external cuticle of the insect and not by ingesting conidia on foliage. Allee *et al.* **(1990)** found penetration of ***B.*** *bassiana* conidia through the gut in only one larva of CPB starved prior to per os inoculation, suggesting that the rate of food passage through the gut may prevent infection through that route. There is some evidence of ***B.*** *bassiana* infection through the alimentary track on aquatic insects. After **24-48** hours after exposure abundant germinated ***B.*** *bassiana* conidia have been observed in the gut of the larvae of the mosquito *Aedes aegypti,* but not in the hemolymph (Miranpuri and Khachtourians, **1991).**

In addition to better penetration sites, sprayed insects experienced a higher rate of germination of conidia than those exposed to treated foliage. As germination of conidia on insects dipped in a conidial suspension was also slower than observed on sprayed

insects, perhaps the pressure of the spray enhance conidial lodging within cuticular folds, facilitating germination and penetration. The proportion of germinated conidia varied in the four body parts and for all treatments with the highest proportion of germinated conidia observed on the dorsal surface of the abdomen. Wraight ***et al.*** **(1990)** observed the percentage germination of conidia of ***Erynia radicans*** on the dorsal surface of potato leafhoppers feeding on cowpea leaves. They found higher germination rates on the abdomen than in the head and thorax, areas of heavy sclerotization that may prevent penetration. They attribute those differences to higher concentrations of chemical cues stimulating germination on the abdomen compared with the other body parts. We found a lower overall proportion of germinated conidia on the ventral side of the insect than on the dorsal side of the abdomen, and the lowest proportion on the head (consisting mainly of mouth parts). It is possible that glandular secretions inhibit conidial germination. Gochnauer ***et al.*** **(1979)** found that geraniol and citrol present in the Nassanoff scent-gland secretions of adult worker honey bee inhibit or kill the chalkbrood fungus, *Ascosphaera* ***apis.*** These glandular antibiotic secretions have also been observed in ants (Brough, **1983).** No studies have been done on the effects of glandular secretions from CPB on ***B. bassiana.*** Exposure to concentrated alkaloids from active feeding on potato foliage may also inhibit germination. Costa and Gaugler **(1989)** found that certain concentrations of the glycoalkaloids tomatine and solanine reduced ***B. bassiana*** colony formation and growth in media.

Insects exposed to sprayed foliage always yield a lower proportion of germinated conidia. The germination of conidia may be delayed by the presence of phytochemicals found on the potato foliage. However, this phenomena could also be due to the insect collecting conidia over the exposure time period on sprayed foliage, so it is expected that the development of the conidia will be correlated to the time in which attachment to the cuticle took place. In sprayed insects, because inoculation is not spread over time, conidial development and germination is more synchronized.

The dorsal surface of the cuticle may produce higher quantities of cuticular wax to prevent desiccation. The conidia germinate in response to the chemical cues but grow along the surface until finding a suitable penetration site. On relatively smooth dorsal surfaces of the abdomen the fungus may need to grow more along the cuticle to reach suitable penetration sites. Germ tubes of conidia on the ventral surface were observed to be significantly shorter than on the dorsal surface. This phenomenon could be due to the loss on viability of the conidia on the ventral surface once germinated due to exposure to plant secondary metabolites or abrasion.

In conclusion, I found that direct applications of ***B.*** *bassiana* results in higher mortality than exposure to fungus inoculated foliage. Although insects moving on sprayed foliage acquire a greater density of conidia on the legs, mouthparts and ventral surface areas, these higher densities of conidia attached contribute little to additional mortality. We hypothesize that these body regions are less susceptible to penetration and subsequent infection by the fungus. However, exposure to treated foliage does result in additional CPB larval mortality. An important implication of this analysis is that prolonged persistence of conidia in field applications would enhance the efficacy of foliar treatments by adding mortality to that portion of the population which does not experience contact with the direct spray.

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MANUSCRIPT 2

PRODUCTION OF *BEAUVERIA BASSIANA* (BALS.) VUILL. CONIDIA ON COLORADO POTATO BEETLE LARVAL CADAVERS

Introduction

Uncontrolled populations of the Colorado potato beetle (CPB) can defoliate potato (*Solanum tuberosum*) plants causing total yield loss (Hare, 1980). With rapidly increasing pest resistance to chemical insecticides (Forgash, 1985; Grafius, 1997) and increasing concern for public health and environmental impact of chemicals, alternatives to chemical control of the CPB are being evaluated.

Beauveria bassiana is a Deuteromycete which has proven to be successful in lowering populations of the Colorado potato beetle (CPB), *Leptinotarsa decemlineata*, after foliar applications (Galani, 1984; Anderson et al., 1988; Drummond and Groden, 1996; Poprawski et al., 1997). However, the true capacity of fungal pathogens for long-term pest suppression may be in exploiting their epizootic potential.

Although humans have been unsuccessful in creating epizootics (rapid increases in disease incidence) (Fuxa and Tanada, 1987) to control pests, fungal epizootics in insect populations have been observed in nature and can be spectacular (Carruthers and Soper, 1987). Epizootics in insects can be manifested if the pathogen is widely distributed in the host environment (Bird and Elgee, 1957; Tanada, 1961); or if high densities of infected insects are present, increasing the probability of contact between healthy and diseased individuals (Anderson and May, 1980). In general terms, the higher the inoculum (or infective units of the pathogen population) resulting from secondary cycling, the higher the probability of disease progression. Pathogen population densities are determined by, among other factors, the pathogen's reproductive rate and its capacity to survive (Fuxa and Tanada, 1987).

Most reports of fungal epizootics of insects have involved pathogens in the order Entomophthorales (Goh et al., **1989;** Harcourt et al., **1990;** Steinkraus et al., **1998).** Some of these fungi, while having great potential for use as classical biological control agents, are presently difficult to grow and produce inefective units in artificial medium, making their augmentation in fields costly.

Entomopathogenic Deuteromycetes are a group of fungi that replicate in an insect population by means of asexual spores or conidia (infective units) and which have great potential as microbial insecticides. Some of these fungi are already registered with the EPA for control of many pests including several moths, weevils, whiteflies, aphids, grasshoppers and turf pests *(B. bassiana* ATCC produced by Troy Biosciences Inc., Phoenix, AZ; Mycotrol, *B. bassiana* strain GHA produced by Mycotech, Butte, MT; and *Paecilomyces fumosoroseus* Apopka strain **97** produced by Thermo-Trilogy Corp. Columbia, MD). Entomopathogenic Deuteromycetes can be easily mass produced in a variety of liquid and solid media (Desgranges et al., **1993;** Jackson et al., **1997;** Vidal et al., **1998)** and applied as foliar applications using conventional equipment. However, epizootics of entomopathogenic Deuteromycetes are uncommon, and thus most of the research on these fungi has focused on their insecticidal properties: effect of dose on mortality and time to kill, and performance of different isolates for insect control (Fargues et al., **1994;** Milner and Prior, **1994;** Adane et al., **1996;** Valera and Morales, **1996;** Milner et al., **1998;** Wraight et al., **1998;** Poprawski et al., **1999).** As naturally occurring pathogens in insects, entomopathogenic Deuteromycetes have the potential to replicate in their host inducing secondary cycling of the disease. Conidia produced by cadavers constitute the inoculum necessary for transmission of the disease in the insect population. The amount of conidia produced and their survival in the environment will determine the rate with which the disease spreads.

Research on conidia production by entomopathogenic Deuteromycetes has concentrated mainly on the performance of the fungi on artificial media (Feng et al. **1994).** The influence of factors including fungal dose and host age on sporulation of cadavers from

infected insects has been investigated for *Metarhizium anisopliae, **B.** bassiana* and *Paecilomyces* sp. infecting *Schistocera americana,* diamondback moth, coffee berry borer and silver leaf whitefly, among others (de la Rosa et al., **1997;** Sieglaff et al., **1998;** Vandenberg et al., **1998;** Wraight et al., **1998).** However, data concerning quantity of conidia produced per infected host is scarce. Luz and Fargues **(1998)** investigated ***B.*** *bassiana* conidia production by different life stage cadavers of *Rhodnius prolixus.* They found that conidia production by cadavers increases with cadaver size. It is likely that host and pathogen factors that impact insect mortality and sporulation also have an effect on conidia production by cadavers.

This study explores the effect of host and pathogen factors on density of conidia produced by CPB larval cadavers resulting from ***B.*** *bassiana* infections and their potential role in transmission of the disease. ***B.*** *bassiana* is likely to be transmitted in CPB populations by insects encountering sporulating cadavers on or in the soil (Long et al., 2000a) or by infection through the build up of inoculum in the soil. To predict transmission by either mechanism, the amount of conidia generated from sporulated cadavers is important. The results derived from these experiments will provide fundamental information that will be incorporated in a simulation model constructed with the purpose of understanding secondary cycling and transmission of the disease in the beetle population (Long et al., 2000b; Jorgensen, 2001).

Materials and Methods

Fungal Culture

The fungus used for bioassays was ***B. bassiana*** strain GHA (supplied as technical powder by Mycotech Corporation, 630 Utah Av. Box 4109, Butte MT 59702). Conidia were applied with a sterile rod to Sabouraud's dextrose agar (SDA) and grown at 25°C in an incubator at 16:8 h light:dark cycle. After 10 days, plates were transferred to 4°C until used in bioassays. The conidia used in the experiments were harvested from the first or second subcultures from technical powders and were less than 1 month of age. Viability of the conidia was examined prior to every experiment and was always ≥ 99%.

Insect Culture

Insects used in these experiments were obtained from a laboratory colony. A stock culture of CPB was established from field collected adults. Colony adults were kept at a ratio of 4 females to 2 males in 300 cc paper cups and maintained in an environmental chamber at 25ºC, 68-70% relative humidity (RH), and a 16:8 h light:dark cycle. Adults were fed fresh greenhouse grown potato foliage daily. Eggs were collected daily and stored for up to 8 days at 12°C. For assays, egg masses were transferred to 25°C, and upon eclosion, larvae were fed fresh potato foliage daily until reaching the second stadium. Eggs from new field collected adults were added to the colony annually.

Number of Conidia Produced per Cadaver

The production and viability of conidia on CPB cadavers was assessed through a series of experiments conducted in 1995-96. Production and viability was assessed over time after death for CPB larvae inoculated as second instars.

Dry ***B. bassiana*** conidia collected from SDA plates were suspended in 0.1% Tween 20® (Sigma Chemical Company, P.O. Box 14508, St. Louis MO, 63178) by vortexing. Conidia concentrations were adjusted following estimation using a hemacytometer. Groups

of 50 or less second instars placed in 9 cm diameter petri dishes with moist filter paper were sprayed with 80 μl of a conidia suspension in five trials in 1995 and 160 μl in one trial in 1996 trial using a Paasche airbrush sprayer (Paasche Airbrush Co., Hartwood Heights, IL). The density of conidia/mm^2 was estimated by spraying suspensions on plates of SDA and observing conidia at 400X magnification. Conidia suspensions ranged from 1.39 x 10^8 to 1.59 x 10^8 conidia/ml and resulted in estimated sprayed concentrations ranging from 117 to 159 conidia/mm^2 in the five trials conducted on 1995, and 1.07 x 10^8 conidia/ml resulting in 384 conidia/mm^2 in 1996. Fifty-six to 99 larvae were sprayed per trial in 1995 and 238 were treated in the 1996 trial. Inoculated insects were held individually in 3 cm diameter petri dishes with fresh potato foliage in an environmental chamber at 25ºC, 68-70% RH, and a 16:8 h light:dark cycle. Insects were given fresh foliage daily. The status of the insects was observed every 12 hours.

Upon death, foliage was removed and dishes with cadavers were placed in 100% humidity chambers (bell jars with water) at 25ºC to induce sporulation. Initial weight and weight at death were recorded in the last three trials in 1995. Days to death, stage at death, and days from death to initial sign of sporulation (visible fungal growth on the external surface of the insect) were recorded for all trials in 1995 and 1996. Sporulation of cadavers was monitored every 12 hours for all trials in 1995 and every **24** hours for the 1996 trial. Ten to 43 sporulating insects were randomly sampled per day from 1 to 10 days after initial signs of sporulation in the 1995 trials. In the 1996 trial, nine to 39 sporulating cadavers were randomly sampled 4, 6, 9, 12, 15, 18, and 21 days from the first sign of sporulation. Sampled cadavers were individually submerged in **0.4**ml 0.1% Tween-20® solution and vortexed for 1.5 minutes. The resulting conidia concentration was determined with a hemacytometer by averaging the conidia counts of 5 randomly chosen 0.2 mm^2 squares.

Viability of conidia on cadavers was assessed by placing 0.1 ml of the conidia suspensions from sporulating cadavers on SDA. The conidia solution was evenly distributed on the surface of the SDA culture plate with a sterile glass rod and incubated at

25°C for 24 hours. Proportion of germinated conidia was assessed under the microscope at 200X. Only conidia that exhibited a germ tube of length equal to the diameter of the conidia were considered germinated.

Initial Inoculum Concentration

The impact of *B. bassiana* inoculation dose on conidia produced by second instar CPB larvae was assessed in 1996. In each of two trials, 50 second instar CPB were inoculated per dose of *B. bassiana*. Larvae were sprayed with a Burkard computer controlled spraying apparatus set at **8** Kg/cm^2 (Burkard Manufacturing Co. Ltd., Rickmansworth Hertfordshire, England) and held in an environmental chamber as described for the previous experiment. Conidia concentrations included 3.0, 11.2, 18.1, 71.0, and 123.2 conida/mm^2 in trail 1 and 3.0, 10.0, 27.2, 67.1, and 114.5 conididmm' in trial 2. Conidia concentrations were assessed by counting the density of conidia on ten randomly selected sections (0.2 mm^2 each) of sprayed water agar plates at 400X magnification. Days to death, stage and weight at death, and days to sporulation from death were recorded. Mummified cadavers were sampled 6 days from the first sign of sporulation and conidia production was determined as previously described.

Stage at Time of Infection

To assess the effect of host life stage at the time of *B. bassiana* infection on the number of conidia produced by CPB larval cadavers, a series of conidia suspensions were applied to the different instars. For each instar a total of 50 insects per dose were sprayed with 116,280 or 971 conididmm'. Third and fourth instars were also treated with the concentration of 327 conidia/mm^2. Conidia suspensions were prepared as previously described and applied using the Burkard computer controlled spraying apparatus set at **8** Kg/cm^2. Two 2% water agar plates per spray run were observed at 400X to determine the density of conidia per mm^2 achieved. Insects were held individually in 5 cm diameter petri

dishes as previously described. Days to death, stage and weight at death, and days to sporulation from death were recorded. Seventeen to 35 sporulating cadavers were randomly sampled per day 4, 12, 24 and 36 days from the first sign of sporulation and the number of conidia per cadaver was determined as described for previous experiments.

Host life stage effect on conidia production was again evaluated in a second series of experiments in which conidia production by larvae treated as first, third and fourth instars were assessed in separate trials in which each instar was compared to a standard second instar. For each trial groups of 30 first, third and fourth instars were sprayed with five concentrations ranging between 2.0 and 836.8 conidia/mm^2. Thirty second instars per trial were treated with a single concentration (49.6 to 65.3 conidia/mm^2 depending upon the trial). Conidia production was assessed 10 days after initial sporulation. One trial was conducted for CPB larvae treated as first instars, and two replicate trials were each conducted for larvae treated as third and fourth instars. Insects were held and conidia production was quantified as previously described.

Data Analysis

Nominal logistic regression (JMP, **SAS** Institute Inc., **SAS** Campus Drive, Cary, NC 27513) was used to determine the effect of dose on mortality and sporulation of treated insects. Multiple regression and ANOVA was used to test for significant relationships between host parameters (weight and stage at death, days from death to sporulation and incubation time of mummified cadavers) and conidia production. Data were transformed to the logarithm (based 10) of the cadaver weight (g) and the logarithm (based 10) of the conidia produced per insect prior to analysis. Data on the effect of conidia concentration on conidia production were transformed to the square root of conidia produced per insect prior to analysis. When determining the effect of instar at inoculation time on conidia production, the few insects that died prior to four days post-treatment were excluded from the analysis because of the extremely low numbers of conidia produced by these cadavers. Separate

analyses were conducted for each of the initial stadia in order to detect variable effects on time to death and conidia production.

Results

Conidia Production by Cadavers

In the 1995 and 1996 trials on conidia production over time, insects died 1-8 days post inoculation, with the majority dying between days 3 and 4 (median = 4 days; mean ± se = 3.64 ± 0.04 days). Insect weight at death ranged from 0.0014 to 0.0597 g (mean ± se = 0.0115 ± 0.0008), and initial signs of sporulation on cadavers were observed within 0 to 15 days after death, with most cadavers beginning to sporulate 1-2 days after death (median = 1 days; mean ± se = 2.47 ± 0.12 days). In both 1995 and 1996 trials, the longer it took for larvae to die the larger the size of the resulting cadaver. Weight at death increased significantly with time to death ($p < 0.001$; df = 1, 153; $F = 298.5$; $r^2 = 0.66$) in 1995, and stage at death increased significantly with time to death ($p = 0.001$; df = 1, 185; $F = 76.79$) in 1996. In the 1995 trials weight and stage at death had no significant effect on the number of conidia produced by cadavers (stage at death, $p = 0.82$; time to death, $p = 0.77$; and weight at death, $p = 0.91$). However, in the 1996 trial conidia produced by cadavers increased with stage at death ($p < 0.001$; df = 1, 198; $F = 76.79$). Time from death to initial sporulation also had no significant effect on production of conidia in 1995 ($p = 0.54$), but in 1996 conidia production significantly decreased with time to first sign of sporulation ($p < 0.001$; df = 1, 192; $F = 84.01$; $r^2 = 0.30$) with maximum conidia production observed for cadavers which started sporulating 1 to 2 days from death.

Conidia production by cadavers increased significantly with incubation time (time from initial sporulation to conidia sampling). In 1995, 70% of the cadavers sporulated by the sixth day after inoculation. Conidia per cadaver increased from one to 10 days after the first sign of sporulation ($p < 0.001$; df = 4,269; $F = 86.63$), with time explaining 40.1% of the variation of conidia produced per cadaver (**Fig. 2.1.A).** This relationship between days after first sign of sporulation and conidia per cadaver varied between trials (interaction: $p < 0.001$; df = 4,269; $F = 7.29$), however, in all trials, except trial **4,** conidia produced per cadaver increased with time. Maximum conidia production in the 1995 trials ranged from a

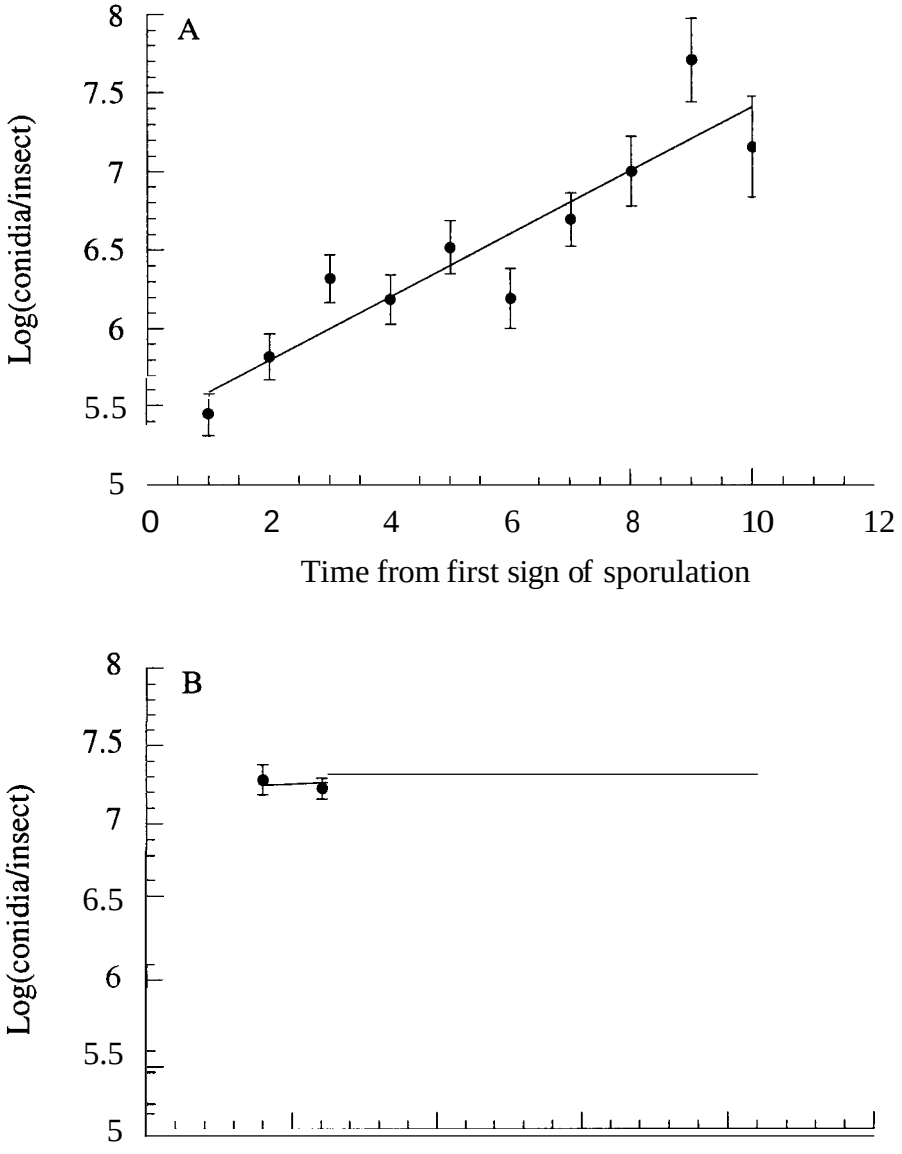

Figure 2.1. Relationship between days from first sign of sporulation and conidia produced per insect from cadavers of ***B. bassiana*** treated second instar Colorado potato beetle (express in log of the conidia produced per insect). **A)** All 5 trials conducted in 1995, and in which conidia production was observed over 10 days from first sign of sporulation (linear fit: $\mathbf{Y} = 5.4 + 0.2X$; $r^2 = 0.84$). B) 1996 trial in which observations were made over 21 days from first sign of sporulation (linear fit: $\mathbf{Y} = 7.21 + 0.008X$; $r^2 = 0.37$). Analysis was conducted with individual observations; graph depicts curve fit to the means.

mean of 6.36 x 10^7 conidia per insect in trial 1 to a mean of 4.89 x 10^6 in trial 3. Maximum conidia production was observed when insects died 3 days post infection and initiated sporulation 1 day after death. In 1996 trial, 92% of the cadavers sporulated by the sixth day after inoculation. Conidia production also significantly increased between 4 and 21 days after the first sign of sporulation ($p = 0.03$; df = 1, 190; $F = 4.86$; for 1996) (Fig 2.1.B), with the maximum production averaging 2.47 x 10^7 conidia per insect.

The proportion of viable conidia produced by cadavers was evaluated from 4 to 21 days after the first sign of sporulation in the 1996 experiment. The proportion of viable conidia did not change significantly over this period ($p = 0.69$) and ranged from 0.75 ± 0.6 (mean ± se) on day 4 to 0.93 ± 0.02 on day 9.

Initial Inoculum Concentration

The relationship between mortality and concentration varied with trial ($p < 0.001$; df = 1,596; $\chi^2 = 37.68$) (Table 2.1). In trial two, mortality increased with increasing inoculum, however, in trial one, no relationship between inoculum and mortality was observed. The proportion of cadavers which sporulated increased with increasing inoculum ($p < 0.001$; df = 1,597; $\chi^2 = 117.35$). However, the number of conidia produced per cadaver significantly decreased with increasing inoculum concentration ($p = 0.006$; df = 1, 139; $F = 7.86$) and increased with cadaver weight ($p < 0.001$; df = 1, 139; $F = 45.31$; $r^2 = 0.39$). When holding cadaver weight constant (partial correlation analysis), the correlation between inoculum concentration and conidia production was -0.18, and when holding inoculum concentration constant, the correlation between cadavers weight and conidia produced per insect was 0.51. The standardized partial slopes for inoculum concentration and cadavers weight, which conidia production as a dependent variable, were $\beta = -0.16$ for inoculum concentration and $\beta = 0.52$ for weight. Cadaver weight is the overriding factor explaining conidia production (Fig. 2.2.A). Conidia production also increased with cadaver instar at death (stage: $p < 0.001$; df = 1, 141; $F = 39.89$; $r^2 = 0.23$) (Fig. 2.2.A).

TABLE 2.1

Relationship between *B. bassiana* concentrations applied to second instar Colorado potato beetle and mortality, sporulation, time to death, weight at death and conidia produced by cadavers.

Trial	Inoculum density conidia/mm^2	Prop. mortality observed[a]	Prop. sporulation from total dead[b]	Days to death[b]	Cadavers weight g[b]	Conidia/insect[b] (x 10^7 ± x 10^6)
1	0	0.54 ± 0.07	0			
	3	0.34 ± 0.02	0			
	11	0.60 ± 0.07	0.06 ± 0.03	9.00 ± 1.00	0.0348 ± 0.0062	3.19 ± 2.32
	18	0.32 ± 0.06	0.31 ± 0.06	6.00 ± 0.63	0.0137 ± 0.004	3.03 ± 7.94
	71	0.50 ± 0.07	0.44 ± 0.07	7.27 ± 0.43	0.0218 ± 0.0049	3.10 ± 3.73
	123	0.64 ± 0.07	0.64 ± 0.07	5.63 ± 0.38	0.0151 ± 0.0029	3.03 ± 4.54
2	0	0.14 ± 0.05	0			
	3	0.26 ± 0.07	0.46 ± 0.07	6.33 ± 0.49	0.0263 ± 0.0059	3.74 ± 5.00
	10	0.32 ± 0.06	0.37 ± 0.07	5.50 ± 0.43	0.0259 ± 0.005	4.06 ± 7.13
	27	0.70 ± 0.06	0.77 ± 0.06	5.66 ± 0.17	0.0219 ± 0.0023	3.09 ± 2.85
	67	0.76 ± 0.06	0.55 ± 0.07	5.48 ± 0.23	0.0224 ± 0.0029	3.12 ± 4.86
	114	0.98 ± 0.02	0.94 ± 0.03	4.00 ± 0.10	0.0091 ± 0.0006	1.34 ± 2.40

[a] Proportion mortality (± **SE)** observed in both trials (means not corrected for control mortality)
[b] Mean ± **SE**

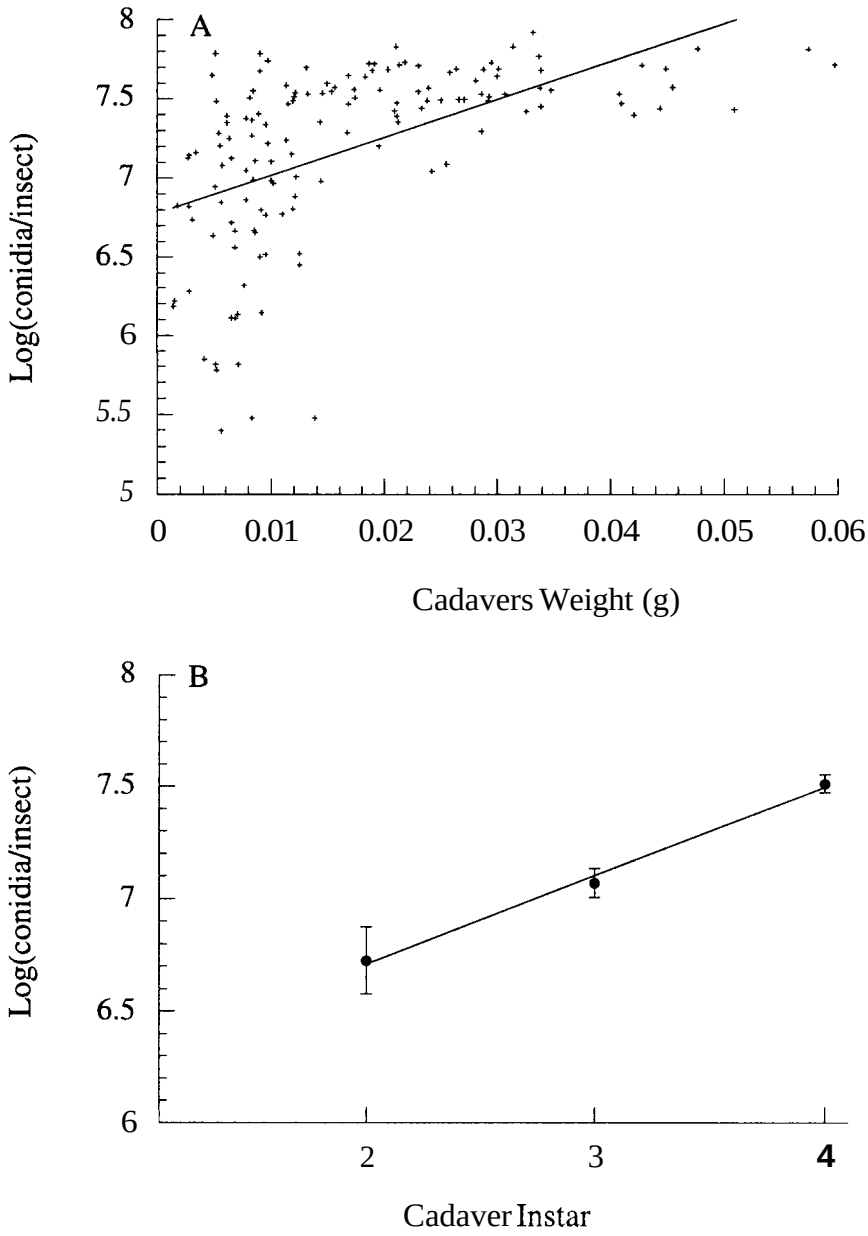

Figure 2.2. Relationship between cadaver size and conidia produced per insect from cadavers of ***B. bassiana*** treated second instar Colorado potato beetle. **A)** Cadaver's weight (g) (linear fit trial 1: $\mathbf{Y} = 6.78 + 23.85X$; $r^2 = 0.27$). B) Stage at death (linear fit: $\mathbf{Y} = 5.92 + 0.39X$; $r^2 = 0.99$). Analysis was conducted with individual observations; graph depicts curve fit to the means.

Stage at Time of Infection

A significant increase in mortality and sporulation of cadavers was observed with increasing instar at inoculation time ($p = 0.031$; $df = 1,700$; $\chi^2 = 8.84$ for mortality and $p = 0.023$; $df = 1,561$; $\chi^2 = 9.55$ for sporulation) (Table 2.2). Time to death, larval weight and stage at death increased with increasing stage at time of inoculation (Table 2.2). The larger instars took longer to sporulate than the smaller instars. Yet more conidia were produced from inoculations during the early stadia than those inoculated during the later stadia ($p < 0.001$; $df = 3,406$; $F = 6.02$; Table 2.2). Within each instar, conidia production per insect increased with cadaver weight (Fig. 2.3). Total conidia production decreased over time when cadavers were incubated for up to 32 days ($p < 0.001$; $df = 1,398$; $F = 44.10$; $r^2 = 0.13$).

In the second series of experiments where conidia production was assessed at a single incubation time, proportion mortality ranged from 0.5 ± 0.07 (mean $\pm$ se) for second instars in trial five to 1.0 for second, third and fourth instars in trial two, three, and four, respectively. The proportion mortality did not differ between instars within a trial except in trial 5 where treated seconds instars experienced lower mortality than fourth instars ($p < 0.002$; $df = 1, 60$; $\chi^2 = 9.71$) (Table 2.3). The proportion of sporulating cadavers ranged from 0.6 ± 0.1 for first instars in trial one to 0.96 ± 0.03 for fourth instars in trial four (Table 2.3). Only in trial four was there a significantly difference in the proportion sporulating cadavers between fourth and second instars. Conidia production by first instars was not significantly different than seconds ($p = 0.59$). However second instars produced significantly more conidia than third instars in both trials ($p = 0.02$; $df = 1, 140$; $F = 5.77$ for trial 2 and $p = 0.009$; $df = 1, 49$; $F = 7.25$ for trial 3) and fourth instars in trial **4** ($p < 0.001$; $df = 1, 91$; $F = 11.91$). In trial 5 no differences in conidia production were detected between second and fourth instars ($p = 0.67$) perhaps due to the low number of second instars observed (Table 2.3). Dose did not have a significant effect on conidia production for third or fourth instars ($p = 13$ for thirds and $p = 0.48$ for fourths).

TABLE 2.2

Relationship between stage of Colorado potato beetle at time of inoculation with ***B.*** *bassiana* and days to death, cadaver weight, stage at death, days from death to first sign of sporulation and conidia produced by cadavers.

Stage[a]	***N***	prop. mortality[b]	Prop. sporulation[bc]	Days to death[d]	Cadaver weight $x10^{-2}$ g[b]	Stage at death[b]	Days to sporulation[d]	Conidia/insect[d] (x 10^7 ± $x10^6$)
1	84	0.79f0.04	0.77 ± 0.06	5.62 ± 0.25ab	0.64 ± 0.06	2.14 ± 0.08	1.05± 0.08a	1.29± 1.06a
2	91	0.73 ± 0.09	0.91 ± 0.04	4.04 ± 0.23a	1.06± 0.10	2.78 ± 0.04	1.32± 0.08a	1.28± 1.10a
3	104	0.81 ± 0.03	0.85 ± 0.05	6.37 ± 0.29b	2.56 ± 0.19	3.73 ± 0.04	2.67 ± 0.18b	1.22 ± 1.76b
4	129	0.85 ± 0.05	0.93 ± 0.02	8.08 ± 0.22c	6.38 ± 0.30	4	2.88 ± 0.18b	1.02 ± 0.99b

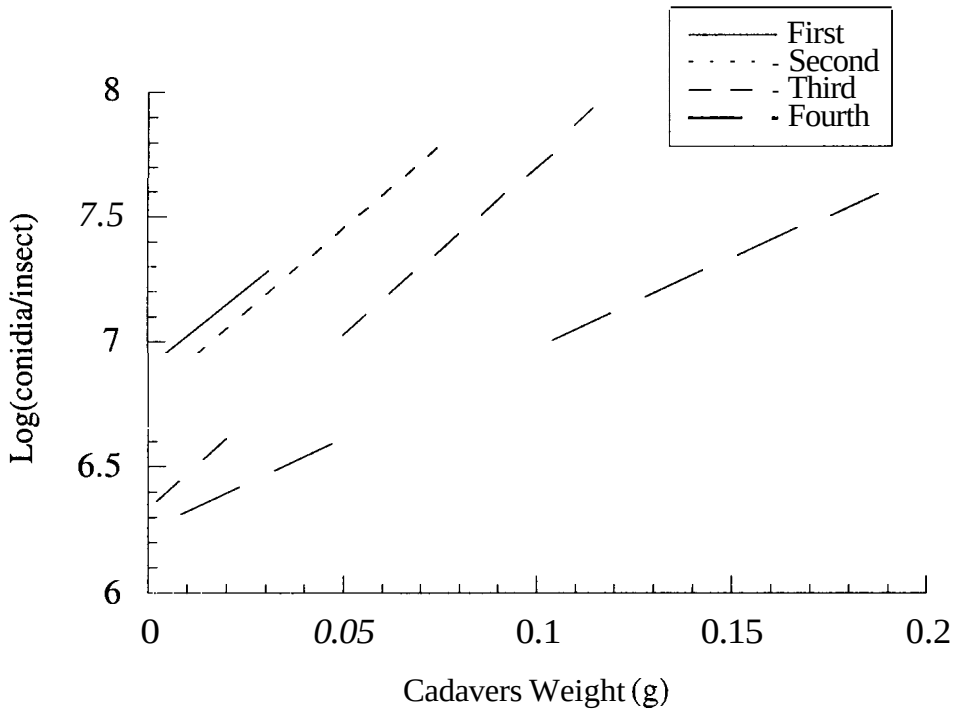

Figure 2.3. Relationship between Colorado potato beetle larval cadavers weight (grams) following treatment with ***B. bassiana*** conidia suspensions and conidia produced per cadaver (expressed as log(conidia/insect)) for each instar. Graph depicts linear fit of second degree to the mean log(conidia/insect) for each instar at inoculation time: first instar: $\mathbf{Y} = 6.86 + 18.81X$; $r^2 = 0.26$; second instar: $Y = 6.78 + 13.34X$; $r^2 = 0.29$; third instar: $\mathbf{Y} = 6.33 + 13.87X$; $r^2 = 0.41$; and fourth instar: $\mathbf{Y} = 6.24 + 7.28X$; $r^2 = 0.44$.

TABLE2.3

B. bassiana conidia production from Colorado potato beetle larvae treated at first, third and fourth instar, with each instar compared to a second instar standard. First, third and fourth instar were inoculated with *B. bassiana* concentrations ranging between 2.0 to 836.8 conidi/mm^2. Second instars were inoculated with a single concentration of *B. bassiana* ranging between 49.6 to 65.3 conidia/mm^2.

Experimental trial	Stage at inoculation time	*N*	Proportion mortality"	Proportion sporulation[ab]	Conidia per insect' $1 \times 10^6 \pm 1 \times 10^5$
1	L1	83	0.83 ± 0.07 a	0.60 ± 0.10 a	7.87 ± 11.00 a
1	L2	25	0.86 ± 0.06 a	0.85 ± 0.07 a	5.89f9.20 a
2	L2	22	1 a	0.83 ± 0.07 a	10.86± 20.28 a
2	L3	119	0.96 ± 0.03 a	0.69 ± 0.09 a	7.70 ± 8.46 b
3	L2	27	1 a	0.90 ± 0.05 a	28.05 ± 80.72 a
3	L3	36	1 a	0.70 ± 0.08 a	13.84± 26.59 b
4	L2	21	0.96 ± 0.04 a	0.76 ± 0.08 a	7.65 ± 10.07 a
4	L4	72	1 a	0.96 ± 0.03 b	3.42 ± 4.34 b
5	L2	5	0.5 ± 0.09 a	0.73 ± 0.11 a	132.86± 772.4 a
5	L4	32	0.97 ± 0.03 b	0.89 ± 0.06 a	118.55± 331.9 a

[a] Letters correspond to nominal logistic analysis comparing proportion mortality and sporulation between the two instars per trial.
[b] Proportion of cadavers sporulating.
[c] Mean ± **SE.** Letters correspond to ANOVA means comparison using Student t-test, significant difference $\alpha < 0.05$ for each repetition.

Discussion

Factors influencing mortality in **CPB** larvae treated with ***B.*** *bassiana* may deferentially influence the quantity of conidia produced by cadavers. **As** expected, and reported by others, proportion mortality increased with inoculum concentration (Fargues, **1972;** Ignoffo et al., **1983)** as did the proportion of cadavers which sporulated. However, time post infection and size of cadavers in most trials influenced the number of ***B.*** *bassiana* conidia produced by **CPB** larval cadavers. Conidia production decreased as time between death and first signs of fungal growth increased. This period between death and the initiation of the fungus' sporulation phase may be critical for ***B.*** *bassiana* competing with other microbes for the availability of nutrients provided by the cadaver (Woodring et al., **1995).** The delay in sporulation could also be caused by the death of the host before the fungus had the opportunity to ramify throughout the body (Woodring et al., **1995).** In contrast, in the **1995** and **1996** experiments, conidia production by cadavers increased over time following the initiation of external fungal growth. Luz and Fargues **(1998)** also observe an increase in ***B.*** *bassiana* conidia production on cadavers of infected first instar ***R.*** *prolixus* from 2 to **4** days of incubation at **25°C** and **97%RH** after which production does not significantly increase for the remaining time of observation (up to 20 days). In our study, production appeared to increase more slowly over time in the **1996** trial where production was evaluated for **21** days than in the **1995** trials. However, in **1996,** production of conidia on the initial sample dates at days **4** and **6** after the first sign of sporulation was already ca. **50%** greater **(2.17×10^7** conididinsect) than that observed at this time in the **1995** experiments (conidia production at day **4-5** was ca. **1.4** x 10^7 conididinsect).

Size of cadavers, whether expressed as stage at death or weight at death, had a significant impact on conidia production in all experiments except the **1995** trials. However, in the experiment conducted in **1996,** there were only **17** insects that died as third instars compared with **181** that died as second instars. In the experiments evaluating the impact of initial ***B.*** *bassiana* concentrations on conidia production there was a slight, direct, negative

effect of concentration, but cadaver weight had a greater impact on the number of conidia produced per cadaver. Lower conidia concentrations were less effective and increased the time the inoculated insect remained alive, thereby increasing weight at time of death, and consequently increasing the production of conidia. This same trend of increase in conidia production with increasing size was observed in the experiment where insects were inoculated at different stages. For each instar an increase in days from inoculation to death, for the first 8 days, resulted in an increase in the cadaver weight and a subsequent increase in conidia production within each inoculated instar. Larger cadavers probably result in greater amounts of nutrients for the fungus, as well as a larger surface area from which the fungus can ramify.

Luz and Fargues **(1998)** observed an increase in ***B.*** *bassiana* conidia production with increasing instar of *Rhodnius prolixus* exposed to a single dose. Fifth instar cadavers produced on average 68% more conidia than third instar. Contrary to what was expected, in this study, mortality of CPB larvae exposed to the same ***B.*** *bassiana* concentrations increased or did not differ with instar at inoculation and larvae that were treated as third and fourth instars, produced significantly fewer conidia than larvae inoculated as first and second instars. Other researchers have shown increases in susceptibility to infection by ***B.*** *bassiana* and other fungi with later life stages at inoculation for several insect species (Mohamed et al., **1977;** Feng et al., **1985;** Glare and Milner, **1991;** Glare, **1994;** Vandenberg et al., **1998).** It may be possible that at equal dosages of conidia/mm^2, larger instars receive a much higher dose per insect because of their larger surface area. However, our observations and others on increased time to death (Fargues et al., **1972)** and over conidia production with increasing larval stadium suggest that fungal growth is somehow limited. **As** such, the fungus is less able to exploit the greater resources provided by the larger instars. Yet, conidia production increased with the cadaver's weight at the time of death within each initially treated instar, the larger quantity of conidia produced by early

(first and second) compared with later (third and fourth) instars might result from different immune responses during the initial stages of infection.

Immune responses of CPB infected with ***B. bassiana*** have only been compared in third and fourth instars (Seryczynska and Bajan, 1974), both of which have lower hemocyte counts than that observed in untreated controls. However, the observed density of hemocytes was higher in the third instars than the fourth instars, leading the authors to suggest that third instars may have a much more developed immune system than fourth instars. This limited evidence, and the mortality data does not indicate that a stronger immune response in larger versus smaller instars might account for the differential success in fungal growth.

However, differences in feeding rates between CPB instars may impact fungal growth. The third and fourth instars are responsible for the majority of total food consumption (94%) by immature CPB (Logan et al., 1985). Potato foliage is high in glycoalkaloids (Friedman and McDonald, 1997) and greater consumption of foliage will result in a larger intake of glycoalkaloids. It is not known if consumption of glycoalkaloids results in their incorporation into insect tissue, but glycoalkaloids do limit ***B. bassiana*** growth (Costa and Gougler, 1986b). If these toxic secondary metabolites from the foliage are incorporated into CPB tissue, the tissue of the larger instars may provide a poorer growth medium for the fungus. Also when the fungus invades the cuticular lining of the midgut, the gut contents are released into the body cavity. Again, this would be greater in the larger instars and could be a source for toxic secondary metabolites, as well as, bacteria which could interfere with growth of ***B. bassiana.***

All factors investigated in this paper were responsible for a range of production from 1.25×10^4 to 8.7×10^8 conidia/insect. Host size (stage at inoculation and weight of cadaver) was highly correlated with production. Pathogen inoculation dose had a slight negative impact on production, but more importantly, directly affected the weight of the cadavers. Lower doses resulted in longer survival times and consequently the larger, heavier

cadavers that resulted produced greater amounts of conidia per cadaver. However, as pathogen dose increased, so did proportion mortality and the proportion of cadavers which sporulated. Taking these data into account, **a** greater overall production of conidia for persistence and recycling of the disease would result from a mid (trial 2) to a high (trial 1) inoculum dose. In contrast, the increase in mortality and proportion sporulation observed with increasing instar at infection is balanced by the greater number of conidia produced per cadaver when infected during the first and second stadium. Given the longer times to death from infection and the high consumption ratio and damage potential of larger larvae, treatments of ***B. bassiana*** conidia targeted against smaller **CPB** larvae will not only optimize immediate pest reduction, but optimize production of secondary inoculum. Based on the modeling results of Long et al. (2000b), targeting foliar treatments against small larvae also optimizes timing of encounters between sporulating cadavers and susceptible prepupa, and hence represent the best strategy for managing this pest with ***B. bassiana***.

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MANUSCRIPT 3

ENVIRONMENTAL FACTORS IMPACTING CONIDIAL PRODUCTION BY *BEAUVERZA BASSZANA* (BALS.) VUILL. INFECTED COLORADO POTATO BEETLE LARVAE

Introduction

The impact of environmental conditions in insect-fungal disease interactions has been well reported. Temperature, humidity, light intensity and quality, soil and rainfall, among others, are factors that influence: survival, germination, infection, reproduction and propagation of entomopathogenic fungi (Glare et al., 1986; Krueger et al., 1991; Moore et al., 1996; Oduor et al., 1996; James et al., 1998; Luz and Fargues, 1998; De Croos and Bidochka, 1999; Hallsworth and Magan, 1999; Inglis et al., 1999; Inglis et al., 2000), effectiveness of host defenses (Blanford and Thomas, 1999), and disease incidence in insect populations (epizootics) (Hayek et al., 1993; Giles et al., 1994). Natural fungal epizootics occur when certain environmental conditions are meet. Giles et al. (1994) observes that epizootics of *Zoophthora phytonomi* in *Hypera postica* populations are associated with above average rainfall. Models that simulate insects epizootics often include weather data that trigger or regulate these dynamics (Carruthers, et al., 1985; Weseloh et al., 1993; Hajek et al., 1993; Long et al., 2000a; Joergensen, 2001).

The information available on environmental factors, cultural practices and formulation affecting survival of *Beauveria bassiana,* a naturally occurring pathogen of Colorado potato beetle, and other entomopathogenic Deuteromycetes in the soil (Gaugler et al., 1989; Storey et al., 1989; Studdert and Kaya, 1990; Groden and Lockwood, 1991) and in the plant canopy (Inglis et al., 1993; Inglis et al., 1995; James et al., 1995; Inglis et al., 2000; Joergensen et al., 2001) is plentiful. The effects of temperature and humidity on the efficacy of the organisms for microbial control of pests have also been studied (Carruthers et al., 1985; Rath et al., 1995; Hegedus and Khachatourians, 1996; James et al, 1998;

Vandenberg, et al, 1998; Inglis, et al., 1999). Other studies have focused on environmental factors affecting germination, growth and survivorship of the fungus *in vivo* and *in vitro.* Research on conidia production by *B. bassiana* has concentrated mainly on the performance of the fungus on artificial media (James et al., 1998; Hallsworth and Magan, 1999). Information regarding quantity of conidia produced per infected host is scarce, but is potentially important for understanding transmission of the disease (Long et al., 2000b).

Luz and Fargues (1998) investigated production of ***B.*** *bassiana* conidia by cadavers of *Rhodnius prolixus* over a range of environmental conditions. They found that conidia production by cadavers is optimal at 25ºC, with humidity levels higher than 96.5%, and it increased with cadaver size. However, Ramoska (1984) reports that cadavers of *B. bassiana* infected chinch bugs, *Blissus leucopterus,* are able to produce conidia at humidities as low as 75%.

Understanding the environmental requirements for pathogen replication in the host population is essential for maximizing the potential for epizootics. This study explores the effects of temperature, relative humidity and the presence of soil on conidia production by CPB larval cadavers resulting from *B. bassiana* infections.

Materials and Methods

Temperature Experiments

The effect of temperature on sporulation and conidia production by cadavers was assessed in three trials. Temperatures and inoculation procedures differed in each trial.

Conidia suspensions were prepared and second instar CPB were sprayed with the Burkard computerized spray apparatus, as described in Manuscript 2. In trial 1,400 insects were sprayed with a density of 146 conidia/mm^2, and inoculated insects were held individually in 3 cm diameter petri dishes and fed daily. In trial 2 and 3, approximately 1,500 second instar per trial were sprayed with 214 and 198 conidia/mm^2 respectively. One hundred and fifty to 200 inoculated insects were held per plastic container with fresh foliage and moist paper towels in 42 x 29 x 15 cm plastic containers. Plastic containers were covered with 4 layers of cheesecloth to permit airflow yet prevent insects from escaping.

For all three trials, sprayed insects were held at 25ºC, 68-70% RH and a 16:8 h light:dark cycle and fed greenhouse foliage daily. Upon death, cadavers were placed individually in 10 x 75 mm test tubes enclosed in 100% humidity chambers. Humidity chambers containing cadavers were held in environmental chambers set at the following temperatures: trial 1: 15, 20, 25, and 30°C; trial 2: 2, 7, 14, 20, 25, 30, 35 and 40°C; and Trial 3: 4, 7, 14, 20, 25, 32, 34 and 36°C. Only cadavers produced on the fourth day after inoculation were used in trials 2 and 3. The number of cadavers per temperature treatment ranged from 78 to 91 for trial 1; 84 to 89 for trial 2 and 81 to 85 for trial 3.

Sporulation of cadavers was monitored daily and 14 to 21 mummified cadavers were randomly sampled at 6, 12, 18 and 24 days from the first sign of sporulation in trial 1. Two to 23 mummified cadavers were randomly sampled at 5, 10, 20, 40 and 60 days from first sign of sporulation in trial 2 and 3 (lower sampling numbers correspond to temperatures between 2-5ºC and 35-40ºC). At each sampling day, 1 ml of 0.1% Tween 20® was added to each test tube containing a cadaver. Cadavers were macerated and the solution vortexed for one to two minutes. If the conidia solution obtained was too concentrated for microscopic

observation, it was diluted prior to quantification. Resulting conidia concentrations were as determined with a hemacytometer by averaging the conidia counts of 5 randomly chosen 0.2 mm squares subdivisions within a microscope field. The number of conidia produced per insect was calculated by multiplying by the dilution factor.

Ambient Relative Humidity Experiments

Chambers with relative humidities of 0 to 100% were established by adding the following saturated salt solutions to sealed glass desicators held at 25°C: Na_2SO_4 (Hammond, W. A. Drierite Co., Ltd. P.O. Box 460, Xenia, OH 45385) for 0%, $MnCl_2$ for 56%, NaCl for 75%, $(NH_4)_2SO_4$ for 80%, KCl for 85%, $MgSO_4$ for 89%, $NaHPO_4$ for 95% (Sigma Chemical Corporation, 630 Utah Av. P.O. Box 14508, St Louis, MO 63178) (Winston and Bates, 1960) and distilled water for 100%. Conidia suspensions were prepared and insects were inoculated with 222 conidia/mm^2 using a Burkard® computer controlled spraying apparatus as described previously.

Sprayed insects were placed individually in 3 cm diameter petri dishes and kept in environmental chambers at 25°C, 68-70% RH and 16:8 h light:dark until their death. Upon death, foliage was removed and insects were weighed. Dishes with cadavers were randomly allocated to the humidity treatments. Between 50 and 55 cadavers were placed in each of the eight humidity chambers. Cadavers were monitored daily for signs of sporulation. Mummified cadavers were randomly sampled 6, 12, 18, and 24 days from the first sign of sporulation. Conidia per cadaver were assessed as previously described. Cadavers that did not sporulate after 32 days were transferred from their original humidity chamber to a chamber with 100% RH. Cadavers were then monitored for 14 days for signs of sporulation.

Soil Effects on Conidia Production

Fungal material, insects and conidia solutions were obtained as described previously. Caribou gravelly loam (fine loamy, mixed, frigid, Typic Haplorthods) soil was collected from the University of Maine Potato Research Farm in Presque Isle, Maine. This soil contained 29.7% sand and 28.35% moisture at field capacity (Long et al., 2000a). Soil was sieved though a 2 mm mesh (U. S. Standard Sieve Series #10) and 10g portions were placed in 22.17 ml paper cups (American Convenience Products, Milwaukee, WI 53212) for the experiment.

A total of 400 second instars were treated in groups of 50 larvae per 9 cm diameter petri dish. Dishes containing insects were sprayed with 80 µl *B. bassiana* conidia suspension using a Paasche airbrush sprayer (Paasche Airbrush Co., Hartwood Heights, IL) resulting in 233 conidia/mm^2. Sprayed insects were held individually in 3 cm diameter petri dishes and fed potato foliage daily. Upon death the following was measured and recorded: days to death, stage and weight at death.

Cadavers were randomly placed individually in one of the following treatments: 1) soil surface (n = 71); 2) buried in soil at a depth of ca. 0.5 cm (n = 67); and 3) petri dishes (3 cm diameter) without soil (n = 133). Cups of soil and petri dishes with cadavers were held at 100% relative humidity and 25°C. Conidia produced by cadavers held without soil were assessed either directly or indirectly. Directly assessment entailed vortexing cadavers in 10 ml of 0.1% Tween 20' and counting conidia using a hemacytometer as described previously (n = 69). Indirect assessment entailed adding 10 g of soil at the time of sampling and determining conidia production by drop plating soil on selective media as described below (n = 64). Conidia per cadaver was sampled at 6, 8, and 10 days from first sign of sporulation as described previously.

To evaluate the conidia produced by cadavers exposed to soil, and cadavers incubated with no soil but soil added at sampling time, the contents of each paper cup, containing a sporulated cadaver plus soil, were blended in 200 ml of 0.1 % Tween 20'

solution. The blended solution was diluted twice in a series of ten fold dilutions. Two replications per dilution were plated. For each replication a 0.4 ml aliquot of the soil solution was pipetted into a 9 cm petri dish containing approximately 30 ml of wheat germ selective media (Sneh, 1991). Dishes were incubated for 9 days in the dark at laboratory ambient conditions (20-23°C and 50-75%RH). The density of ***B.*** *bassiana* colony forming units (CFUs) growing on plates of selective media was observed under a dissecting microscope at 5, 7 and 9 days after the inoculation of media. In order to account for the possible presence of ***B.*** *bassiana* conidia in the soil used in this experiment, a 10 g sample of soil with no cadavers was added to 200 ml of 0.1% Tween 20® solution, plated and incubated as described above. Direct hemacytometer counts of conidia per cadaver from insects held without soil were compared with the number of CFUs recovered when these cadavers were mixed with a soil solution and plated on selective media. This comparison was used to estimate conidia per cadaver from CFUs recovered from cadavers incubated with soil.

Data Analysis

Least square regression and nominal logistic regression (JMP, **SAS** Institute Inc., **SAS** Campus Drive, Cary, NC 27513) was used to test for significant linear relationships between environmental parameters (temperature, relative humidity, and soil treatment) and proportion sporulation and conidia produced by cadavers. Because of the limitation on number of temperature and humidity chambers, we will assume no chamber effect so each cadaver held in its test tube will be considered an independent observation or replicate. Proportion sporulation was transformed by the arcsin square root of and weighted by the number of cadavers per treatment for the temperature experiment. Nominal logistic analysis was conducted to detect relative humidity and soil effects on sporulation.

Relationships between host parameters (weight and stage at death, days to death, days to sporulation and mummified cadavers incubation in time) and conidia production by

cadavers was also investigated. Prior to analysis, data were transformed to the logarithm (based 10) of the number of conidia produced per insect. When analyzing for effects of days to death on cadaver weight, cadaver weight (g) was transformed with a logarithm function. For the analyses of the time to sporulation of cadavers transferred from lower humidity levels to 100%RH the data was transformed to the square root of days from death to first sign of sporulation.

Results

Temperature

Temperature significantly influenced the proportion of sporulated cadavers ($p < 0.001$; $df = 2, 1723$; $F = 57.85$) (Fig. 3.1). Proportion sporulation did not differ significantly between 7 and 33°C ($p = 0.50$). Above and below this range, the proportion of sporulating cadavers dropped rapidly to 0.04 at 2°C and 0.05 at 36°C. No cadavers sporulated at 40°C. As only four out of 84 individuals at the lowest temperature (2°C) sporulated, these data were removed from the data set prior to analyzing the effect of temperature on time to sporulation (but mean time to sporulation for the four cadaver was 1.5 days). Time to sporulation decreased with increasing temperature from 6.6 days at 4°C and 2 days at 15°C to a minimum of 1 day at 25°C, and increased to 2 days from 30 to 35°C. A significant nonlinear regression of temperature on time to sporulation was detected ($p = 0.001$; $df = 2, 18$; $F = 16.29$; $r^2 = 0.73$) (Fig. 3.2).

The amount of conidia produced per insect was significantly related to temperature ($p < 0.001$; $df = 2, 1129$; $F = 27.46$). The relationship is best described by a sigmoid curve with an upper boundary (Logan et al., 1976) (Fig. 3.3). Conidia production increased in the range from 2 to 25°C, but declined at temperatures of 30°C and higher. The curve fit predicts an upper limit of 43°C for conidia production, but the data indicates that the limit might be reached at 36-38°C.

The average number of conidia produced by cadavers was lowest at 5 to 6 days after first sign of sporulation at almost all temperature levels tested in all three trials. There was no difference in conidia production between 10 to 60 days after first sign of sporulation ($p = 0.44$). Only at 30°C in trial 1 was conidia production highest at day 5 after first sign of sporulation (interaction between temperature and incubation time: $p = 0.01$; $df = 1,1095$; $F = 6.27$; $r^2 = 0.37$). Conidia production increased significantly with stadium of cadaver at death ($p < 0.001$; $df = 1, 1095$; $F = 28.53$), and significantly decreased with days from death to sporulation ($p < 0.001$; $df = 1, 1095$; $F = 63.91$).

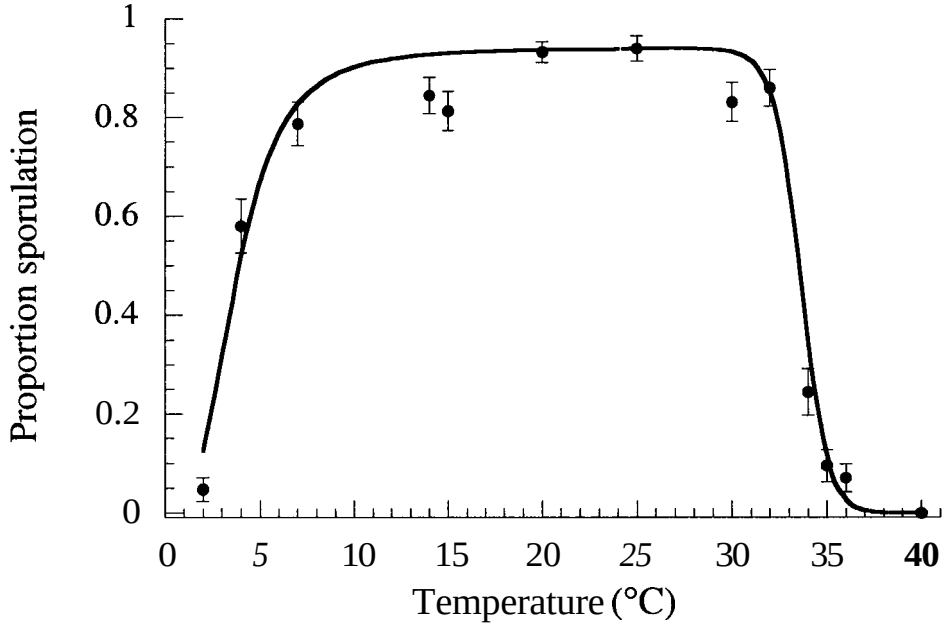

Figure 3.1. Proportion of sporulated cadavers from ***B. bassiana*** treated second instar Colorado potato beetle held at different temperatures. Analysis was conducted with individual observations: graph depicts curve fit to the means. Curve fit in two parts: ascending to 25°C: $Y = 0.94/[1 + e^{(35.91 - 32.07 X^{0.086})}]$, $r^2 = 0.94$; and descending from 25°C: $Y = 0.94/[1 + e^{(-20 \quad 0.0065 X^{3.2})}$ $(-0.74 + 0.$ $3,$ $r^2 = 0.98$.

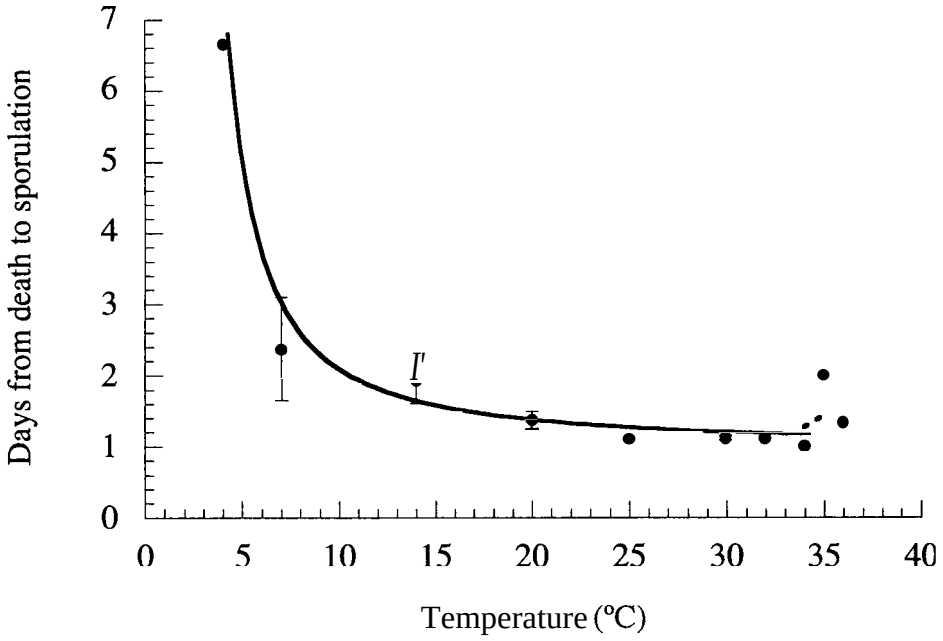

Figure 3.2. Days from death to sporulation for cadavers from ***B.*** *bassiana* treated second instar Colorado potato beetle held at different temperatures. Analysis was conducted with individual observations; graph depicts curve fit to the means (curve fit from 4'' to 34ºC: $Y = 0.9212 * e^{[0.0128/(0.00[illegible]X)]}$, $r^2 = 0.97$).

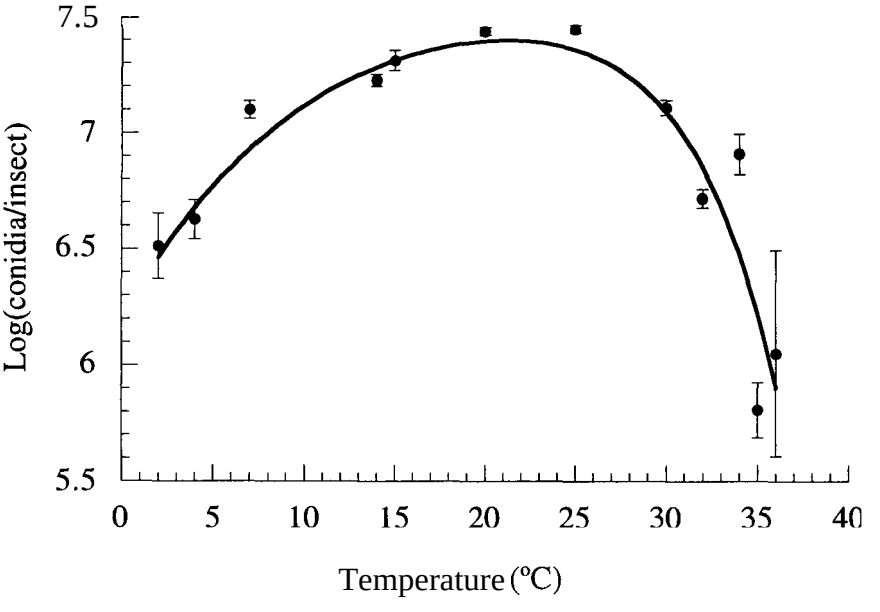

Figure 3.3. Production of conidia by cadavers from ***B. bassiana*** treated second instar Colorado potato beetle (expressed as log(conidia/insect)) held at different temperatures. Curve fit is a sigmoid curve with an upper boundary (Logan et al., 1976): $Y = 7.6(1/(1 + e^{(-1.5 - 0.12X)}) - e^{-(43.1 - X)/4.7)})$, with an $r^2 = 0.85$. Analysis was conducted with individual observations; graph depicts curve fit to the means.

Ambient Relative Humidity

Cadavers showed signs of mycelial growth only when held at humidities greater than 56% and only produced conidia at 89, 95 and 100% RH (Fig. 3.4). The proportion of cadavers that exhibited mycelial growth increased significantly with humidity ($p = 0.011$; $df = 1,432$; $\chi^2 = 13.43$; $r^2 = 0.64$) (Fig. 3.4.A). At 89% RH only two cadavers produced conidia, therefore the analysis of conidia production was limited to cadavers held at 95 and 100% RH (Fig. 3.4.B). Conidia production increased significantly with cadaver size, expressed as either stage or weight at death (stage at death: $p < 0.001$; $df = 1, 93$; $F = 17.0$, and weight: $p < 0.001$; $df = 1, 92$, $F = 20.56$).

Days from inoculation to death had no effect on conidia production ($p = 67$). Conidia production decreased at a faster rate with increasing time from death to the first sign of sporulation for cadavers held at 95% RH than for those held at 100% RH (interaction between RH and time to sporulation: $p < 0.001$; $df = 1, 89$; $F = 19.95$), and was significantly higher for cadavers held at 100% RH (Fig. 3.4.B). Conidia production significantly decreased over time between **6** and 24 days from the first sign of sporulation to sampling ($p < 0.005$; $df = 1, 89$; $F = 12.93$).

Cadavers that did not exhibit signs of mycelial growth at humidities lower than 95% for 32 days of exposure were then transferred to 100% RH. Initial humidity level significantly influenced the proportion of cadavers sporulating after their transfer to 100% RH ($p < 0.003$; $df = 1, 188$; $F = 9.05$). A higher proportion of those originally held at 0 and 85% sporulated when transfered than those originally held at the medium relative humidities of 56% (Fig. 3.5.A). The time to sporulation after the transfer also was less for cadavers initially held at 85 and 89% and the slowest by those initially held at 56% RH ($p < 0.001$; $df = 1, 122$, $F = 290.77$) (Fig. 3.5.B).

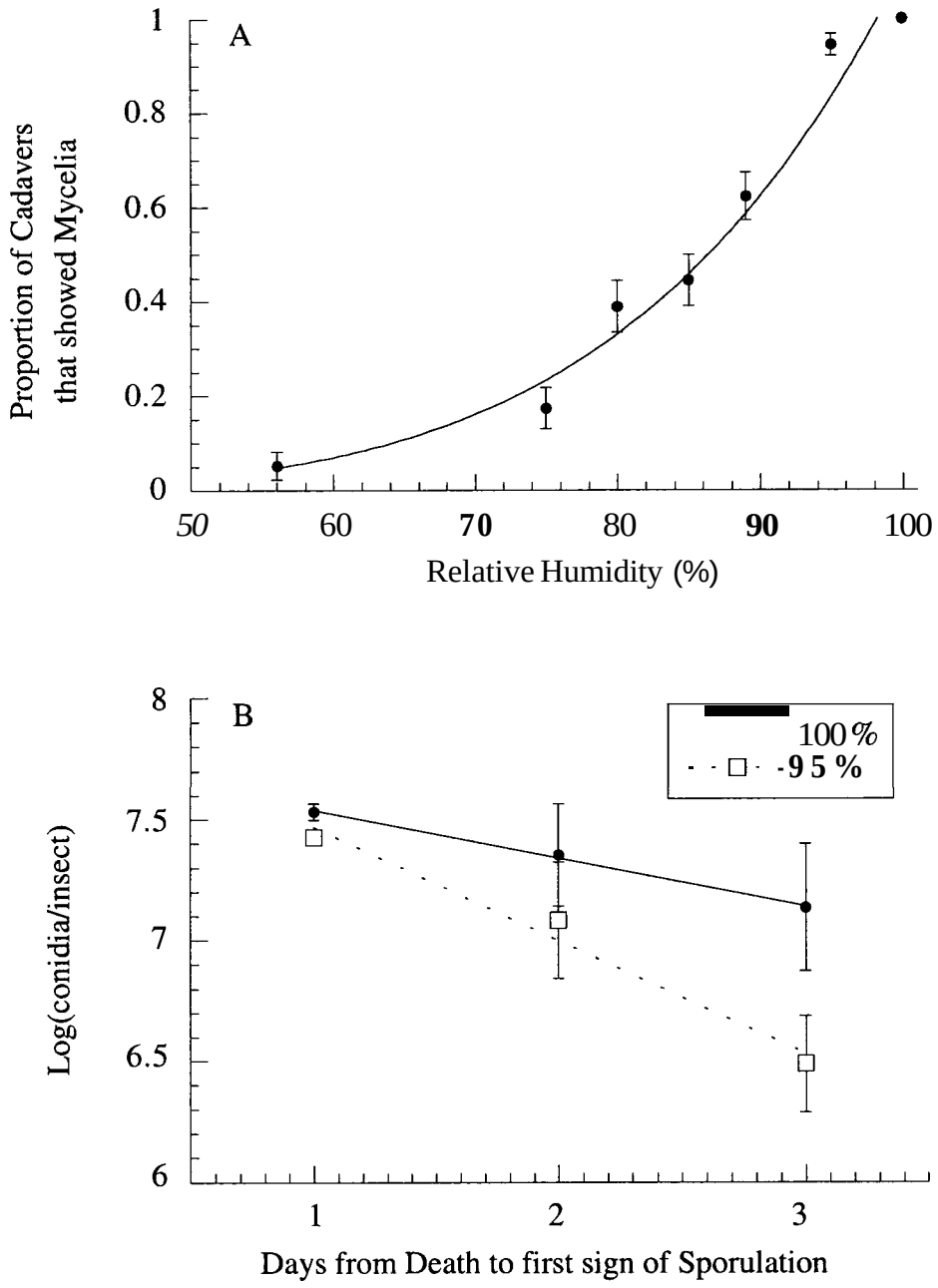

Figure **3.4.** Relationship between relative humidity and fungal growth on cadavers from ***B. bassiana*** treated second instar Colorado potato beetle. **A)** Proportion of cadavers that exhibited mycelia growth (power curve fit: $Y = 1.83 * e\text{-}11X^{5.39}$; $r^2 = 0.96$). **B)** Conidia production (log(conidia/insect) by cadavers held at **95** and 100%relative humidity (100% RH linear fit: $Y = 7.74 - 0.2X$; $r^{2=} = 0.99$; and **95%** RH linear fit: $Y = 7.94 - 0.47X$; $r^2 = 0.98$). Analysis was conducted with individual observations; graph depicts curve fit to the means.

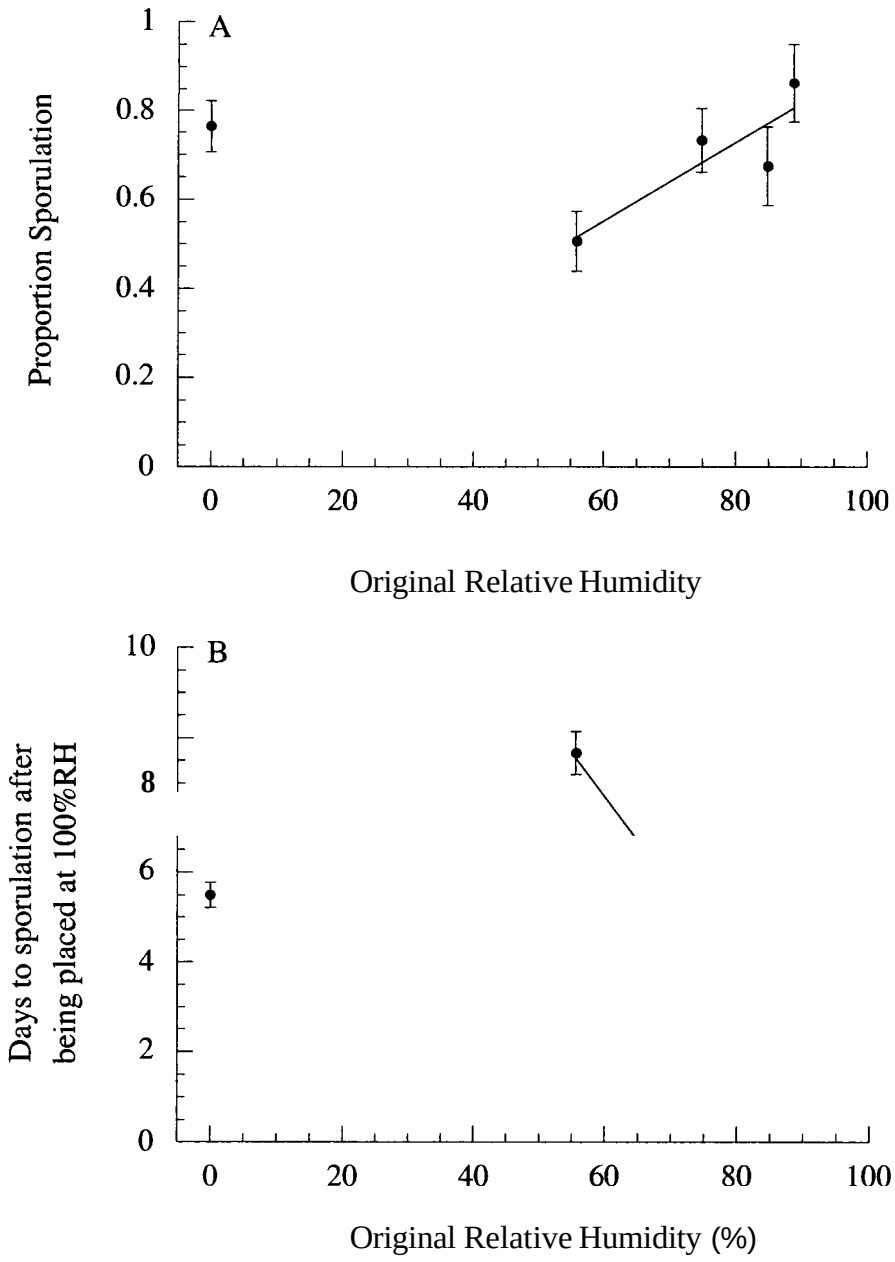

Figure 3.5. Sporulation by cadavers from ***B. bassiana*** treated second instar Colorado potato beetle held at humidities lower than 95% for 32 days and then transferred to 100% RH. **A)** Proportion of cadavers sporulating (curve fit: $\mathbf{Y} = 0.02 + 0.009X$; $r^2=0.88$). **B)** Days to sporulation (curve fit: $\mathbf{Y} = 19.5 - 0.19X$; $r^2 = 0.99$). Analysis was conducted with individual observations; graph depicts curve fit to the means.

Soil Presence

The presence of soil surrounding or beneath cadavers had a significant effect on the proportion of cadavers which sporulated ($p = 0.006$; $df = 1,375$; $\chi^2 = 7.42$). Cadavers in direct contact with soil sporulated at a higher proportion (0.85 ± 0.03) than those incubated without soil (0.74 ± 0.03). Conidia production by cadavers (measured as colony forming units (CFU's) recovered) also varied between treatments. Cadavers incubated with soil produced significantly more CFU's than those incubated without soil ($p < 0.001$; $df = 1, 190$; $F = 25.01$) (Table 3.1). There was no significant difference in proportion of sporulated cadavers or CFUs per cadaver between cadavers burrowed in the soil and cadavers incubated on the soil surface. Cadavers exposed to soil (either burrowed or incubated on the soil surface) produced on average 52% more CFUs than cadavers incubated without soil. Colony forming units per cadaver did not change with increasing time to death and sporulation of the cadavers burrowed or placed on the soil surface. However, for those cadavers incubated without soil no sporulation was observed one day after death, and CFU's decreased with increasing time to sporulation (interaction: $p = 0.040$; $df = 2, 190$; $F = 3.25$) (Fig. 3.6).

TABLE **3.1**

Estimated conidia production by cadavers from ***B.*** *bassiana* treated Colorado potato beetle second instar held in, on, and without soil. Conidia production was estimated by drop plating solutions of cadavers and soil onto plates **of** selective media and reading resulting colony forming units (CFUs).

Soil Treatment	*N*	Mean CFUs/cadaver $(x\ 10^7)^b$
Surface	70	4.78 a
Buried	66	5.49 **a**
No Soil"	60	2.68 b

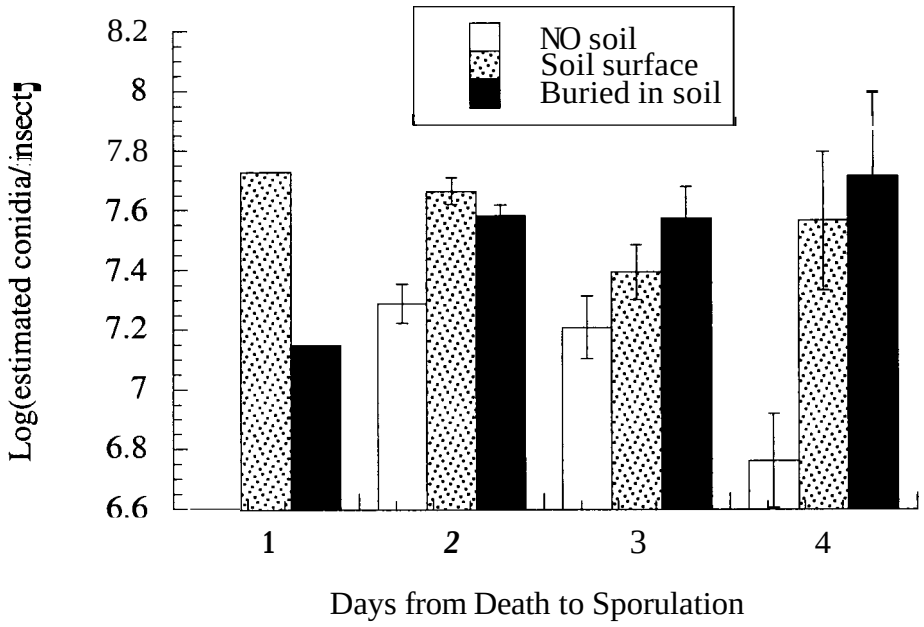

Figure 3.6. Conidia production over 4 days by cadavers from ***B. bassiana*** treated second instar Colorado potato beetle incubated under three soil treatments: **1)** No soil (soil added at time of plating), 2) cadavers placed on the soil surface, and 3) cadavers buried in soil. CFU/insects were adjusted by the difference between direct counts of conidia with the hemacytometer and CFU obtained by sporulated cadavers incubated without soil.

Discussion

Temperature has been determined to be the dominant abiotic factor affecting disease incubation period, and data describing temperature dependent effects on disease dynamics are essential for constructing a quantitative epizootiotic model (Carruthers et al., 1985, Long et al., 2000a). Germination, infection and survival of entomopathogenic Deuteromycetes are all dependent on temperature. *B. bassiana* is capable of germinating *in vitro* at a range of temperatures from 8 to 35°C, with an optimum at 25-32°C (Hallsworth and Magan, 1996; Fargues et al., 1997). Infection of CPB with *B. bassiana in vivo,* however, has been observed at a broader range of temperature levels from 0 to 40°C with an optimum range of infection of 20-30°C (Schaerffenberg, 1964; Fargues, 1972; Benz, 1987). We have found that similarly, sporulation of *B. bassiana* infected cadavers occurs over a broad range of temperatures.

Saprophytic development of the fungus was initiated within 1 to **2** days on a high percentage (> 70%) of CPB larval cadavers held between 7 and 33°C after death. Mycelial growth was observed on 58% of the cadavers held at 4°C, but was not visible until more than 6 days after death. Similarly, the rate of saprophytic growth on cadavers increased above 34°C. Four and 33°C may represent the thermal limits for saprophytic growth of *B. bassiana* on CPB cadavers, as signs of spomlation (mycelial growth) decreased rapidly below and above these temperatures respectively. *In vitro,* the lower and higher thermal threshold for *B. bassiana* growth is 2-4°C and 35-37°C respectively depending on the isolate (Fargues et al., 1997).

All of the cadavers from *B. bassiana* infected second instars which showed signs of saprophytic growth between 2 and 36°C produced conidia. Greater than ten million conidia per insect were observed between 8 and 28°C with the maximum production reached at 25°C. Luz and Fargues (1998) also found maximum *B. bassiana* conidia production by infected *Rhodnius prolixus* at temperatures between 20 and 25°C, after which production rapidly declined. However, Studdert and Kaya, (1990) found no difference in mean ***B.***

bassiana colony diameters in soil from Spodoptera exigua between 13 and 28ºC, although colonies expanded more rapidly between 20 and 28°C.

The optimal range of temperature levels for the production and germination of conidia (15-28°C) is also the range in which in vitro *dry* conidia lose viability faster (Alves et al., 1996; Jayaramaiah and Veeresh, 1984; Daoust and Roberts, 1983; Walstad et al., 1970). Synchronization between germination, faster development of the disease, and production of infective units at moderate temperatures may be an evolutionary strategy for which the fungus will remain dormant until optimum temperature is reached, therefore, aiding in the survival and spread of the fungus.

If temperature is the single most important factor for the development of entomopathogenic Deuteromycetes fungal diseases, relative humidity is the single most important limiting factor for the reproductive phase of these fungi. The infection and incubation phases of these fungal diseases are not affected by relative humidity (Ferron, 1977). *In* vitro requirements for ***B.*** bassiana conidia germination seem to be much higher than for in vivo, 97.5% compared to less than 50% relative humidity (Walstad et al., 1970; Fargues, 1972; Studdert and Kaya, 1990; Lingg and Donaldson, 1981; Ramoska, 1984; James et al., 1998). Infections at low humidities occur because ambient humidity may be much lower than the humidity experienced by a phytophagous insect in proximity of leaf surfaces, or from transpiring water through their cuticle (Ramoska, 1984; James et al., 1998). When the insect dies, the cadaver's exterior loses the moisture generated through respiration, creating a dependency on ambient humidity to trigger the formation of mycelia and therefore conidia.

Our results indicate an increase in the proportion of ***B.*** *bassiana*-killed insects showing mycelial growth with increasing humidity level from 55 to 100%. These results coincide with Ramoska (1984) who reports that mycelia grows from cadavers of ***B.*** bassiana-killed ***B.*** leucopterus at humidities between 75 and 100%, but not lower than 50%. Cadavers from the migratory grasshopper Melanoplus *sanguinipes* never exhibit mycelial

growth at humidities lower than 100% (Marcandier and Khachatourians, 1987). Conidia production requires at least 95% RH, and increases significantly at 100% RH. The effect of high humidity on production of conidia has been observed previously by Luz and Fargues (1998), who report a minimum of 96.5% for formation of conidia, and a maximum conidia production at 100% with ***B.*** bassiana-killed cadavers of ***R.*** *prolixus* incubated at 25°C. Studdert and Kaya (1990) were unable to observe conidiophores from cadavers of S. *exigua* buried in soil at water potential lower than the equivalent to 86% relative humidity.

Initially, intermediate humidities appeared to be detrimental for the development of the saprophytic phase of ***B.*** bassiana in our study. However, the fungus persisted within cadavers incubated at sub-optimal humidity conditions for sporulation, and when optimum conditions (100% RH) were provided, a considerable proportion of cadavers produced conidia. This fungal persistence and delayed conidia production may represent an important addition to the overall inoculum in the environment of the beetle population.

The amount of time for which cadavers are exposed to intermediate humidity levels before transfer to more favorable conditions for sporulation may also be important for the rate of sporulation. **A** lower proportion of the cadavers incubated at intermediate humidity levels (lower than 85%) for 32 days sporulated when transferred to 100% RH and those that did sporulate, took longer to do so. Marcandier and Khachatourians (1987) observe that only a very small fraction of cadavers of Melanoplus sanguinipes killed by ***B.*** bassiana produced mycelia when transferred to 100% RH after spending 3 months at humidity levels between 12 and 76%. The detrimental effect of intermediate humidity has been reported in several studies on survival of entomopathogenic Deuteromycetes (Teitell, 1958; Clerk and Madeline, 1965; Ferron, 1977; Daoust and Roberts, 1983; Jayaramaiah and Veeresh, 1984). The same requirements for humidity are observed in the literature for in *vitro* germination. Teitell (1958) argues that intermediate humidities (below the requirements for germination) change the metabolic activity of the conidia to the point where dormancy is lost, but the amount of moisture is insufficient to complete germination. **As** mentioned for temperature,

synchronization between germination and conidia production at high humidity levels may be an evolutionary strategy by which possibilities of secondary infection are increased when environmental conditions are favorable.

The effect of a soil substrate on sporulation and conidia production in cadavers is particularly relevant for the transmission of *B. bassiana* in **CPB** populations. In the field infected **CPB** larvae feeding on foliage die, fall to the ground and sporulate on the soil surface. **A** significant amount of transmission of *B. bassiana* in **CPB** populations occurs when late fourth instars descend from the potato canopy and wander on the soil surface searching for a suitable site to burrow and pupate (Long et al., 2000b). In the process, pre-pupae encounter sporulated cadavers and become infected. Gottwald and Tedders **(1984)** measured *B. bassiana* colonies growing a distance of up to 8.55 cm in diameter from infected pecan weevil cadavers. Our results indicate that insects exposed to soil produce ca. **48%** more conidia than insects that were incubated in the absence of soil. The soil used in these experiments had a high moist content (field capacity) and hence provided a good environment for sporulation. This may have contributed to the significant number of conidia produced in soil compared to no soil treatments This contradicts the findings of Pereira et al. **(1993)** who report the lack of *B. bassiana* conidia development from inoculated *Solenopsis invicta* exposed to non-sterile soil. **A** very common soil fungus, *Penicillium* sp., has been shown to produce metabolites detrimental to *B. bassiana* (Lingg and Donaldson, **1981;** Shields et al. **1981).** However, although *Penicillium* sp. have been isolated from soils in this study, the presence of soil appeared to enhance conidia production.

As noted by Gottwald and Tedders **(1984)** *B. bassiana* colonies from cadavers in soil were able to grow three-dimensionally, spreading through the soil. Mycelial growth on cadavers incubated in petri dishes remained in close proximity to the cadaver and spread almost insignificantly across the dish surface. Greater mycelial development may lead to greater conidia production. However, more extensive mycelial growth may also lead to an

over estimation of conidia production if mycelial fragments broken into small pieces during the blending and plating process produce colony forming units. It is not certain if these fragments would represent infection potential for insects. Krueger et al. **(1992)** found that the potential for metabolizing fragments of ***Metarhizium anisopliae*** produce infective conidia in soil, but clear evidence has not been produced for ***B. bassiana.***

This study points to the importance of environmental factors for conidia production. Relative humidity appears to be the limiting factor for sporulation. **A** specific threshold of relative humidity needs to be reached in order to trigger the production of conidia, whereas temperature has a progressive impact on conidia production. Cadavers produced conidia at a wide range of temperatures and in the presence of soil. The potential for disease transmission in the soil may be enhanced by elevating the level of soil humidity in the field with irrigation during synchronized periods of high cadaver density (following foliar applications of ***B. bassiana)*** and activity of susceptible life stages on the soil (prepupal burrowing **,** emergence of adults).

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MANUSCRIPT 4

THE SPORULATION AND VIABILITY OF *BEAUVERIA BASSZANA* (BALS.) VUILL. ON INFECTED CADAVERS IN POTATO FIELDS

Introduction

Insect pathogens are increasingly being considered as potential biological control agents of insect pests (Carruthers et al., 1999). While some fungal pathogens are known to cause widespread epizootics in nature (Hajek, 1997; McLeod et al., 1998; Steinkraus et al., 1998; Harcourt et al., 1990), most attempts to reproduce these insect disease outbreaks to control undesired insect populations have been unsuccessful (Carruthers and Soper, 1987; Hajek and Webb, 1999; Geden et al., 1993). These failures can in part be attributed to the lack of knowledge about the relationships between host, pathogen and the environment (McCoy et al., 1988; Carruthers et al., 1999).

Beauveria bassiana is an entomopathogenic fungus that has been proven effective in lowering Colorado potato beetle populations (CPB) (*Leptinotarsa decemlineata*) after foliar applications (Galani, 1984; Anderson et al., 1988; Drummond and Groden, 1996; Poprawski et al., 1997). As a naturally occurring disease agent the fungus may persist and replicate through secondary cycling in the pest populations. However, research on ***B.*** *bassiana* has focused on its potential as a bio-insecticide (Legaspi et al., 2000; Lacey et al., 1999; Drummond and Groden, 1996) and has involved the study of the virulence of certain isolates (Todorova et al., 2000; Jones et al., 1996; Varela and Morales, 1996) and of mass production and survival (Lane et al., 1991; Fargues et al., 1997; Inglis et al., 1995ab; Edington et al., 2000). Therefore, research on factors that affect disease transmission: conidia production by cadavers and persistence of both in the field will improve our understanding of ***B.*** *bassiana* potential as an effective biological control agent.

In the recent years more information on factors (host, pathogen and environmental) influencing in vivo production of conidia (Manuscripts 2, and 3 of this dissertation; Luz and

Fargues, 1998); transmission of the disease (Manuscript 5 of this dissertation; Long et al., 2000a; Gottwald and Tedders, 1984; Pereira and Stimac, 1992; Grace and Zoberi, 1992); and persistence of conidia (Daoust and Pereira, 1986) has become available. However, most of the studies of production and persistence of the pathogen have been conducted under controlled laboratory conditions. In the field, the environmental factors to which insects are exposed may differ substantially from those simulated in the laboratory. Thus, field validation studies of production and viability are necessary. This manuscript seeks to fulfill that aim by addressing sporulation and persistence of cadavers of ***B. bassiana*** killed CPB larvae under field conditions. In sum, the objectives of this paper are to study (1) conidia production on cadavers under field conditions, and **(2)** the viability over time of conidia generated by cadavers. These findings will be compared with those obtained from laboratory studies described in Manuscripts 2 and 3.

Materials and Methods

Arena Preparation and Shade Treatments

Potatoes, *Solanum* ***tuberosum*** cv. Katahdin, were started in 26 cm diameter plastic pots in a greenhouse. In mid June 1998, when plants were approximately 40 cm in height, they were transplanted 35 cm apart in a bare field at the University of Maine Potato Research Farm in Presque Isle, Maine. Circular sections of galvanized steel stove pipe (25 cm diameter and 12.5 cm in height) were pushed ca. ***5*** cm into the soil between the plants to create experimental arenas. Arenas were placed to establish the following shade treatments: 1) full potato canopy cover; 2) reduced canopy cover, where potato plants were trimmed to half their number of leaves; **3)** no cover, where arenas received no shade from plants; and 4) full artificial cover, where arenas were kept covered at all times with a white plastic potting bowl. The bottoms of the arenas were covered with nylon mesh (1 mm^2) to exclude ground beetles and the mesh was covered with **a** 2 cm thick layer of sieved soil (U. **S.** Standard Sieve Series #10, 2 mm^2 mesh). Six arenas per treatment were established for each of the three trials set up to measure conidia production and two arenas were established for each of three trials set up to monitor changes over time in viability of conidia produced by cadavers. In addition, eight arenas per treatment were established for a fourth trial of the conidia production experiment and four arenas for a fourth trial of the viability experiment, but only the full canopy cover and the no cover treatments were compared in this trial. The experimental design was a randomized block design with trial as a block.

Cadaver Preparation

CPB second instars were collected from a laboratory colony for infection with a conidia suspension of ***B.*** *bassiana* strain GHA (Mycotech Corporation, Butte MT). Dry conidia were harvested from colonies grown on Sabouraud Dextrose Agar (SDA) (Difco Laboratories, Detroit, MI) plates, and were suspended and vortexed in 0.1% Tween 20® (Sigma Chemical Company, P.O. Box 14508, St. Louis MO, 63178). Conidia

concentrations were adjusted following estimation with a hemacytometer (VWR Scientific, Boston, MA). Groups of 50 larvae were placed in 9 cm diameter petri dishes with moist filter paper and sprayed with 160 μl of the suspension using a Paasche airbrush sprayer (Paasche Airbrush Co., Hartwood Heights, IL). The conidia suspensions ranged from 2.08 x 10^8 to 3.55 x 10^8 conidia/ml and resulted in estimated sprayed densities ranging from 220 to 340 conidia/mm^2. Approximately two thousand larvae were sprayed per trial. Inoculated insects were held in petri dishes at 25°C, 68-70% RH, and a 16:8 h light:dark cycle. Insects were given fresh foliage daily and all cadavers found were removed and discarded for the first 3 days. Insects that died at four and five days after infection were more likely to sporulate and hence were used in the experiments.

Environmental Monitoring

Two arenas per treatment were established to monitor environmental conditions using two Campbell Scientific data loggers@. The environmental parameters monitored within the arenas for each treatment were: soil temperature at depth of 2 cm; air temperature and relative humidity; and solar radiation. Soil samples (approximately 5 g) were collected to a depth of 0.3-0.5 cm using a weighing spatula in two additional arenas per treatment three to eight times a day to gravimetrically determine percent moisture in the surface soil. For all the experimental arenas, leaf area index (amount of shading provided by the canopy over the arenas) was measured on overcast days throughout the experiment with a LAI-2000 Plant Canopy Analyzer (LI-COR Inc., Lincoln, Nebraska).

Sporulation and Conidia Production

Cadavers were placed at 5°C for up to two days prior to their release in the experimental arenas in order to prevent sporulation. Only mummified cadavers that did not show signs of sporulation were used in the experiments. Between 22 and 25 cadavers per

trial where placed on the surface of the sieved soil in each arena. A control group of cadavers was held in the laboratory in humidity chambers at 100% RH and 25°C.

Trials were conducted at weekly intervals between June 26 and August 7. In the first three trials the arenas were covered at night and during rainfall events with a 30 cm diameter white plastic bowl to prevent precipitation from reaching the cadavers. In the fourth trial, arenas were left uncovered for the duration of the experiment. Arenas were checked and the proportion of cadavers showing signs of sporulation (growth of the fungus on the cuticle) was recorded every 2-5 days during trials 3 and 4. The number of conidia produced per sporulating cadaver was sampled in all trials (1-4). In trial one, two cadavers per arena were sampled to assess conidia densities at 4, 8, 12, 18 and 24 days after placement of the cadavers in the arenas. In the remaining trials three cadavers per arena were sampled at 12, 18 and 24 days.

Cadavers were carefully removed from the field with soft forceps and individually placed into test tubes. Those with visible signs of sporulation at the time of collection were processed immediately by adding 1 ml of 0.1% Tween 20® to the tubes and vortexing for 1 min. Previous experiments demonstrated that one minute was sufficient for removal of all the conidia from the cadaver's cuticle. One drop of the suspension was then placed on a hemacytometer and conidia were counted in five 0.2 mm^2 squares to estimate the mean conidia per ml (cadaver). The cadavers that did not show signs of sporulation at time of field collection were placed in chambers in the laboratory set at 25°C and 100% RH. The density of conidia on the cadaver was assessed 12, 18, and 24 days after collection.

Viability of Conidia

Cadavers were held at 25°C in the laboratory in containers with moist sieved soil (2 mm^2 mesh) for 10 days prior their release into the experimental arenas. Between 22 and 25 sporulated cadavers per treatment were placed in each arena at the beginning of trials conducted at weekly intervals between July 3 and August 7, 1998. Conidia were sampled

from two randomly selected cadavers per arena following 1, 3, 6, 12 and 24 days of field exposure. Conidia were harvested from cadavers with a sterile metal loop and directly spread onto plates of SDA. Inoculated plates were incubated at 25°C for 24 hours, and then observed at 400X with a light microscope to assess the proportion of germinated conidia. Five to seven microscope fields were randomly selected and a total of ca. 25-50 conidia were counted per field. A conidia was considered germinated if the germ tube measured at least the diameter of the conidia.

Statistical Analysis

Two-way analyses of variance (ANOVAs) were used to test for shade treatment and time effects on environmental conditions, the proportion of cadavers sporulating over the sampling interval, the density of conidia produced by cadavers, and the change in viability of conidia over the sample interval. Then, for each sporulation response variable (proportion, density and viability), a series of multiple regressions (SAS Institute, 1994) was run using one measure each of moisturehumidity, temperature, and solar radiation to determine which combination of variables best predicted the response. Environmental variables were calculated over the interval of one sample date to the next and correlated with the response measured at the end of the interval. Variables evaluated for moisturehumidity included: average relative humidity, hours of relative humidities > 95%, total rainfall, and cumulative average, maximum and minimum percent soil moisture. Temperature variables included hours of ambient temperature > 33°C, hours of soil temperature > 33°C, cumulative ambient degree days (base 10°C) within the interval; and cumulative soil degree days (base 10°C). Prior to analysis, the data was transformed to the arcsin square root of the proportion of sporulating cadavers and to the logarithm of conidia per insect. Change in viability of conidia over the interval was expressed as a proportion of the viability at the beginning of the interval ((final proportion viable minus initial proportion viable) / initial proportion viable).

Results

Shade Treatment Effects on Sporulation. Conidia Production and Viability

Data for cadavers under a full canopy and under a reduced canopy were pooled into a single canopy treatment category because there were no significant differences in the leaf area index over the arenas ($p = 0.80$), in the proportion sporulation ($p = 0.8$), or in the proportion of viable conidia in the field ($p = 0.70$). With the pooled canopy treatment, neither shade treatment nor time of field exposure had a significant effect on proportion sporulation by CPB cadavers in the field ($p = 0.93$ for treatment and $p = 0.76$ for time of field exposure). **As** proportion sporulation was only measured over two trials, the sample size was low and variability was high. There was, however, a trend of lower sporulation in the cadavers with no cover compared to those shaded by the potato canopy or artificial covers. When a nominal logistic regression was conducted to examine shade treatment effects on the total proportion of cadavers that sporulated by the end of the experiment, a significant effect of shade treatment was detected ($p = 0.008$; $df = 1,423$, $\chi^2 = 9.66$). By the end of the experiment, a larger proportion of the cadavers under the potato canopy or artificially covered had sporulated than for cadavers without any cover. The maximum proportion of sporulated cadavers in any arena reached 0.93 on the last sampling date in an arena under the canopy. In the other two shade treatments the maximum proportion was 0.5 for an arena with artificial cover and **0.4** for an arena with no cover.

Shade treatment and time of field exposure, did significantly impact the quantity of conidia produced by cadavers on the soil ($p = 0.038$; $df = 1, 12$; $F = 4.32$ for treatment, and $p = 0.026$; $df = 1, 12$; $F = 6.40$ for time of field exposure) (Fig. 4.1). Shaded cadavers produced more conidia than unshaded cadavers, and conidia production increased over time for all treatments. Cadavers that did not show signs of sporulation in the field sporulated when they were brought into the laboratory and placed in favorable conditions for sporulation (25°C and 100% RH) regardless of shade treatment. Shade treatment and exposure time in the field had no effect on the quantity of conidia produced by these

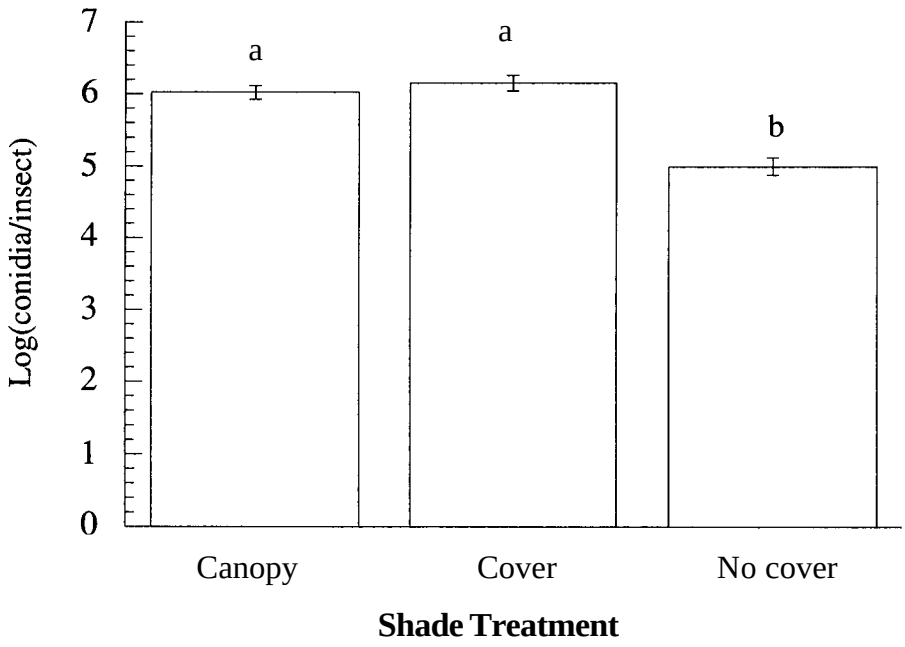

Figure **4.1.** ***Beauveria bassiana*** conidia produced by cadavers under different amounts of cover. Canopy: cadavers placed under the potato canopy; Cover: cadavers artificially covered; No cover: cadavers placed on bare soil. Letters correspond to ANOVA mean comparison using Tukey-Kramer **(HSD),** significant differences $a < 0.05$.

cadavers, and production by these field cadavers did not differ from that of control cadavers maintained at favorable conditions in the laboratory since death ($p = 0.67$ for treatment and $p = 0.34$ for exposure time to field conditions).

Cadavers that did not show signs of sporulation in the field, sporulated when they were brought into the laboratory and placed at 25°C and 100% RH, regardless of shade treatment. Shade treatment had no effect on the density of conidia produced by these cadavers ($p = 0.44$).

Change in the proportion of viable conidia on cadavers in the field did not vary between all trials ($p = 0.82$), or between treatments ($p = 0.38$), or through time of field exposure ($p = 0.19$) when examined across the entire experiment. However, time effect on viability were observed when each treatment was examined independently.

Viability of conidia declined significantly on cadavers under the canopy and on those without covers from July 4 through August 6 (for canopy $p = 0.006$; $df = 1, 31$; $F = 8.69$ and for uncovered $p = 0.073$; $df = 1, 24$; $F = 3.51$) (Fig. 4.2). However, on the August 12 sampling date, viability in these two treatments increased significantly from a mean of 71% on August 6 to 86% on August 12 for cadavers in the canopy and a mean of 45% to 87% for uncovered cadavers (Fig. 4.2). This resulted in a positive change in viability in these treatments between August 6 and 12. This increase in viability followed a major rain event which occurred on August 11 (Fig. 4.2). After August 12, again viability of conidia on cadavers under canopy and uncovered cadavers declined. The decline was more rapid on the uncovered cadavers ($p = 0.008$; $df = 1, 4$; $F = 23.25$ and canopy $p = 0.1$). Viability of conidia on cadavers that were maintained under artificial covers did not change throughout the experiment ($p = 0.8$). Viability for this treatment consistently averaged 97% (Fig. 4.2).

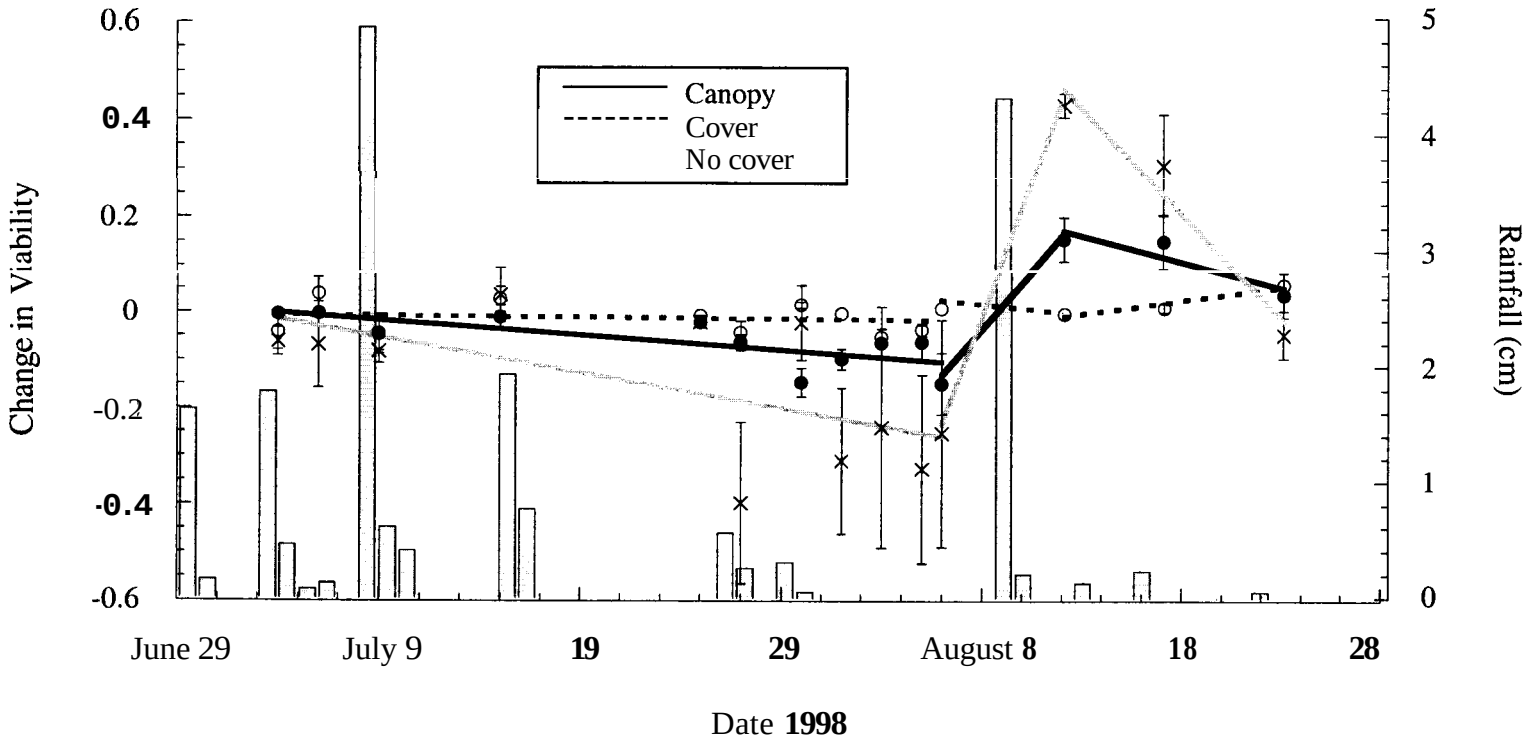

Figure **4.2.** Relationship between rainfall and viability of ***B. bassiana*** conidia on Colorado potato beetle cadavers in the field. Canopy: cadavers placed in arenas under the potato canopy; Cover: cadavers artificially covered; No cover: cadavers placed in arenas and no covered. Bars correspond to rainfall events (cm). Change in viability for the interval of time = ((final proportion of viable conidia – initial proportion of viable conidia)/final proportion of viable conidia).

Shade Treatment Effect on Envir Conditions

Environmental conditions under shade treatments varied significantly throughout the field season. However, shade treatment significantly influenced the environmental conditions that cadavers experienced. Average relative humidity decreased as shade cover (artificially cover < canopy < no cover) decreased ($p < 0.001$; $df = 2, 41$; $F = 21.65$). However, there was a significant interaction between shade treatments and time between periods of high humidity ($p < 0.001$; $df = 2, 37$; $F = 18.68$). During the first half of the season, cadavers under the canopy experienced more hours at humidities higher than 95% than cadavers under the other two treatments. After July 29'' it appears that cadavers under all three shade treatments experienced same periods of time at humidities higher than 95% (Fig. 4.3). Shade treatment had a moderating effect on temperature (Fig. 4.4). Shaded cadavers experienced significantly fewer hours of detrimental air and soil temperatures than unshaded cadavers ($p = 0.001$; $df = 2, 39$; $F = 8.13$) for hours at air temperatures > 33°C; and $p < 0.001$; $df = 2, 36$; $F = 8.09$ for hours at soil temperatures > 33°C). However, shade treatment had no significant effect on the cumulative air or soil degree days (based 10°C) for the observation period ($p = 0.83$ for air and $p = 0.72$ for soil).

Relationship Between Environmental Variables and Sporulation. Conidia Production and Viability:

The proportion of sporulated cadavers did not vary between trial 3 (arenas covered at night) and 4 (arena left uncovered at night) ($p = 0.81$), but because the methodology differed between the two trials we analyzed the effect of environmental parameters on sporulation for each trial separately. In trial 3 the environmental factors most highly correlated to proportion of cadavers sporulating across all three shade treatments (cadavers placed under canopy, covered or not covered) were average soil moisture and air degree days (Table 4.1). Proportion sporulation increased with increasing soil moisture and decreased with increasing degree days. These two variables explained 42% of the variation

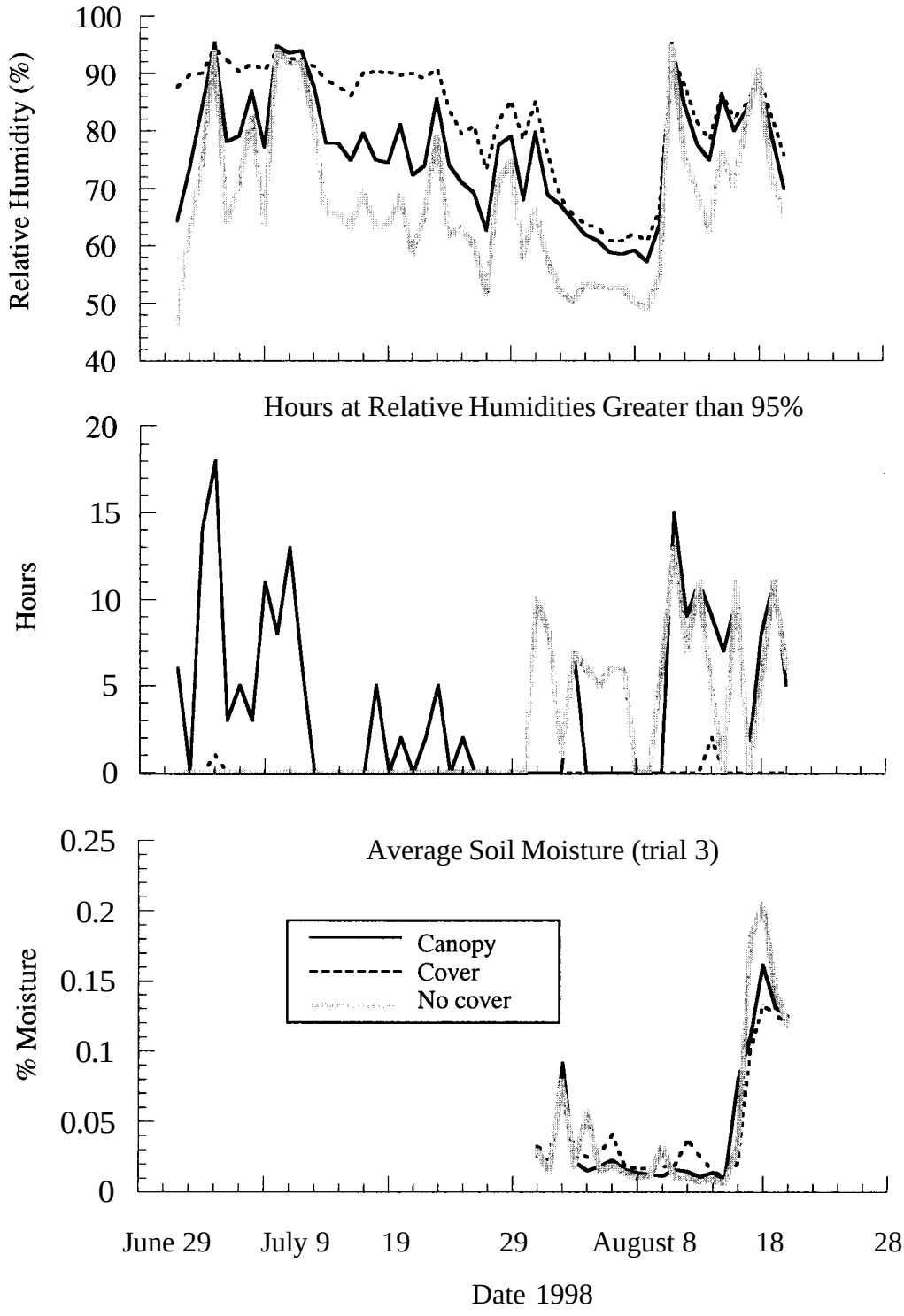

Figure **4.3.** Relationship between humidity/moisture parameters and shade treatments. Average soil moisture estimated gravimetrically.

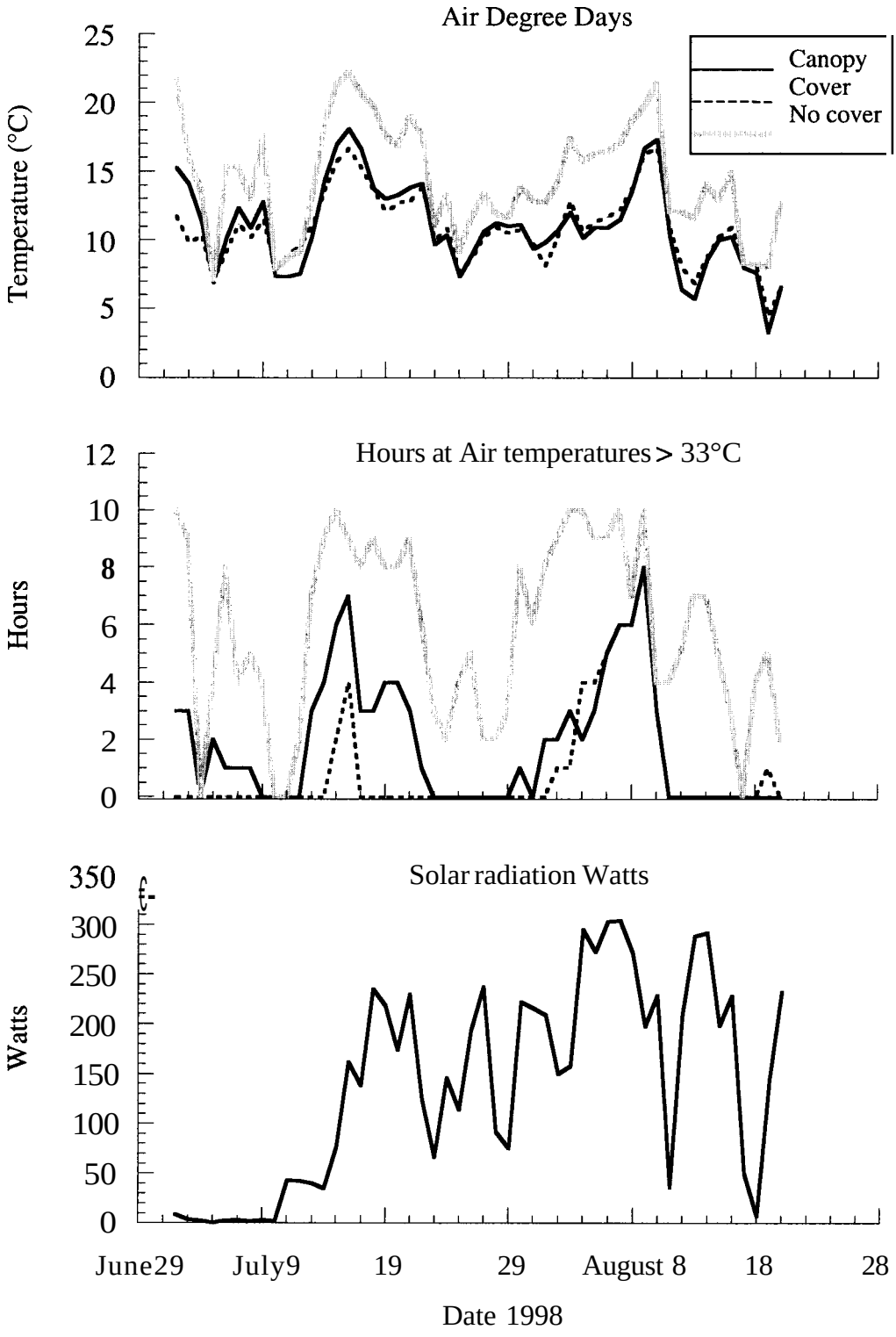

Figure **4.4.** Relationship between air temperature parameters and solar radiation (watts) and shade treatments.

TABLE4.1

Multiple regression results showing the relationship between significant environmental factors and sporulation of ***B.*** *bassiana* killed Colorado potato beetle cadavers under different shade treatments in the field.

Response Variable	Trial #	Shade Treatment	Environmental Variable	df	Coefficient	P	r^2
Sporulation[a]	3	All	Ave. soil moisture[b]		3.38	0.0001	
			Cum. air degree days	1, 119	-0.003	0.0001	0.42
		Canopy	Ave. RH	1, 59	0.004	0.0001	0.73
		Cover	Ave. soil moisture		2.51	0.01	
			Cum. air degree days	1, 27	-0.004	0.004	0.35
		No cover	Rainfall[c]		-0.01	0.02	
			Hours air temp. > 33°C		0.02	0.0001	
			Cum. solar radiation[d]	1, 27	$-0.97e^{-7}$	0.0001	0.46
	4	All	Rainfall		-0.03	0.0004	
			Cum. Solar radiation	1, 69	$0.3e^{-6}$	0.0004	0.15
		Canopy	Rainfall		-0.03	0.001	
			Cum. Solar radiation	1, 45	$0.4e^{-6}$	0.0006	0.20
		No cover	Ave. soil moisture		6.89	0.006	
			Hours air temp. > 33°C		0.02	0.002	
			Cum. solar radiation	1, 20	$-4.9e^{-7}$	0.002	0.32

[a] Data transformed to the arcsin square root of the proportion of sporulating cadavers over the interval.
[b] Average % soil moisture estimated gravimetrically.
[c] Rainfall measured in total cm during the interval of cadaver exposure.
[d] Cumulative solar radiation measured in Watts / m^2.

in sporulation. In trial 4, rainfall and soil degree days were most highly correlated with the proportion of cadavers sporulating across all shade treatments, with sporulation increasing with solar radiation and decreasing with rainfall (Table 4.1). These two variables explained only 15% of the variation in sporulation in trial 4.

Multiple regressions were also performed, separately by shade treatment, to determine if predictability could be improved by assessing relationships between environmental parameters and sporulation within shade treatments. In trial 3 this was the case for cadavers under the canopy and those without covers, but not for cadavers under artificial covers. Seventy-three percent of the variation in the proportion of cadavers sporulating under the canopy was explained by average daily relative humidity alone, with sporulation increasing with relative humidity. For cadavers without any cover, 46% of the variation was explained by total rainfall (cm), hours of air temperature greater than 33ºC, and cumulative solar radiation (watts) over the interval. Under artificial cover, sporulation of cadavers increased significantly with daily average percent soil moisture and decreases with cumulative air degree days, but only 35% of the variation was explained. In trial 4 (arenas uncovered at night), regressions by shade treatment also improved predictability for cadavers under canopy and without covers, but only slightly.

Relationships between environmental parameters and conidia production were examined separately for trials 1 through 3 in which arenas were covered at night and before rain and trial 4 in which arenas were not covered. In trials 1 through 3, average relative humidity, air degree days and solar radiation explained 51% of the variation in conidia production by cadavers (Table 4.2). Conidia production increased with average relative humidity over the sampling interval and cumulative air degree days, and decreased with exposure to cumulative solar radiation (Table 4.2). In trial 4 (uncovered arenas), cumulative air degree days was the environmental parameter that explained most of the variation in conidia production across both shade treatments (cadavers under canopy and uncovered)

TABLE4.2

Multiple regression results showing the relationship between significant environmental factors and ***B. bassiana*** conidia production by Colorado potato beetle cadavers under different shade treatments in the field.

Response Variable	Trial #	Shade Treatment	Environmental Variable	df	Coefficient	P	r^2
Conidia Production[a]	1, 2, and 3	All	Ave. RH		0.21	0.006	
			Cum. air degree days		0.04	0.0001	
			Cum. solar radiation[b]	1, 69	$-0.1e^{-4}$	0.0001	0.51
		Canopy	Rainfall[c]		1.05	0.006	
			Hours air temp. > 33°C	1, 30	1.05	0.003	0.79
		Cover	Not significant	-	-	-	-
		No cover	Rainfall	1, 16	0.07	0.01	0.31
	4	All	Cum. air degree days	1, 44	-0.008	0.005	0.15
		Canopy	Cum. air degree days	1, 37	-0.01	0.03	0.1
		No cover	Not significant	-	-	-	-

[a] Data transformed to the logarithm of conidia produced per cadaver.
[b] Cumulative solar radiation measured in Watts / m^2.
[c] Rainfall measured in total cm during the interval of cadaver exposure.

(Table 4.2), with production decreasing with increasing cumulative air degree days. However, this variable only explained 15% of the variability in conidia production.

Separate regressions performed by shade treatment also improved predictability in the first set of trials for conidia production on cadavers under the canopy, but not for cadavers under artificial covers or with no covers. Seventy-nine percent of the variation in conidia production by cadavers under the canopy was explained by rainfall and hours of air temperature greater than 33°C. Conidia production increased with increasing rainfall and exposure to hours of air temperatures greater than 33°C. Predictability of conidia production did not improve when shade treatments were analyzed separately for trial 4.

Relationship between environmental parameters and viability of conidia were also examined separately for trials 1 through 3 and trial 4. In trials 1 through **3,** hours of relative humidity higher than 95%, and hours of soil temperature greater than 33°C were the environmental factors most highly correlated to viability of conidia across all three shade treatments (cadavers placed under canopy, covered or not covered). The predictability of viability increased with increasing hours at high humidities and decreased with increasing hours at soil temperatures greater than 33°C. These two variables explained 26% of the variation in the viability of conidia (Table 4.3). In trial 4, average soil moisture was the most highly correlated environmental variable with viability across all shade treatments. Viability increased with increasing soil moisture, and this variable alone explained 35% of the variation in viability of conidia (Table 4.3).

Separate regressions performed by shade treatment also improved predictability in the first set of trials for viability of conidia on cadavers under all three shade treatments. Sixty-five percent of the variation in viability of conidia under the canopy was explained by rainfall, hours of soil temperature greater than 33°C, and cumulative solar radiation. Viability increased with rainfall and solar radiation but decreased with hours of soil temperatures greater than 33°C. The predictability of viable conidia improved somewhat when regressions where preformed separately by shade treatment (canopy and no cover) for

TABLE4.3

Multiple regression results showing the relationship between significant environmental factors and viability of ***B. bassiana*** conidia on Colorado potato beetle cadavers under different shade treatments in the field.

Response Variable	Trial #	Shade Treatment	Environmental Variable	df	Coefficient	P	r^2
Viability[a]	1, 2, and 3	All	Hours of RH > 95%		0.002	0.0004	
			Hours soil temp. > 33°C	1, 63	-0.003	0.0002	0.26
		Canopy	Rainfall[b]		0.05	0.0001	
			Hours soil temp. > 33°C		-0.04	0.0001	
			Cum. Solar radiation[c]	1, 21	$5.8e^{-8}$	0.0009	0.65
		Covered	Hours of RH > 95%		0.03	0.008	
			Cum. air degree days	1, 22	$-0.4e^{-3}$	0.02	0.25
		No cover	Ave. soil moisture	1, 24	1.96	0.01	0.22
	4	All	Ave. soil moisture[d]	1, 23	0.68	0.0009	0.35
		Canopy	Ave. soil moisture	1, 18	0.37	0.0005	0.49
		No cover	Ave. soil moisture	1, 6	5.77	0.06	0.44

[a] Relative change in viability of conidia over the interval expressed as a proportion of the viability at the beginning of the interval ((final proportion viable – initial proportion viable) / final proportion viable.
[b] Rainfall measured in total cm during the interval of cadaver exposure.
[c] Cumulative solar radiation measured in Watts / m^2.
[d] Average % soil moisture estimated gravimetrically.

trial 4. For both shade treatments viability increased with increasing soil moisture and this variable explained **49** and 44% of the variation in viability of cadavers under the canopy and with no cover, respectively (Table **4.3).**

Discussion

Shade provided by the potato canopy or artificial cover had a profound influence on the microclimate of *B. bassiana* infected larval cadavers and hence on the environmental parameters that affect sporulation, conidia production and viability. By altering the humidity and temperature of the environment of the fungus, shade treatments produced more favorable conditions for the three processes of fungal development investigated in this study. In laboratory studies sporulation was limited to relative humidities greater than **95%** and the maximum number of conidia produced per insect was at **100%** relative humidity (Manuscript **3).** In addition, sporulation was severely limited at continuous temperatures greater than **40°C** (Manuscript 3) and conidia production declined above **33°C.** The higher humidity and cooler temperature conditions favoring sporulation and likely transmission occurred much more frequently in the shaded treatments.

There are several references to optimal humidity and temperature conditions for sporulation of *B. bassiana* in the laboratory (Ramoska **,1984;** Luz and Fargues, **1998),** but there are limited studies evaluating sporulation under field conditions. Inglis et al. **(1997)** found sunlight to impair mycosis in *B. bassiana* infected grasshoppers. Long et al. (2000a) also found that transmission of *B. bassiana* infection from sporulating cadavers to healthy **CPB** prepupae increased with decreasing temperatures from 25 to **15°C** in the laboratory. With favorable temperatures and humidities for sporulation and transmission, epizootics are more likely to occur. Humidity and temperature conditions have also been shown to be important for initiating epizootics of entomophthoralean fungi. Hayek et al., **(1996)** found that rainfall was positively related to the epizootics of *Entomophaga maimaiga* in gypsy moth and Hayek et al. **(1990)** found that conidiophores of *E. maimaiga* did not develop or survive at constant temperatures greater than **30°C.**

Although proportion sporulation and conidia production were significantly correlated with measures of humidity, temperature, and/or solar radiation across all shade treatments, the relatively low r^2 values indicate poor predictability of these responses from

environmental variables. However, for those cadavers protected from the direct impact of rainfall in trials 1 through 3, regressions revealed good predictability for sporulation and conidia production for cadavers under the potato canopy. Average relative humidity alone explained 73% of the variation in proportion sporulation for these cadavers. Although the cadavers were covered at night and before periods of rain and hence protected from the direct impact of rainfall. Rainfall and hours of air temperature greater than 33°C explained 79% of the variation in conidia produced by cadavers. This high level of predictability was not achieved for sporulation of covered or unshaded cadavers protected from the rain or for any of the treatments where rain was not excluded (trial **4).** This may have been due to the low sample sizes for sporulated individuals in the unshaded arenas.

Shade also had a positive effect on persistence and viability of conidia on cadavers. Exposure to solar radiation has been shown to have a detrimental effect on persistence of conidia on foliage (Inglis et al, 1993; Inglis et al, 1995b;l James et al., 1995; Jorgensen, 2001). Daoust and Pereira (1986) investigated the viability of ***B. bassiana*** conidia on cadavers of cowpea pests (*Cerotoma* sp. and ***Diabrutica speciosa***) in shaded and unshaded conditions. They found that conidia on cadavers protected from rain and sunlight persisted for up to 12 weeks with 95% or greater viability. Conidia on unprotected cadavers, however, remained viable for only **2** weeks. Decline in viability of conidia in sunlight has been attributed to exposure to W radiation (Fargues et al., 1983). Solar radiation had a positive effect on viability on cadavers under the canopy. It is not clear why the viability of conidia on cadavers underneath the canopy was positively correlated with cumulative solar radiation in trials 1 through 3. These cadavers were protected from the direct exposure to W radiation by the potato foliage. Indirect effects on solar radiation from the microclimate are likely responsible.

High temperatures have also been found to directly reduce viability of conidia (Lingg and Donaldson, 1981) and viability under the canopy in trials 1 through 3 was negatively correlated with hours of high soil temperatures. Again, the shade treatments

resulted in considerable moderation of high temperature extremes, and hence had a protective effect on maintenance of viability.

The predictability of relative changes in viability of conidia between sampling intervals was particularly poor over all shade treatments. However, again, predictability improved when examining changes in viability just for those cadavers under the potato canopy. Although cadavers were protected from rainfall in trials **1** through 3, this measurement in combination with hours of high soil temperature and exposure to solar radiation explained *65%* of the variation in viability.

Viability of ***B.*** *bassiana* conidia on CPB cadavers in this study did decline faster in the unshaded treatment than on those under the canopy or cover. However, the average viability of conidia in the unshaded treatments did not decline below 75% over the entire experimental period of 28 days. Viability of conidia actually increased following periods of heavy rain or prolonged high moisture. Rainfall may have improved our measured viability of conidia by removing an outer layer of nonviable conidia from the outer sampled areas of our cadavers. Daoust and Pereira (1986) suggested that the zig-zag shape of the conidia bearing phialides of ***B.*** *bassiana,* may result in protection of the basal conidia by the more distal portion of the phialides and the apical conidia. Rain may wash the outer conidia away from the cadaver exposing the inner more viable conidia for sampling. However, in trial 1 through 3, cadavers were protected from the direct impact of rainfall. Alternatively, high humidity associated with rainfall may induce cadavers to produce additional conidia. Bosch and Yantorno (1999) studied the secondary conidiation process of ***B.*** *bassiana* (conidiation without vegetative phase) under different levels of carbon and nitrogen. They found that the secondary conidia produced by secondary conidiation were viable and capable of repeating the cycle for at least two generations. Thus, environmental factors such as presence of carbon and nitrogen can have the effect of stimulating additional sporulation. It is possible that rainfall may be responsible for **a** similar effect since rainfall is highly correlated with high humidities which are beneficial for sporulation and conidia production (Manuscript 3).

Foliar feeding CPB larvae infected with ***B.*** bassiana drop from the plant to the soil at or just prior death (unpublished observation). Long et al. (2000b) observed that the vast majority of these cadavers were distributed under the canopy of the potato plant. The microclimate created by the canopy provides conditions favorable for sporulation, persistence, and eventual transmission of the fungus to other CPB hosts. Long et al. (2000a) found that in a single encounter with a sporulating cadaver enough inoculum can be acquired to kill a prepupa. However, if cadavers are exposed to environmental conditions which do not favor conidia production, the fungus appears to remain protected within the integument of the cadaver and will readily sporulate when conditions are favorable. Mature CPB larvae have the potential to encounter cadavers when they leave the plant and burrow into the soil to pupate, and newly eclosed adults may encounter them when they emerge back up through the soil. However, carabids have been observed interfering with cadavers during this study. On some occasions cadavers were found buried, and at other times they were moved along the surface or unaccounted for. It is not known whether insect scavengers will feed on sporulating or on pre-sporulating ***B.*** bassiana-killed insects, but ants have been shown to feed on nematode killed prey (Baur et al., 1998). Given the capacity of *B. bassiana* infected larval cadavers to produce abundant conidia, to maintain a high level of viability over time, and to persist during periods of unfavorable environmental conditions, they provide considerable potential for transmission of the fungus to other individuals in the host population, if they remain on the soil. The activity of scavengers which may affect persistence of cadavers needs to be further investigated to determine what impact they have on disease transmission.

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MANUSCRIPT 5

HORIZONTAL TRANSMISSION OF *BEAUVERZA BASSZANA* (BALS.) WILL. IN THE COLORADO POTATO BEETLE, *LEPTZNOTARSA DECEMLZNEATA*, FEEDING AS LARVAE IN THE CANOPY

Introduction

The Colorado potato beetle (CPB), *Leptinotarsa decemlineata* (Say), is the most important insect defoliator of potato in North America. Because the beetle has become resistant to most synthetic pesticides (Bishop and Grafius, 1996), alternative control methods such as the use of the insect pathogenic fungus *Beauveria bassiana,* are being explored. Many researchers have demonstrated successful control of CPB with foliar applications of this fungus (Hayek et al., 1987; Drummond and Groden, 1996, Poprawski et al., 1997). B. *bassiana* also has the potential to induce additional mortality through secondary cycling of the disease when healthy beetles become infected by conidia produced from cadavers (Long et al., 2000a). The process of disease transmission from infected to healthy members of an insect population within **a** season is referred to as horizontal transmission and has been given considerable attention by researchers studying insect diseases and their progression using empirical and mathematical models (Gottwald and Tedders, 1984; Carruthers et al., 1986; Onstad and Maddox, 1989; Kaaya and Okech, 1990; Dwyer and Elkinton, 1995; Thomas, et al., 1995; Brown and Hasibuan, 1995; Drummond et al., 1996; Knell, et al., 1996; Long et al. 2000b). Understanding the processes governing the transmission can enhance the potential of fungi as biological control agents of arthropods pests.

Horizontal transmission of *B. bassiana* may occur through direct contact of healthy individuals with sporulated cadavers (Long et al., 2000a) or indirectly through healthy individuals contacting conidia released into the environment by weathered sporulated cadavers (Drummond and Groden, 1996; Jorgensen, 2001). In either case, mortality due to

disease transmission is a density dependent process (Anderson and May, 1980), which can be influenced by the behavior of the host; changes in behavior of infected hosts; density of infected units (inoculum) produced by infected hosts; and inoculum persistence and dispersal.

Horizontal transmission of ***B.*** *bassiana* in CPB prepupal populations on the soil surface was studied by Long et al. (2000a). They reported that after foliar applications, diseased CPB larvae die and drop to the ground where cadavers sporulate if the environmental conditions are favorable. Prepupae search for sites to burrow and pupate, contact the sporulated cadavers and become infected. Long et al. (2000a) conclude that the density of sporulated cadavers on the soil surface have a significant effect on prepupal mortality and sporulation.

Although most of the potato beetle's life cycle occurs on foliage, ***B.*** *bassiana* transmission at the foliar level has not been addressed. This paper investigates the relationship between density of infected larvae and mortality of healthy CPB larvae and emerging adults.

Materials and Methods

Transmission in Larval Populations

To determine the degree of transmission of the disease in CPB larval populations, a series of field cage experiments were conducted at the University of Maine Agricultural Experiment Station in Presque Isle, Maine during the summers of 1995 to 1997. The experimental design of the transmission experiments varied from year to year.

In June 1995, a complete randomized design was used to test different ratios of infected to healthy larvae on ***B. bassiana*** transmission. Four replicate 0.1 x 1.5 x 0.9 m^3 field cages per treatment were placed over three potato plants, *Solanum tuberosum* cv. Katahdin. Cages consisted of a wooden frame covered with 1 mm^2 nylon mesh. The tops of the cages were removable, and sealed with weather striping to prevent escape of larvae. Prior to the initiation of the experiment, all plants and soil within each cage were examined and any CPB or predators were removed.

Groups of 50 early second instars were placed in 9 cm diameter petri dishes with moist filter paper and sprayed with a ***B. bassiana*** conidia suspension resulting in concentrations ranging from 180 to 230 conidia/mm^2 using a Paashe® airbrush sprayer as described in Fernandez (2001). Sprayed larvae were kept in an incubator at 25°C for 24 h prior to their release into the field cages. Release of infected larvae was conducted by placing individual larvae on potato foliage with soft forceps.

Four days after the release, the number of live larvae remaining and number of sporulating cadavers observed in the soil were recorded. At this time healthy-unsprayed first instar CPB were also released. A total of fifty larvae were released into each cage at the following numbers of infected to healthy larvae: a) 0:50; b) 17:33; c) 25:25; d) 33:17; and e) 50:0. Eight days after the release of healthy first instars, all larvae remaining on the foliage in each cage were collected, set up individually in 9 cm diameter petri dishes with moist filter paper and food, and monitored for 10 days for mortality and sporulation.

A second experiment was conducted on July 26, 1995 to evaluate transmission of ***B. bassiana*** to CPB larvae in different parts of the potato canopy. The experiment consisted of a complete randomized block design with six individual plants as blocks. Two replicate nylon mesh sleeve cages (15 x 20 cm^2 per block were placed around insects in the lower portion of the canopy, one per block in the mid canopy and one per block at the top of the plant canopy. Second instars were sprayed as described above and allowed to die in the laboratory. Fifty cadavers per plant were randomly distributed at the base of the 6 plants (blocks). Following the release of cadavers, the potato plants were irrigated 1 h/day for 3 d with a rotating garden sprinkler with a single nozzle. Two days after the cadavers were placed in the field, five CPB first instars were enclosed in each sleeve cage. Bagged insects were collected 7 days after their release.

In 1996, the experiment was conducted in a complete randomized design with treatments consisting of different densities of infected larvae released into cages 4 days prior to the release of a single density of healthy larvae with 10 replicates per treatment. Seed potatoes, *S. **tuberosum*** cv. Katahdin, were planted in 26 cm diameter plastic pots and maintained in a greenhouse. Thirteen days prior the release of infected larvae, potted plants were transplanted into a newly tilled field plot. Each plant was individually covered with a 0.9 x 0.8 x 0.9 m^2 field cage and watered daily. Applications of the fungicide copper hydroxide (ac. 0.05%) were used weekly with an air pressurized hand sprayer to prevent outbreaks of the foliar disease ***Phytophthora infestans***.

New second instar CPB were sprayed with a ***B. bassiana*** conidia suspension yielding 188 conidia/mm^2. To ensure infection, inoculated insects were kept in an incubator at 25° C and 68-70% relative humidity for 24 h prior their release into cages. Foliage with infected larvae was pinned to potato foliage in the upper portion of the canopy in each of the cages using insect pins. Infected second instars were released on July 17, at densities of 0, 10, 20, 30, and 40 per cage into ten cages per density treatment. Four days after the release of infected second instars, 20 healthy second instars were introduced into each cage in the

same manner as infected larvae. The maturity of all surviving larvae and density of sporulating cadavers on the foliage and soil was measured 10 days after the release of the healthy larvae. Surviving larvae from five cages per density treatment were removed from the cages, brought to the laboratory, and observed for mortality for 7 days. In the remaining five cages per density treatment, surviving larvae were left in the cages and allowed to burrow into the soil for pupation.

Three additional cages per treatment, where only infected larvae were released, were used to monitor the density of conidia released by sporulating cadavers (explained below in "recovery of conidia on foliage and soil"), and to observe mortality of infected larvae. Survival of infected larvae was observed 10 and 14 days after their release. An additional cage was used for environmental monitoring.

In 1997 the potato plants, the field cage establishment and the fungicide regime were the same as described for the 1996 experiments. The experiment consisted of a randomized block design with the amount of irrigation as a blocking variable. The amount of irrigation reaching each cage was determined by measuring the depth of water accumulated after 8 min. in a 443 ml plastic container on top of each cage. Cages were allocated to one of three blocks based on the relative amount of water received with three to four replicates per treatment in each block. Treatments consisted of different densities of infected second instar CPB released on July 10 and 11, followed by the release of a single density of healthy second instars on July 30 and 31.

CPB second instars were treated with ***B. bassiana*** conidia suspensions yielding 212 and 234 conidia/mm^2 on July 8 and 9 respectively. The treated larvae were held with potato foliage as previously described for 2 days before their release into the field cages. Larvae on pieces of foliage were released as in 1996 at densities of 0, 15, 30, 45, and 60 infected larvae per cage with ten cages per density treatment. Groups of 15 and 20 healthy second instars were introduced into the cages 20 and 21 days after the release of the infected larvae. Observations of densities of live larvae were made on August 12, 31-32 days after the

release of the infected larvae and 12 days after the release of the healthy larvae. Surviving larvae were collected and maintained as described previously, and monitored for 10 days for mortality and mycosis.

Transmission in Prepupal Populations

Transmission of ***B. bassiana*** from different densities of infected second instar larvae to prepupae in the soil was evaluated in two experiments conducted in 1995 and 1996. In 1995 second instars were sprayed with a Paashe® airbrush sprayer with 215 conidia/mm^2 and allowed to die in the laboratory as described previously. Each experiment replicate consisted of cadavers randomly distributed about the base of three consecutive potato plants, and six replicates were established per density treatment on August 15 with the release of 0, 17, 25, 33, and 50 cadavers per 3 plants. The plot where cadavers were released was irrigated 1 hour a day for 3 days with a rotating garden sprinkler with a single nozzle. Bottomless soil cages consisting of circular sections of galvanized steel stove pipe measuring 25 cm in diameter and 12.5 cm in height were pushed into the soil at the base of the plants where the cadavers were previously placed. Three days after the placement of cadavers (on August 18), fifteen field collected CPB prepupae were placed on the soil in each of the soil cages and covered with 1 mm nylon mesh held in place with elastic loops to keep the prepupae from crawling away and/or to capture adults. Twenty days following the release of prepupae, emerging adults were collected, placed individually in petri dishes with potato foliage and moist filter paper, and survival was monitored daily for 10 days.

In 1996, ***B. bassiana*** transmission from larval cadavers to prepupae was evaluated using the same field cages as in the study of transmission in larvae. Fifteen days after the release of infected second instars (on July 31), the remaining healthy larvae from the foliar experiment were removed from the field cages and 20 late fourth instars (prepupae) were placed on the soil in each cage. In the remaining five cages, surviving larvae from the foliar experiments were left to burrow down into the soil to pupate. Cages were checked for

newly emerging adults every day for 15 days and live adults were held in dishes to assess post-emergence mortality for 10 days.

Density of *Beauveria bassiana* Conidia Produced by Cadavers in Foliage and Soil

In both experiments in 1995 the density of ***B. bassiana*** conidia produced by released infected larvae was monitored on foliage and soil surrounding the cadavers. Ten plastic cover slips per plant were placed within the potato canopy inside each of the four cages at the highest ratio of infected to healthy larvae (50:0). Cover slips were placed randomly in one of three regions of the canopy a day after healthy first instars were released. Five, three, and two cover slips were attached to the lower, middle and upper leaves, respectively. Cover slips were changed every 12 hours for 7 days and stored at -20°C until processing. Conidia attached to the cover slips were recovered by submerging the cover slips into test tubes containing 5 ml of 0.1% Tween® 20 solution and vortexing for 2 minutes. Half of 1 ml of the vortexed solution was pipetted onto petri plates 9 cm in diameter containing ca. 30 ml of wheat germ selective media (Sneh, 1991). Inoculated plates were incubated in the dark at 23-25°C for 9 days. Colony forming units (CFUs) were observed in each plate after 5, 7, and 9 days.

To estimate conidia density on soil in 1995, four soil cores per cage (2-2.5 cm in depth) from the four cages with the highest ratio of infected to healthy (50:0) were pooled together for a single sample from each cage. Soil samples were sieved and 20 g of soil per sample were added to 180 ml of 0.1% Tween@**20** and blended for one minute. Two serial dilutions were used for each soil sample and two plates of selective media (Sneh, 1991)(replicates) per dilution were inoculated with 0.4 ml of solution. Inoculated plates were stored **as** mentioned above, and CFUs were observed at 5, 7, and 9 days after inoculation.

In 1996 and 1997, leaf samples were collected from 3 additional cages per density treatment in which only infected larvae were introduced. Leaf samples consisted of ten leaf

discs (2 cm in diameter) from each plant with five leaf discs from the lower leaves and five from the remainder of the plant. Leaf samples were collected 5, 8, 11 and 14 days after the release of infected larvae in 1996, and 15, 20, and 25 days after the release in 1997. Leaf samples were kept at -20°C until processing.

The leaf samples were blended in 150 ml of 0.1% Tween® 20. The blended solution was diluted twice and aliquots (0.4 ml per plate) of each of the 3 dilutions were used to inoculate 3 plates of selective media (Sneh, 1991) per dilution. The plates were held at 23-25°C in the dark and CFU observations were made at 5, 7, and 9 days by counting the number of CFUs in five 3.61 cm^2 areas of the plate under 4X magnification.

In 1996 and 1997 soil samples were collected from 3 cages per infected larval density. Samples were collected 5, 8, 11, and 14 days post release in 1996 and 11, 15, and 20 days post release in 1997, four soil cores per cage per density treatment were pooled together in a single sample and stored at -20°C prior to plating. Samples were plated as described in the 1995 experiment except 15 g of soil per sample were added to 150 ml of 0.1% Tween® 20 and 3 plates per dilution were inoculated. CFUs per plate were sampled with the same procedure as described for the leaf samples. In order to determine the proportion of ***B. bassiana*** recovered as CFUs on the selective media 3.5 $x10^6$ ***B. bassiana*** conidia from a colony culture grown in **SDA** and incubated for 10 days were added to 150 ml of soil/Tween® 20 suspensions. This inoculated soil was plated and the CFUs sampled as described above.

Environmental Monitoring

Environmental monitoring was conducted using a Campbell Scientific data logger®. Two sets of probes were used to monitor conditions inside and outside the cages. The environmental parameters monitored were: soil temperatures under the potato canopy at depths of 1 cm and 4-5 cm; ambient temperature and relative humidity inside and at the top

of the potato canopy; leaf wetness (inside and at the top of the canopy); solar radiation and wind velocity.

Statistic Analysis

Linear regression **(SAS** Institute, **1994)** was used to determine the relationship between the ratio of infected:healthy or the density of infected larvae and a) the density of sporulating cadavers found in the cages, b) the proportion of surviving infected second instars at the time of release of healthy larvae, c) the proportion of surviving healthy larvae released at time of collection from the field, and d) the proportion of emergence of adults and their survival. Regressions were weighted by the sample size based on the dependent variable. Additional regressions were conducted to elucidate the relationship between the density of sporulated cadavers observed in the cages and the survival of healthy larvae. Independent variables were transformed to the log(density sporulated cadavers + 1) and proportions were transformed to the arcsin square-root of the proportion prior to analysis. Linear regressions were also used to determine the relationship between the density of infected larvae released, and the density of sporulated cadavers in the cages where only infected larvae were released, and the CFUs recovered from leaf and soil samples. Prior to analysis the data were transformed to log(CFUs + 1).

Results

Transmission in Larval Populations

In 1995, the number of cadavers found sporulated in the cages was positively correlated with the release density of infected larvae ($F = 39.22$; $df = 1, 18$; $p < 0.001$; $r^2 = 0.67$). On average, 26% of the infected larvae released were found sporulated on the soil surface with no observed sporulation on the foliage. However, survival of healthy larvae 8 days following release was positively correlated with the ratio of infected:healthy ($F = 8.99$; $df = 1, 14$; $p = 0.01$; $r^2 = 0.35$, weighted by the number of healthy larvae released) (Fig 5.1), and was not correlated with the number of sporulated cadavers found inside the cages ($p = 0.67$). When the surviving healthy larvae were brought to the laboratory and monitored for 10 days, their survival was also found to be significantly positively correlated with the number of infected larvae released ($\chi^2 = 15.91$; $df = 1,247$; $p < 0.001$) and the number of sporulated cadavers found in the cages ($\chi^2 = 15.75$; $df = 1,247$; $p < 0.001$). In the 1995 experiments where transmission was evaluated in different portions of the potato canopy, survival of healthy larvae released into mesh sleeve cages did not differ between position of the bags in the canopy ($p = 0.44$).

In 1996 the density of sporulated cadavers was again correlated with the density of infected larvae released ($F = 132.01$; $df = 1, 47$; $p < 0.001$; $r^2 = 0.73$). On average 18% of the cadavers were found sporulating on the foliage with the remaining 82% sporulating on the soil surface. Survival of healthy larvae in these cages decreased but not significantly with the increasing density of infected larvae ($p = 0.07$) (Fig. 5.2). However, survival of healthy larvae decreased significantly with increasing density of sporulated cadavers ($F = 5.57$; $df = 1, 47$; $p = 0.02$) (Fig. 5.3). In the three cages per density treatment where only infected larvae were released, the proportion surviving increased significantly with increasing density 14 days after their release ($F = 18.92$; $df = 1,10$; $p = 0.001$). No surviving larvae were recovered in cages with 10 and 20 infected larvae released, but 1.3 ± 0.33 (mean $\pm$ **SE)** larvae (proportion 0.04 ± 0.01) were recovered in the cages with 30

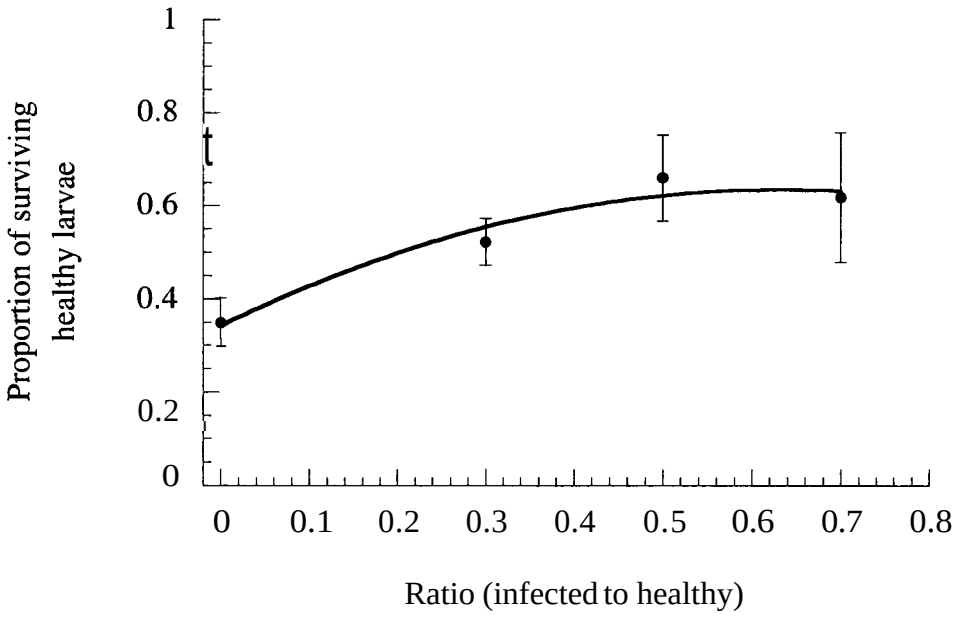

Figure 5.1. Field survival of healthy Colorado potato beetle larvae following exposure to ***B.*** *bassiana* infected Colorado potato beetle larvae (1995). Insects released at different ratios of infected:healthy. Analysis was conducted with individual observations; graph depicts curve fit to the means: $y = 0.34 + 0.92x - 0.73x^2$; $r^2 = 0.95$.

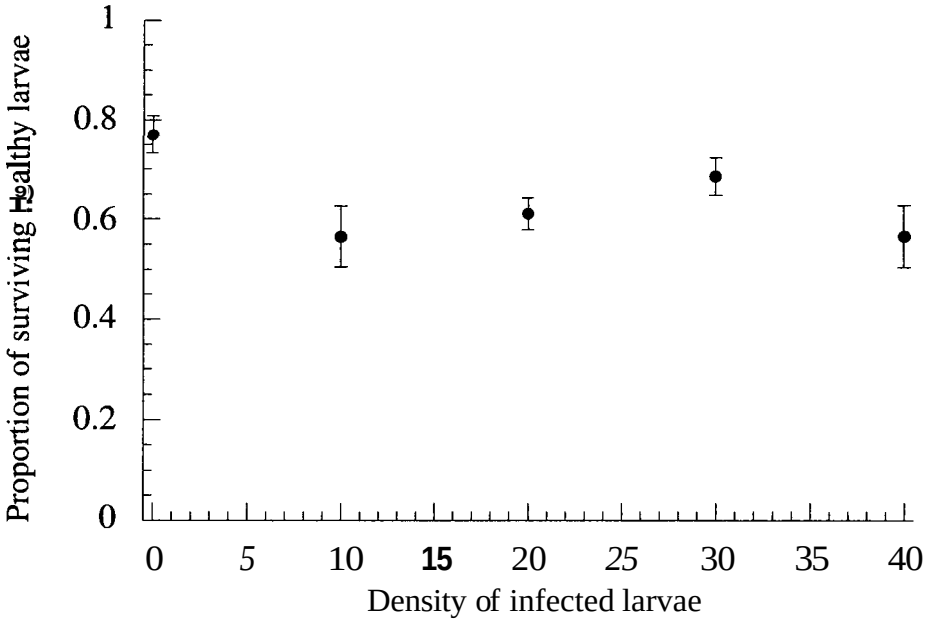

Figure 5.2. Field survival of healthy Colorado potato beetle following exposure to different densities of *B. bassiana* infected larvae: total surviving healthy larvae (1996).

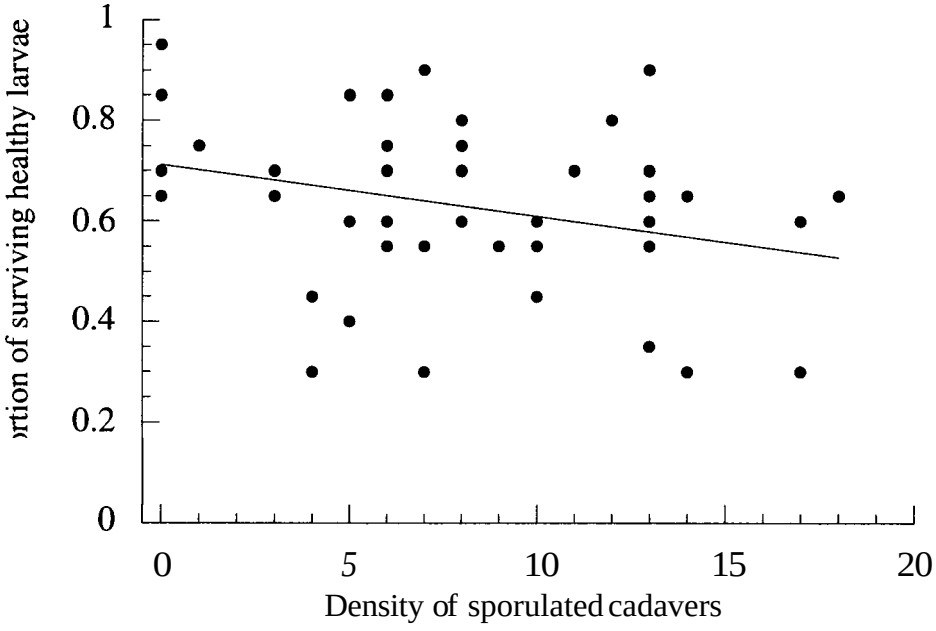

Figure 5.3. Relationship between *B.bassiana* sporulating Colorado potato beetle cadavers observed per cage and the proportion of released healthy Colorado potato beetle larvae which survived following 12 days of exposure (1996): $y = 0.7 - 0.01x$; $r^2 = 0.1$

larvae, and 9.0 ± 4.58 (proportion 0.22 ± 0.11) were recovered in the cages with 40 larvae released.

In 1997, the densities of sporulated cadavers found within the cages were greater at 11-12 days than at 21-22 days post release. On both dates, cadaver density increased in direct proportion to the densities of infected larvae released ($F = 146.09$; $df = 1, 43$; $p < 0.001$; $r^2 = 0.74$ for 11-12 days and $F = 83.03$; $df = 1, 43$; $p < 0.001$; $r^2 = 0.63$ for 21-22 days). Approximately 25% of the released infected larvae had sporulated after 11-12 days and ca. 80% of these were still observed 10 days later. However, neither density of infected larvae released nor the density of sporulated cadavers on both sample days had a significant relationship to the proportion of released healthy larvae surviving during this experiment ($p = 0.6$ for density of infected larvae; and $p = 0.29$ and $p = 0.41$ for 11-12 and 21-22 days after release, respectively). Neither irrigation block nor day of release of infected larvae had any effect on the survival of infected larvae ($p = 0.34$ and $p = 0.12$, respectively)

Transmission in Prepupal Populations

In 1995, thirty percent of the ***B. bassiana*** infected CPB cadavers generated in the laboratory and placed in the field sporulated before the release of the prepupae, and 60% sporulated by the fourth day after the release. A high proportion of the released prepupae were not recovered. The number of cadavers released per plant did not have a significant effect on the number of emerging adults ($p = 0.54$) nor on the number of dead insects in the soil ($p = 0.29$). However, the highest proportion of dead insects (both adults and prepupae) was detected in the treatments with the highest number of cadavers placed at the base of the plants (0.38 ± 0.04).

The densities of infected larvae released, the density of sporulated cadavers found in the cages, and of CFUs recovered from soil samples' had no significant effect on the

[1] CFU data from **5** day incubation of second dilution series of soil samples collected from those **3** cages per density with only infected larvae released.

proportion of adults emerging from the 20 prepupae released per cage ($p = 0.46$, $p = 0.42$ and $p = 0.13$, respectively) in the 1996 experiment. Similar results were observed for the proportion of adults emerging in those cages where insects were released as second instars ($p = 0.31$ for density of infected larvae; and $p = 0.51$ for density of sporulating cadavers; and $p = 0.06$ for density of CFUs per cage). Post emergence mortality of adults from either group of cages was also not affected by either the density of infected larvae released ($p = 0.37$ for prepupae released cages and $p = 0.64$ for second instar release cages) or the density of cadavers found on soil in the cages with release of prepupae ($p = 0.58$) and with release of healthy second instar ($p = 0.30$).

Density of *Beauveria bassiana* Conidia Produced by Cadavers of Infected CPB Second Instars

In 1995 and 1996, no *B. bassiana* CFUs were recovered from the plastic cover slips placed in the canopy. In 1995 the CFU density from soil samples increased significantly with the number of infected larvae released ($p = 0.004$; $df = 1, 19$; $F = 11.07$; $r^2 = 0.35$) (Fig. 5.4). However, there was no relationship between the density of sporulated cadavers found on soil and the density of CFUs for any of the dilutions and incubation times ($p = 0.32$). Statistical analyses on CFUs from soil samples were conducted using all dilutions at each incubation time. The best dilution for observing *B. bassiana* CFUs and the one to yield the highest correlation coefficient was the second dilution observed after 5 days of incubation.

In 1996 no CFUs were recovered from soil collected 5 days following release of infected larvae, but some were recovered 8, 11, and 14 days post release. CFUs recovered from soil samples increased with time from release of infected larvae ($F = 4.99$; $df = 1,33$; $p = 0.03$), but did not increase with density of released infected larvae ($p = 0.18$). Using observations from plates where a known amount of conidia was added to the soil solution prior to inoculating the plates, the proportion of conidia recovered as CFUs on the second

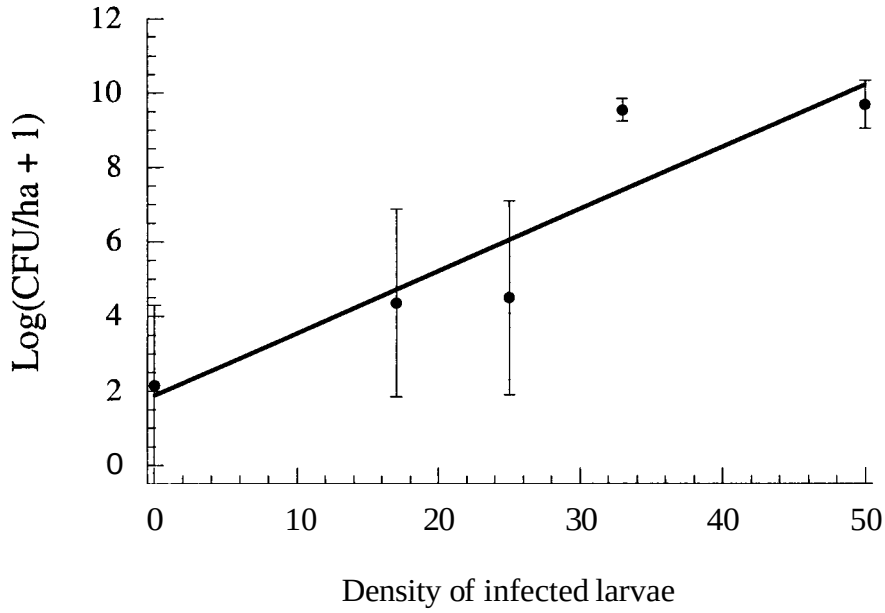

Figure 5.4. Relationship between density of ***B. bassiana*** infected second instar Colorado potato beetle released in cages and density of ***B. bassiana*** colony forming units recovered from soil samples (1995). Analysis was conducted with individual observations (Log(CFU/ha+1)); graph depicts curve fit to the means: $y = 1.87 + 0.17x$; $r^2 = 0.83$.

dilution plates incubated for 5 days was estimated to be 31.42%. Based on CFU recovery, *B. bassiana* conidia density was ca. 1.74×10^{12} conidia/ha 14 days following release.

In 1997 *B. bassiana* was recovered from leaf samples collected from the foliage in the monitoring cages. CFUs increased with increasing density of infected larvae only in the cages that received moderate irrigation (0.43-0.55 cm/min). CFUs declined with increasing density of infected larvae with low (0.19-0.3 cm/min) or high (0.79-1.04 cm/min) levels of irrigation (interaction effect: $F = 4.83$; $df = 2, 34$; $p = 0.02$) (Fig. 5.5). Time after release of infected larvae had no significant effect on the CFUs ($p = 0.35$).

Analyses of CFUs from soil were conducted with data collected from the second dilution observed five days after the inoculation of plates. Neither density nor time after release of infected larvae had a significant relationship with the density of CFUs recovered from soil samples ($p = 0.27$ and $p = 0.35$, respectively). CFUs were significantly affected by the amount of irrigation received ($F = 26$; $df = 2, 33$; $p = 0.02$; $r^2 = 0.18$) (Fig. 5.6).

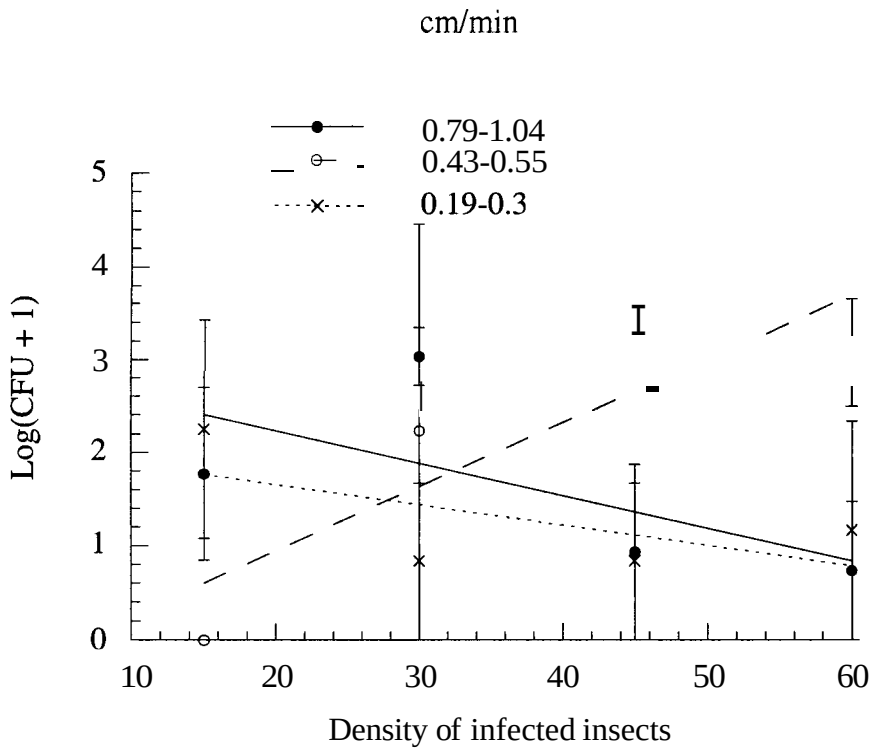

Figure 5.5. Relationship between the density of ***B.*** *bassiana* infected second instar CPB and the density of ***B.*** *bassiana* colony forming units (Log(CFU+1) from foliage samples in blocks receiving different amount of water received through irrigation (cm/min) (1997).

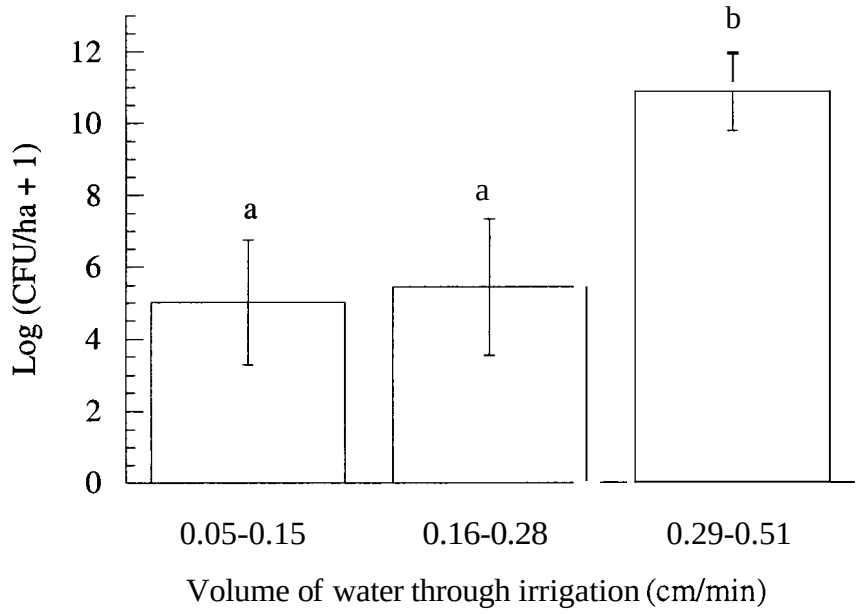

Figure 5.6. Effect of amount of water received through irrigation (cm/min) on the density of ***B. bassiana*** colony forming units (Log(CFU/ha + 1))from soil samples (1997). Letters correspond to ANOVA means comparison using Tukey-Kramer, significant difference $\alpha < 0.05$.

Discussion

Transmission of pathogens in insect populations is a complex process that requires a good comprehension of the biology of both the insect and the pathogen and their interaction. Gottwald and Tedders (1982) investigated the release of ***B. bassiana*** conidia by sporulated pecan weevil cadavers under changing relative humidity, light and vibration. They found that cadavers release conidia at low humidity levels, or at high humidity with vibration, regardless of light conditions. These experiments were conducted in chambers in the laboratory, with sporulated cadavers attached to microscope slides inverted over a spore trapping device. As most CPB larval cadavers sporulate on soil surface, it is difficult to extrapolate the results obtained by Gottwald and Tedders (1982) to the upward movement of conidia to the canopy under field conditions. However, results from this study designed to evaluate the amount of transmission of ***B. bassiana*** from infected CPB larvae to healthy larval and prepupal populations were inconclusive. Although some conidia were detected in 1997, the average density was 15 conidia/cm^2 of leaf surface. **A** density of 973 conidia/cm^2 of leaf surface is needed to cause ca. 50% mortality of second instar CPB in the laboratory (Fernandez et al., 2001). The amount of conidia that was carried up to the foliage from cadavers was insufficient to have a measurable impact on CPB mortality.

Impacts of sporulating cadavers on the soil surface on the survival of larvae in the potato canopy was slight. In 1995, transmission in larval populations was higher at lower densities of released infected larvae. Mortality of healthy larvae in cages with noninfected larvae (0:50) may have been due to density dependent factors such as competition among healthy first instars. In 1996 and 1997 to avoid the confounding effects of density dependent mortality, a set number of healthy second instars were released into the cages. With this modification a small but not significant ($p = 0.07$) amount of ***B. bassiana*** transmission to healthy larvae was detected in 1996. In 1997, even with higher densities of infected larvae released no transmission of the pathogen was detected ($p = 0.6$). A possible reason for the lower mortality of healthy larvae in 1997 was the later timing of their release

after the release of the infected larvae. Maximum mortality of infected larvae was predicted to occur ca. 70 degree-days after exposure to *B. bassiana* conidia. This occurred within 3-4 days after release in both years. Sporulation of cadavers of *B. bassiana* infected larvae in the laboratory occurs between 12 and 24 hours at 25°C and 100% relative humidity, but the time to sporulation in the field is uncertain. In 1996, healthy larvae were released ca. 4 days before the predicted maximum mortality, whereas in 1997 they were released ca. 17 days after predicted maximum mortality. Although the proportion of cadavers that sporulated in 1996 was higher than in 1997, the density of sporulated cadavers in the highest infected density treatment was the same in 1996 and the first observation **4** days before the release of healthy larvae in 1997. However, the density of sporulated cadavers decreased in 1997 between the first and the second observations, twelve days post release of the healthy. In 1997, sporulating cadaver densities may have been below a threshold needed to detect transmission.

Natural precipitation and irrigation may also have been more favorable for ***B.*** *bassiana* in 1996 than in 1997. Long et al. (2000b) found that rainfall between death of infected CPB larvae and exposure of them to healthy prepupae explained variation in disease transmission. In 1996 cages received a total of 3.22 cm of rainfall between the time of release of infected and estimated maximum mortality of infected larvae and a total of 3.52 cm during the experiment. However, in 1997 only 2.03 cm of precipitation occurred during the field experiment. This amount of precipitation may not be sufficient to foster transmission.

Over the two years of this study density of released infected larvae did not affect mortality of prepupae. Long et al. (2000b) also studied the effect of the density of ***B.*** *bassiana* sporulating CPB cadavers on prepupae and adult emergence in Northern Maine. Contrary to our results, they observed 60 to 80% mortality and 20-30% sporulation among the prepupae at the lowest density of sporulated cadavers investigated, 0.015 cadavers/cm^2. In our study, the density of sporulating cadavers ranged from 0-0.003 cadavers/cm^2. In

addition, many of the released healthy prepupae were unaccounted for. Long et al. (2000b) state that high mortality in emerging adults CPB is a typical phenomena in Northern Maine. In our study, the density of sporulated cadavers may have been too low to detect transmission of ***B. bassiana*** against the background of high natural mortality.

Density thresholds alone do not explain the process of transmission. The synchrony of the transmitting stage of infection with the susceptible stage of the healthy larvae during periods of favorable environmental conditions is needed. Onstad **(1993)** emphasizes the importance of adding temporal and spatial components to models of pathogen transmission. Under more natural conditions than in this study both healthy and infected populations occur simultaneously so the degree of transmission might be greater than what we have observed. High humidity levels are essential for the production of conidia by cadavers of ***B. bassiana*** infected CPB. Transmission both on foliage and on soil may be higher in years with higher rainfall. However, transmission on foliage is likely to be minimal compared to that on soil.

Although Long et al. (2000ab) did show measurable transmission from sporulating cadavers to prepupae on the soil, the more significant contribution of sporulating cadavers to secondary cycling of the disease may be through increasing inoculum of conidia in the soil and subsequent infection of pupae and overwintering adults. The number of overwintering colonizing adults in the Maine Potato Ecosystem Project decreased between **1992** and **1994** in the plots previously treated with ***B. bassiana*** as compared to the plots that received conventional insecticides (Drummond and Groden, **1996).** This decrease is likely due to the persistence of ***B. bassiana*** conidia in the soil. An increase in the soil inoculum level can result from an addition of conidia from direct spray applications or from sporulating cadavers. CPB larval cadavers can produce between $\mathbf{1.24} \times 10^{4}$ and $\mathbf{8.7} \times 10^{8}$ conidia (Manuscript **2),** and these conidia have the capacity to persist in the soil for years (Clerk and Madelin, **1965;** Lingg and Donaldson, **1981)** inducing mortality in underground stages of

the beetle. Conidia persistence may contribute significantly to the effectiveness of ***B.*** *bassiana* as a long term biological control agent of CPB.

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BIOGRAPHY OF THE AUTHOR

Silvia Fernandez was born in Granada, Spain on May 22, 1969. She was raised in Palma de Mallorca, Spain and graduated from high school in 1988. She attended the University of Maine and graduated in 1994 with a Bachelor of Science degree in Sustainable Agriculture. She entered the graduate program in Entomology at the University of Maine in the fall of 1994.

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