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The Effect of Digging by the Colorado Potato Beetle (*Leptinotarsa decemlineata*) (Coleoptera: Chrysomelidae) on the Acquisition and Retention of *Beauveria bassiana* Conidia

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ABSTRACT

The fungal pathogen, *Beauveria bassiana*, may play an important role in the management of overwintering Colorado potato beetles. Prediapause beetles dig into the soil in the fall where they overwinter. The loss or removal of conidia from the integument during the digging process may decrease the chance of disease development and subsequent mortality. We evaluated the number of fungal conidia that were acquired and retained by the beetle after burrowing into soil that was surface-treated with *B. bassiana* conidia. In addition, we evaluated beetles for disease initiation after exposure to conidia at the soil surface. Results showed that beetles acquired significant numbers of conidia from the surface treatment; however there was a linear decrease in the number of conidia retained on the integument as the beetles' burrowing depth increased. Almost half the population (54 and 46%) lost all conidia when the burrowing depth was between 21 and 30 cm. *B. bassiana* application method, spraying vs mixing conidia into the soil surface, did not significantly incite disease; however the inoculation concentration significantly affected mortality. More insects became infected when adults were exposed to surface concentrations of 10^7 compared to 10^6 conidia per cm^2. Fall application of *B. bassiana* to reduce overwintering populations may not be a viable option because beetles tend to lose the conidia that are acquired on the surface during digging, thus reducing the chances of disease occurring during the overwintering stage. Soil treatments in the spring may be more beneficial in reducing the Colorado potato beetle colonizing population.

Keywords: depth, disease initiation, overwintering, soil inoculation

INTRODUCTION

The Colorado potato beetle *Leptinotarsa decemlineata* (Say) is a well-established pest on potatoes in Canada and the United States. Insecticides have been the principal method employed by growers to control beetle populations and reduce yield loss. However, this insect has demonstrated the ability to rapidly develop insecticide resistance (Roush *et al.* 1990). Increasing pest pressure due to widespread insecticide resistance has resulted in the search for alternative control methods, especially those that can be used in conjunction with pesticides in an IPM program (Boiteau *et al.* 1995; Cloutier *et al.* 1995; Zhao *et al*. 2000; Noronha *et al.* 2001).

The fungal entomopathogen, *Beauveria bassiana* (Bals.) Vuill. has been studied extensively as a biocontrol agent of the Colorado potato beetle (Campbell *et al.* 1985; Hajek *et al.* 1987; Anderson *et al.* 1988; Drummond and Groden 1996; Poprawski *et al.* 1997; Long *et al.* 2000; Wraight and Ramos 2002; Wraight *et al*. 2009). The efficacy of *B. bassiana* to control Colorado potato beetle larvae and adults has been demonstrated in both field and laboratory studies (Campbell *et al.* 1985; Hajek *et al.* 1987; Long *et al.* 2000; Fernandez *et al*. 2001). Research efforts have mainly focused on foliar applications of *B. bassiana* to control larvae and adults (Campbell *et al.* 1985; Hajek *et al.*1987; Wraight and Ramos 2002). Results from these studies have been varied. Poprawski *et al.* (1997) and Anderson *et al.* (1988) reported significant reductions of 60-76% in Colorado potato beetle populations when compared to the control; but Hajek *et al.* (1987) found no difference in defoliation and total yield between the treated and control plots, irrespective of *B. bassiana* concentration used. Foliar applications of *B. bassiana* are known to have limitations mainly because conidial viability decreases in the presence of solar radiation, fungicide applications, or a rainfall event soon after application (Jaros-su *et al*. 1999; Inglis *et al.* 2000).

Soil is the natural habitat of *B. bassiana* and it offers environmental conditions that are favourable to conidial persistence such as moisture, temperature, and protection from solar radiation. Consequently, persistence in the soil is much longer than on foliage (Lingg and Donaldson 1981; Watt and LeBrun 1984; Gaugler *et al.* 1989; Inglis *et al.* 1993, 1997, 2000). Several studies have demonstrated that conidia mixed in the soil remain viable for one to two years following introduction (Wojciechowska *et al.* 1977; Bajan 1980; Steenberg 1995). Bajan (1980) found that 50% of conidia mixed into the soil in the fall remained viable for two years following introduction. Thus, soil applications of *B. bassiana* may be capable of inciting infections for a longer period of time in contrast to foliar applications.

Although the Colorado potato beetle is a foliage-feeding pest, this insect also has a close association with the soil during two stages of its development: 1) during the summer when mature larvae enter the soil to pupate and subsequently emerge as adults to commence feeding on the potato foliage, and 2) in the fall when prediapause adults enter the soil to overwinter (Noronha and Cloutier 1998, 1999; Noronha *et al.* 2002). Natural infections are mostly encountered in prepupal, pupal, and adult populations within the soil. Soil inoculation trials to control mature larvae when they enter the soil to pupate have shown a decrease in the number of emerging adults (Watt and LeBrun 1984; Cantwell *et al.* 1986; Groden and Dunn 1996; Long *et al.*

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2000). However, very few studies have been conducted on the efficacy of *B. bassiana* against overwintering adults (Bajan *et al.*1977; Watt and LeBrun 1984; Gaugler *et al*. 1989; Cantwell *et al*. 1986). Prediapause adults dig through several centimeters of soil to overwinter; depths can vary with 10-30 cm being typical (Gibson *et al.*1925; Minder and Petrova 1966; Noronha and Cloutier 1998; Noronha *et al.* 2002). Most studies reported low levels of mortality due to *B. bassiana* in overwintering populations in the field (Bajan *et al.* 1977; Watt and LeBrun 1984; Cantwell *et al*. 1986; Gaugler *et al*. 1989).

Hypocrealean fungi such as *B. bassiana* infect insects primarily through the cuticle, however to incite infection and subsequent death, exposure to an established minimum dose of inoculum is required. The loss or removal of conidia from the integument before they have penetrated the cuticle of the beetle can significantly decrease the chance of disease development and eventual mortality. Quintela and McCoy (1998) demonstrated that larvae of the soil-inhabiting root weevil, *Diaprepes abbreviatus*, treated with the insecticide imidacloprid, retained more conidia of *Metarhizium anisopliae* and *B. bassiana* than untreated larvae. They concluded that this was the result of reduced mobility within the soil of the insecticide-treated larvae. The effect of the digging process on conidial retention by Colorado potato beetles following acquisition from inoculated soil and the subsequent development of disease remain unknown. The purpose of our study was to determine the acquisition and retention of *B. bassiana* conidia by beetles digging through surface-inoculated soil, and the number of beetles that become infected after digging through surface-inoculated soil.

MATERIAL AND METHODS

Insect rearing

A laboratory colony of the Colorado potato beetle was established from adults collected at the Agriculture and Agri-Food Canada Research Centre in Lethbridge Alberta, and maintained at 24°C under a 16:8 L: D photoperiod. To maintain genetic diversity, the colony was partly renewed each summer by introducing beetles obtained from egg masses collected in the field. Potato plants (cv. 'Russet Burbank') were grown in a greenhouse and the foliage was used as the food source for the beetles.

Diapause initiation

Newly emerged beetles from the colony were placed into cages with a potato plant (cv. 'Russet Burbank') and placed in a growth chamber under a 12:12 L:D photoperiod coupled with an oscillating temperature of 10 to 18 ± 2°C, conditions conducive to diapause induction in this beetle. A new potato plant was added to the cage as required. After two weeks, beetles that had ceased feeding and consequently were considered ready to enter diapause were removed and used in all subsequent experiments (Noronha and Cloutier 1998).

Soil

Top soil obtained from a field at the Lethbridge Research Centre was used for all experiments. The soil was a clay-loam, dark-brown Chernozem, with a 15-cm A-horizon containing approximately 1.6% organic matter (Janzen 1987). The soil was moistened to 50% of saturation with tap water and then sifted through a 4 mm sieve to remove coarse debris. It was then placed in plastic bags and maintained at 4°C. When needed, the required amount of soil was removed and allowed to reach room temperature for 2-3 h before inoculation with the fungal conidia.

Treatments

Beauveria bassiana isolate GHA (Laverlam International, Butte, MT) was used for all experiments. This isolate has previously been shown to be virulent against the beetle in both laboratory and field assays (Jaros-Su *et al*. 1999; Long *et al.* 2000; Fernandez *et al*. 2001; Klinger *et al.* 2006). Unformulated conidia were obtained from Mycotech (presently Laverlam) and stored at 5°C until used. Condia were suspended in a 0.01 M sodium phosphate buffer amended with 0.05% Tween (80); a Kontes micropestle was used to reduce clumping. Concentrations of conidia were estimated using a haemocytometer, and were adjusted to viable conidia/ml. Conidial viability was estimated using the technique described by Goettel and Inglis (1997). The suspension was then diluted to the desired concentration for application to the soil surface. Control treatments received an application of buffer-Tween solution alone. Treatments consisted of two conidial concentrations, either 10^7 or 10^6 viable conidia per cm^2, and buffer as a control. The conidial suspension was sprayed onto the soil surface using an air brush (Aztek - 3000S Airbrush Kit (Aztek Inc. USA)) (Goettel and Inglis 1997).

Digging

Acrylic tubes 30 cm in length with an inside diameter of 2.9 cm were used to determine the effects of digging on conidial acquisition and retention. Soil (219 g) was placed into digging tubes by gentle tapping, ensuring that density varied by <5% throughout the soil column. The surface of the soil in each tube was sprayed to attain either 10^7 or 10^6 viable conidia per cm^2, and buffer as a control, as described above. The soil was then allowed to dry for 30 min. A single prediapausing beetle was then placed on the soil surface of each tube after being weighed, measured, and sexed. The tubes were placed in an incubator under diapausing conditions (12:12 L:D photoperiod coupled with an oscillating temperature of 10 and 18 ± 2°C) and beetles were allowed to dig. Beetles that did not dig after 12 h (17 beetles at 10^6 viable conidia per cm^2 and 12 beetles at 10^7 viable conidia per cm^2) were removed and processed as surface beetles. After two days, the beetles that had entered the soil were carefully removed and the distance from the surface was recorded. Beetles were placed individually in Petri dishes and allowed to walk for 15 min to remove any excess soil on the surface. Each beetle was then individually homogenized in 1 ml of the buffer-Tween solution in a 1.5 ml microtube. A serial dilution to 10^{-3} was made of the original suspension and each suspension including the original was plated in duplicate on an oatmeal-dodine selective agar medium (Goettel and Inglis 1997). Plates were placed in an incubator at 20°C for 5-7 days and the numbers of colony-forming units (CFU) were enumerated. The experiment was repeated 4 times, for a total of 62 beetles per treatment.

Level of infection

Plastic pots (10 cm height, 10 cm top diameter) were filled with 91 g of soil to maintain compaction level. Two methods of inoculation were used: 1) The soil surface was sprayed with the required conidial concentration or buffer as described above; or 2) After spraying, the conidia were mixed into the top 1 cm of the soil using a spatula.

After spraying or mixing, the soil surface was allowed to dry for 30 min before seeding with beetles. Ten prediapause adult Colorado potato beetles were introduced per pot and allowed to dig. Controls consisted of beetles placed onto non-inoculated soil. The pots were covered with a clear plastic lid to prevent any beetles from escaping. The pots were randomly placed within an incubator under diapausing conditions (12:12 L:D with oscillating temperatures of 10 and 18 ± 2°C). After 12 hours, the five beetles that did not enter the treated soil were removed. Soil moisture was maintained by weighing the pot every second day and adding the required amount of water from the bottom of the pot. The beetles were removed from the pots after 45 days and the numbers of dead and live beetles were recorded. Dead individuals were placed in a moist chamber to allow the fungus to emerge and sporulate to verify cause of death (Goettel and Inglis 1997). All other individuals were surface-sterilised by immersing in an alcohol water series, and then homogenised in 1 ml of the buffer-Tween solution. The resulting suspension was diluted to 10^{-3}; each dilution was then plated in duplicate on oatmeal dodine selective media and placed at 20°C for 5-7 days after which time the numbers of CFU

were enumerated. Each treatment was replicated five times and the experiment was conducted twice over time. None of the control beetles died during the experiments.

Analysis

All experiments, i.e. digging and infection rate, were conducted in a randomized block design with blocks being repeated experiments in time. In the absence of block effects, the data were combined. Data on acquisition and retention following digging were subjected to linear regression to determine the relationship between infection levels and depth reached. The relationship between body length, width, sex and infection level was also subjected to a regression analysis. Data on level of infection were subjected to an analysis of variance followed by an LSD test to determine the effects of conidial concentration and application method on infection. All statistical analyses were performed using SAS statistical software (SAS 2005).

RESULTS

Acquisition and retention of conidia following digging

Results showed a decrease in conidial retention as depth increased irrespective of the initial *B. bassiana* concentration to which the beetles were exposed (**Figs. 1, 2**). Conidial acquisition was higher at soil inoculation concentrations of 10^7 conidia/cm^2 and ranged from 10^2-10^4 conidia per beetle as compared to a range of 10-10^3 conidia per beetle at the lower soil inoculation concentration of 10^6 conidia/cm^2 (**Table 1**). Conidial acquisition was higher at 10^7/cm^2, but conidial loss occurred more rapidly during digging as compared to loss at the lower concentration treatment. This relationship could be modeled by the equation y = b0 + b1 which predicted a steeper slope (3.075 + (-0.049)) r^2 = 0.32 ($F_{1,60}$= 10.56, p<0.001) at 10^7/cm^2 and (2.49 + (-0.031)) r^2 = 0.35 ($F_{3,60}$ = 9.71, p<0.001) at 10^6/cm^2. We found that a higher percentage (75%) of the population acquired conidia in the range of 10^2 at the lower concentration level (10^6/cm^2); however at the higher concentration (10^7/cm^2), there was an equal percentage of beetles that acquired conidia in the order of 10^2 and 10^3 with a smaller proportion at 10^4 conidia/beetle. Retention of these conidia was dependent on the depth of digging.

At both concentrations, the percentage of beetles that lost all conidia increased with increased digging depth (**Table 1**). At the lower concentration, the range of conidial retention at 1-10 cm depths ranged from 0-10^3 conidia per beetle, with 40 and 20% of these beetles showing conidial counts of 10 and 10^2 conidia per beetle. At this depth, only 10% of the population retained 10^3 conidia per beetle. However, as depth increased, spore retention decreased; at depths of 11-20 cm, half of the population lost all conidia and only 30% and 20% retained conidial counts of 10 and 10^2. No beetles at this depth possessed in excess of 10^3 conidia.

At depths of 21-30 cm, a further decrease in the percentage of beetles with counts of 10^2 conidia per beetle was recorded. This decrease in conidial retention was more evident when beetles were exposed to10^7 conidia/cm^2. Conidial acquisition ranged from 10^2-10^4 conidia, with 42% of the population possessing conidia within this range. However, retention of these conidia decreased as soon as beetles started digging, which resulted in 36% losing all conidia between depths of 1-10 cm. A decrease in the percentage of the population retaining 10^2-10^4 conidia per beetle was also noted.

As beetles went deeper into the soil, a further decrease in retention was observed, with only 5% of the beetles retaining counts of 10^3 and an increase in the percentage of the beetles losing all conidia. We observed a further de-

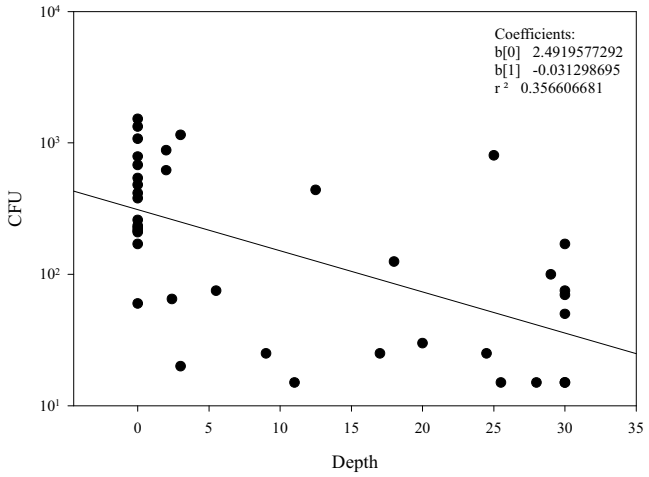

Fig. 1 Regression analysis showing the relationship between spore count per Colorado potato beetle and overwintering depth reached following an initial exposure to 10^6 conidia per cm^2. P< 0.05, CFU= colony forming units.

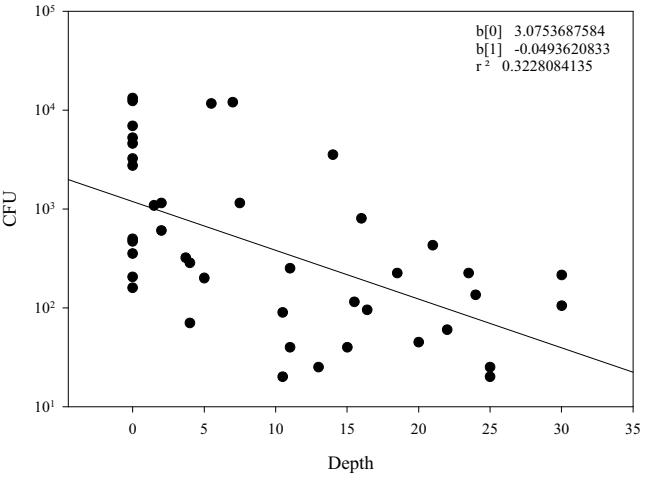

Fig. 2 Regression analysis showing the relationship between spore count per Colorado potato beetle and overwintering depth reached following an initial exposure to 10^7 conidia per cm^2. P< 0.05, CFU= colony forming units.

Table 1 Percentage of prediapause adults showing different levels of conidial retention (CFU[1] per beetle), after digging to different soil depths (N = 62 per treatment).

Inoculum	Depth reached cm (No. of beetles)	Percentage of the population retaining different levels of CFU[1] at the different depths				
		0	**10**	**10^2**	**10^3**	**10^4**
10^6/cm^2	0 (17)		6	75	19	
	1 – 10 (10)	30	40	20	10	
	11 – 20 (10)	50	30	20		
	21 – 30 (25)	54	33	13		
10^7/cm^2	0 (12)			42	42	16
	1 – 10 (19)	36	15	21	15	10
	11 – 20 (17)	41	29	23	5	
	21 – 30 (14)	46	21	35		

[1] Colony forming units

Table 2 Percentage of prediapause adults retaining different concentrations of colony forming units (CFU) following initial exposure to two conidial concentrations (N = 62 adults /concentration).

Inoculum	% adults	CFU[1] recovered
10^6conidia/cm^2	48	0
	34	10
	15	10^2
	2	10^3
10^7conidia/cm^2	40	0
	22	10
	26	10^2
	8	10^3
	4	10^4

[1] Colony forming units

Table 3 Percentage of adults found infected 45 days after digging through soil inoculated with *Beauveria bassiana* conidia.

Treatment	Number of beetles (N)	Infected beetles (%)[1]	SEM
10^6 mix	47	6 b	0.80
10^6 spray	43	7 b	0.51
10^7 mix	46	13 a	0.37
10^7 spray	43	14 a	1.07

[1]Numbers in a column followed by the same letter are not statistically different ($P \leq 0.05$, Protected LSD Test).

crease in conidial retention in individuals that reached depths of 21-30 cm. At these depths, no individuals were found retaining conidia in excess of 10^3 and 10^4 conidia per beetle. Spore retention was lower when the soil surface was treated with a lower concentration of conidia. At both treatment concentrations, a larger proportion of beetles retained conidia between 10 and 10^2 conidia per beetle. At the higher treatment concentration of 10^7, a few individuals retained 10^3 to 10^4 conidia per beetle, although the percentage of the population at this retention level was very low (**Table 2**). None of the beetles were found infected in the control group. None of the other parameters such as sex, width, length, or weight showed any relationship to conidial retention (data not presented).

Infection following soil inoculation

Significantly higher numbers of individuals were found infected when exposed to the higher soil inoculation treatment ($F_{14,43}$= 6.78, p< 0.001). No significant difference was observed whether the inoculum was sprayed on the surface or mixed into the soil at either concentration level; after 45 days, 15 and 14% infection levels, respectively, were obtained when the *B. bassiana* conidia were either applied on the surface or mixed into the top 1 cm of the soil at 10^7 conidia/cm^2 (**Table 3**). However, when the soil was inoculated with 10^6 conidia/cm^2, we found only 4 and 2% of the beetles infected in the sprayed and mixed treatments, respectively. No infection was observed in beetles exposed to buffer-Tween alone.

DISCUSSION

Soil is the natural reservoir of most entomopathogenic fungi and provides a medium for enhanced persistence, dispersal, and growth (Goettel *et al.* 2005). Insects living in and moving through the soil can acquire fungal pathogens which may result in disease development and mortality. However, retention of conidia acquired while moving though the soil medium is important to incite disease. In this study with prediapausing Colorado potato beetles, we found that conidial retention decreased as the adult beetles dug deeper into the soil horizons to overwinter. In the autumn, prediapause Colorado potato beetle adults cease feeding and either fly or walk to the hedgerows, and sometimes within the field, in search of a suitable place to overwinter. They dig into the soil and come to rest at varying depths where they enter diapause (Weber and Ferro 1994; Noronha and Cloutier 1998, 1999; Noronha *et al.* 2002). These beetles will then spend the next nine months in the soil before re-emerging in the spring to colonize newly emerged potato plants. This behaviour of congregating within the hedgerow before entering diapause provides an ideal opportunity to expose prediapause beetles to *B. bassiana* by inoculating the soil surface to increase mortality in the diapausing population. Beetles in Canada and the northern parts of the United States tend to dig to a depth of between 10-30 cm where they form a cell and enter diapause. Temperatures at which the beetles normally commence digging to overwinter would not be a limiting factor for disease development, as they are normally 20°C at 30 cm depths in the fall. We found that temperatures 5 cm below the surface in an open field followed a decreasing trend from 20°C in mid August to 15°C in early September, which should allow a beetle that has acquired conidia from inoculated soil to become infected. However, several studies have shown that the mortality as a result of *B. bassiana* infection is very low in overwintering Colorado potato beetles (Bajan *et al.* 1977; Gaugler *et al.* 1989).

The loss of conidia during digging as seen in our study would account, in part, for the low levels of overwintering mortality found in Colorado potato beetle populations. The main route of infection by entomopathogenic fungi is by penetration through the external integument of the insect, but prior to penetration, it is necessary for the fungal conidia to attach to the surface of the cuticle (Fargues 1984). The loss of conidia from the integument before germ-tube formation and penetration of the host cuticle can significantly decrease the chance of infection and eventual mortality. The process of digging results in the adults moving through several centimeters of soil which is in close contact with the body and can act as an abrasive, removing conidia acquired by the adult on the soil surface. In our study, all of the beetles acquired conidia on the surface, however when beetles were exposed to 10^7 conidia/cm^2, 30% of the beetles lost all conidia by digging to a depth of 10 cm and 50% conidial loss was noted when beetles settled at depths of 11-20 cm. In our laboratory-based study, beetles were on the surface for 12 hours, however, Noronha and Cloutier (2006) found that in the field, prediapause beetles remained on the surface for a few days following cessation of feeding and before they commenced digging. This extra time spent on the surface may influence conidial acquisition and attachment which in turn could decrease the negative effects of digging on conidial retention.

For disease to occur, an adult Colorado potato beetle must be exposed to a threshold number of *B. bassiana* conidia. Several factors influence mortality associated with *B. bassiana* such as temperature, humidity, pesticides, soil moisture etc. (Wojciechowska *et al.* 1977; Anderson *et al.* 1988; Studdert *et al.* 1990; Mietkiewski *et al.* 1992; Jaros-Su *et al.* 1999; Inglis *et al.* 2000). In the Colorado potato beetle, mortality has been observed in larvae in spring and in fall adults (Fargues *et al.* 1994; Groden and Dunn 1996; Long *et al.* 2000; Wraight and Ramos 2002; Klinger *et al.* 2006), however, percent mortality attributed to *B. bassiana* infections in the overwintering populations is very low (Gaugler *et al.* 1989; Mietkiewski *et al.* 1992). Gaugler *et al.* (1989) found that mortality was much lower after the overwintering period in spite of the fact that these beetles tunneled at favorable temperatures through *B. bassiana*-rich soil in the fall. However, they found that soil inoculation in the spring caused significantly higher mycosis in the adults emerging from diapause than when soil was treated with *B. bassiana* in the fall. Survival of conidia was not a factor in the trial as *B. bassiana* conidia have been found to survive for extended periods of time in the soil (Lingg and Donaldson 1981; Studdert *et al.*1990). The loss of a significant proportion of conidia while digging, as seen in our study, may be sufficient for the beetles to overcome any disease caused by the small proportion of conidia still lodged on the integument. In our study, we found that at lower surface concentrations, beetles acquired fewer conidia and thus retained very low levels of between 10 and100 CFU/beetle

while at higher initial exposure, a larger number of beetles (15 and 10% of the population) retained 10^3 and 10^4 CFU/ beetle respectively when resting depth was from 1-10 cm.

Resting depth may also be influenced by soil texture which in turn would affect spore retention. Studies have shown that soil texture and water availability can impact the efficacy of *B. bassiana* inoculations (Studdert *et al.* 1990). Colorado potato beetles have been found to respond to soil density, decreasing their digging depth and speed as soil density increased (Noronha and Cloutier 1998). In our study, we kept the density of the soil (clay loam) constant, however our results show that the digging process was sufficient to dislodge the conidia before penetration could occur.

The method of application of conidia, surface spray vs mixed into the soil, did not influence disease initiation in our study, however a significant increase in disease initiation was noted when beetles were exposed to a higher concentration of *B. bassiana* conidia. Our results demonstrate that 2-4% of the population was infected when beetles were exposed to an initial concentration of 10^6 conidia per cm^2 compared to 15-16% at the 10^7 exposure. Beetles in this study were allowed to dig to a depth of 10 cm. Gaugler *et al.* (1989) reported that treating the soil by either tilling *B. bassiana* conidia to 7 cm depths or a surface application in the fall before the beetles dig did not incite mycosis in the overwintering population, and mortality in overwintering beetles did not differ from the control. They obtained the same results with granular and wettable-powder applications. Cantwell *et al.* (1986) also reported that *B. bassiana* was ineffective in reducing spring emergence when applied in the fall as a soil-surface spray, but they found that similar spring application gave significant mortality.

Our study suggests that the lack of infection in the overwintering population may result from the physical removal of the conidia during the digging process leading to a decrease in disease development in overwintering populations. Furthermore, diapausing individuals may be more resistant to infection by *B. bassiana* than non-diapausing individuals (Noronha and Goettel 2009). Thus, we can conclude that fall populations may not be a viable target for inciting disease by *B. bassiana* inoculations, but treating overwintering sites in the spring may be a better option because beetles walking on the surface will acquire significant levels of conidia that could attach to the integument and remain attached long enough for penetration to occur and incite disease.

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