

Differential Susceptibility between Diapausing and Non-Diapausing Colorado Potato Beetles (*Leptinotarsa decemlineata*) Treated with *Beauveria bassiana*

Christine Noronha^{1*} • Mark S. Goettel²

¹ Crops and Livestock Research Centre, Agriculture and Agri-Food Canada, Charlottetown, Prince Edward Island, Canada

² Lethbridge Research Centre, Agriculture and Agri-Food Canada, Lethbridge, Alberta, Canada

Corresponding author: * Christine.noronha@agr.gc.ca

ABSTRACT

A laboratory-based study was conducted to examine the difference in mortality in non-diapausing and diapausing Colorado potato beetle (*Leptinotarsa decemlineata*) adults, following inoculation with *Beauveria bassiana* conidia. Results showed a higher percentage of mortality among laboratory-reared, non-diapausing beetles as compared to laboratory-reared, diapausing beetles. At the end of 30 days, 85% mortality attributed to *B. bassiana* infection was observed among the non-diapausing beetles and 20% among the diapausing beetles at a 10^6 conidia/cm² inoculation level. Beetles inoculated with 10^4 conidia/cm² showed 20% mortality due to *B. bassiana* infection among the non-diapausing beetles and no mortality among the diapausing beetles. No mortality due to *B. bassiana* was observed in the untreated controls. A similar study conducted with field-collected diapause-ready beetles gave similar results, with higher mortality due to *B. bassiana* infection being observed in the laboratory-reared, non-diapausing beetles. Our results suggest that management of the beetle by targeting pre-diapause beetles with the fungus *B. bassiana* may be a difficult proposition.

Keywords: disease development, mortality, overwintering, physiology

INTRODUCTION

Diapause is the developmental phase in an insect's life cycle which allows it to survive periods of predictable recurring adverse environmental conditions. In order to survive, most insects must synchronize their life cycle to the climatic factors which will promote development and reproduction within their geographical range (Danks 1987). The Colorado potato beetle is one such insect that can persist in northern latitudes only because of the physiological and behavioural changes that accompany winter dormancy. In Canada and other cold regions within its geographical distribution, new-generation adults of the beetle emerge from pupation in mid-summer. Decreasing photophase and temperature interacting with plant maturity act as cues to induce diapause preparation, which typically involves intense feeding to store metabolic reserves (De Wilde 1954; Danks 1987; Noronha and Cloutier 2006). Satiated beetles eventually leave the host plant in search of a suitable site where they enter the soil (Voss and Ferro 1990; Noronha and Cloutier 1999; Noronha *et al.* 2002) and dig to a depth of 10-30 cm, before coming to rest for diapause (Minder 1966; Ushatinskaya 1978; Noronha and Cloutier 1998).

The ability of this beetle to develop resistance to the insecticides used as a control measure has caused major problems for potato producers (Roush *et al.* 1990; Noronha *et al.* 2001). To delay the development of resistance and suppress populations, the use of multiple tactics is considered the best management approach (Hare 1990; Wraight *et al.* 2009). *Beauveria bassiana* is a soil-borne fungal pathogen which infects insects primarily by penetration through the cuticle and is capable of controlling up to 76% of a beetle population when applied as a foliar spray (Poprawski *et al.* 1997). However, foliar applications have limitations because conidial viability decreases in the presence of solar radiation (Hajek *et al.* 1987; Wraight and Ramos

2002) and conidia are easily removed by rain (Inglis *et al.* 2000). Since the soil offers environmental conditions that are favourable to conidial persistence (Lingg and Donaldson 1981; Gaugler *et al.* 1989; Inglis *et al.* 1997), soil inoculations can be used to target the pupae as well as the diapausing adults.

Before entering diapause, the adult beetles undergo several physiological changes which include an increase in lipid and protein content in the hemolymph, synthesis of juvenile hormones, changes to the cuticle, and the elimination of excess water and gut contents (Ushatinskaya 1978; Lefeveres 1989; Boiteau and Coleman 1996). The effects of these physiological changes on the efficacy of *B. bassiana* are unknown. Field observations indicate that mortality caused by *B. bassiana* in overwintering beetles is low (Gaugler *et al.* 1989; Miętkiewski *et al.* 1996). Our study was conducted to examine the difference in mortality between non-diapausing and diapausing Colorado potato beetle adults following inoculation with *B. bassiana* conidia.

MATERIAL AND METHODS

Beetles

Laboratory-reared: A laboratory colony of the Colorado potato beetle was started from adults collected at the Agriculture and Agri-Food Canada Research Centre research farm at Harrington, Prince Edward Island, 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 used as the food source for the beetles.

Field collected: From late August to early September, beetles were collected from field plots that had not been sprayed with

insecticides and were placed within field cages. Potato stalks were collected from the field and placed in the cages for beetles to complete feeding in preparation for diapause. Beetles were maintained in the field cages until late September when they were removed and taken to the laboratory and used for the experiment.

Diapause initiation in laboratory-reared beetles

Newly emerged beetles from the colony were placed into cages which included a potato plant (cv. 'Russet Burbank'). The cages were placed in a growth chamber under a 12:12 L:D photoperiod and a daily oscillating temperature of 10 and $18 \pm 2^\circ\text{C}$, conditions conducive to diapause induction in this beetle. A new potato plant was added to each cage as required. After two weeks, beetles that ceased feeding and showed a propensity to dig, which indicated that diapause preparations were complete, were removed and used in all subsequent experiments (Noronha and Cloutier 1998).

Soil

Top soil obtained from a field at the research farm in Harrington was used for all experiments. The soil was collected from a field which had not been sprayed with either a fungicide or insecticide during the past three years. The soil was a reddish brown sandy-loam, with a 15-cm A-horizon containing approximately 3.3% organic matter (MacDougall *et al.* 1988). 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 in the cold room at 4°C . When needed, the required amount of soil was removed and allowed to reach room temperature for 2-3 h before use.

Treatments

Beauveria bassiana isolate GHA was used for all experiments. Non-formulated conidia obtained from Laverlam International, Butte, MT, were stored at 5°C until use. Conidia were suspended in a 0.01 M sodium phosphate buffer amended with 0.05% Tween (80); a Kontes micropipette was used to reduce clumping. Concentrations of conidia were estimated using a haemocytometer and adjusted to viable conidia/ml. Conidial viability was estimated using the technique described by Goettel and Inglis (1997). The conidial suspension was then diluted to the desired concentration for application. Control treatments received an application of buffer-Tween alone.

Treatments consisted of inoculating beetles with two conidial concentrations; 10^4 or 10^6 viable conidia per cm^2 , and buffer as a control. Beetles in batches of 15-20 were placed in a Petri dish (100×15 mm) and the desired conidial suspension or buffer was sprayed onto the Petri dish using an air brush (Aztek - 3000S Airbrush Kit (Aztek Inc., USA)). Treated adults were allowed to dry for 30 min before being transferred to 29 ml plastic cups containing moist soil. Each adult was placed in a separate plastic cup. A diapausing beetle was placed at the bottom of the cup and soil was placed over it; a non-diapausing beetle was placed on the soil surface in the cup along with a leaf as food. The cups were placed in an incubator at 20°C and 16:8 L:D and were checked every two days for approximately 30 days. Beetles were checked for any

movement by gentle prodding. A beetle was considered dead if no movement was detected. Dead beetles were placed in a moist chamber to allow for fungal growth and sporulation on the cadavers, thus verifying disease (Goettel and Inglis 1997). The experiment was conducted twice over two field seasons with diapause-ready beetles collected in the fall each year, and once with laboratory-reared beetles that were induced into diapause. All non-diapausing beetles were obtained from the laboratory-reared colony.

Statistical analyses

A log-linear analysis was performed on mortality counts using PROC GENMOD with year, diapausing, and their interaction in the model (SAS 2005). Each mortality count was adjusted for the total counts per cell. Since the interaction was not significant, another analysis was performed with only year and diapausing/non-diapausing in the model.

RESULTS AND DISCUSSION

The mortality attributed to *B. bassiana* for the non-diapausing adults was significantly higher ($P < 0.01$) than for the diapausing adults. In the laboratory-reared population, 85% mortality as a result of *B. bassiana* infection was observed in the non-diapausing beetles treated with 10^6 conidia/ cm^2 whereas 20% mortality due to *B. bassiana* was recorded in the diapausing beetles (Table 1). Similarly, in the field-collected population in 2003, 62 and 8% and in 2005, 54 and 4% mortalities attributed to *B. bassiana* infection were recorded in the non-diapausing and diapausing beetles, respectively. When treated with 10^4 conidia/ cm^2 , mortality was lower; 20 and 0% in the non-diapausing and diapausing laboratory-reared beetle populations, respectively. Fifteen and 20% unexplained mortality was recorded in the controls, however none was attributed to *B. bassiana*. In the field-collected beetles treated with 10^4 conidia/ cm^2 , in 2003 and 2005, 16 and 6% mortality, respectively was recorded in the non-diapausing beetles and none in the diapausing beetles. No mortality was recorded when individuals from the field were treated with buffer (control). Laboratory-reared diapausing beetles demonstrated a delay in mortality (12 days), compared to the non-diapausing beetles (9 days). In the field-collected population, mortality generally occurred at the beginning of the trial with no major increase as the days progressed.

Several physiological changes are known to occur as insects prepare for and enter diapause. The preparative phase is thought to be essential to allow for the deposition of extra layers of cuticle for water-proofing and the synthesis of protein in the hemolymph (Danks 1987; Denlinger 2002). In the Colorado potato beetle, the concentration of metabolites in the hemolymph was found to increase during diapause preparation. Lefevre *et al.* (1989) found that the concentrations of free amino acids, glucose, lipids, and protein in the hemolymph were significantly higher in diapausing as compared to non-diapausing individuals. In addition to an increase in metabolites, diapausing insects also produce seve-

Table 1 Percentage mortality in non-diapausing and diapausing adults of the Colorado potato beetle inoculated with *Beauveria bassiana* (Bb).

Beetles	Concentration of conidia/ cm^2					
	10^4		10^6		Control	
	Percentage of dead beetles showing either Bb or other infection					
	Bb	other	Bb	other	Bb	other
2003						
Laboratory-reared Non-diapausing (N=16) ¹	6	0	62	0	0	0
Field collected Diapausing (N=16)	6	0	6	0	0	0
2005						
Laboratory-reared Non-diapausing (N=50)	16	4	54	0	0	20
Field collected Diapausing (N=76)	0	6	4	2	0	15
2005						
Laboratory-reared Non-diapausing (N=20)	20	0	85	5	0	10
Laboratory-reared Diapausing (N=20)	0	5	20	5	0	5

¹ N = population size for each treatment

ral cryoprotectants such as glycerol, sorbitol, and proline (Danks 1987 and references therein; Lefevre *et al.* 1989). The effects of these cryoprotectants on *B. bassiana* germination and growth remain unknown. Studies have shown that not only does the rate of protein synthesis in pre-diapause beetles differ, but that the type of proteins synthesized also differs markedly from reproductively active adults (Dortland and de Kort 1978). Some compounds such as fatty acids are considered to possess fungistatic properties which would prevent the growth and development of *B. bassiana*. Saito and Aoki (1983) found that fatty acids on the surface of the silkworm *Bombyx mori* and the fall webworm *Hyphantria cunea* inhibited conidial germination and hyphal growth of *B. bassiana*. Similar results were obtained from studies with the corn earworm *Heliothis zea* (Smith and Grula 1982). The fungistatic properties of these substances may explain why we found very low mortality in the treated diapausing individuals as compared to the treated non-diapausing individuals, as concentrations of these substances have been found to increase in diapausing adults (Dortland and de Kort 1978; Lefevre 1989).

Changes in water content may also play a role in decreasing pathogenicity of *B. bassiana* conidia. Minder (1966) reported that adult beetles can lose up to 25% of their cellular water and produce chemicals such as glycerol to enhance cold-hardiness. In order to prevent freezing during the winter months, adults must decrease the water content within their hemolymph (Boiteau and Coleman 1996; Danks 2000). Although these changes may prevent beetles from freezing during the winter, they may also be non-conducive to the development and growth of *B. bassiana* after penetration of the cuticle occurs.

In addition to physiological changes within the insect body, changes in the makeup of the cuticle also take place in diapausing beetles. Insect pathogenic fungi penetrate the insect cuticle using different combinations of enzymes and mechanical pressure (Goettel *et al.* 1989). The insect cuticle is multilayered; the epicuticle is a thin layer made up mainly of protein and covered by a waxy layer containing fatty acids, lipids, and sterols (Anderson 1979). Cuticular permeability is reduced in many insects during the diapause phase by an increased thickness of the wax layer (Danks 2000 and references therein). This decrease in permeability is mainly to regulate water loss and dehydration, especially in an insect such as the Colorado potato beetle that undergoes a prolonged dormancy of nine months in its temperate geographical distribution. Below the epicuticle lies the procuticle which is made up of chitin fibers embedded in protein and lipids; protein makes up almost 70% of the cuticle. Mechanical extensibility of the cuticle has also been found to change in diapausing insects with the cuticle of diapausing insects being stiffer than that of non-diapausing ones as was seen in the studies on the bean bug *Riptortus clavatus* (Morita *et al.* 1999). Although penetration of the cuticle by fungal pathogens happens through enzymatic degradation (Goettel *et al.* 1989; Gupta *et al.* 1992), changes in the thickness of the cuticle may prevent or hinder easy penetration by fungal pathogens. Penetration of the thinner more pliable cuticle of non-diapausing individuals may be easier than the thicker cuticle of diapausing individuals.

In a previous study, we demonstrated that digging through the soil affected the retention of *B. bassiana* conidia by the beetles (Noronha and Goettel 2009). Consequently, in the present study we placed the diapausing individuals directly in soil while non-diapausing individuals were placed on the soil surface. The diapausing beetles would have had some movement within the soil as they prepared their overwintering cell, thereby possibly shedding some inoculum, however movement would have been minimal. In addition, the soil microflora may have had some antagonistic effects on conidial viability and germination, thereby affecting the infection rates and subsequent mortality.

The apparent increased resistance to infection by diapausing adults, coupled with the fact that inoculum is lost

while the beetles dig into the ground for overwintering (Noronha and Goettel 2009), may explain why field studies evaluating the efficacy of *B. bassiana* in reducing diapausing adults generally report very low mortality (Gaugler *et al.* 1989; Mietkiewski *et al.* 1996). Thus we can conclude that inciting disease within a diapausing population by targeting pre-diapausing adults may be difficult to accomplish in a beetle-management program using *B. bassiana*.

ACKNOWLEDGEMENTS

The authors would like to thank Dr. Rick Peters for critically reviewing this manuscript Mary Smith for technical assistance, and Toby Entz for statistical analyses of the data. The research was funded by Agriculture and Agri-Food Canada.

REFERENCES

- Anderson SO (1979) Biochemistry of insect cuticle. *Annual Review of Entomology* **24**, 17-24
- Boiteau G, Coleman W (1996) Cold tolerance in the Colorado potato beetle *Leptinotarsa decemlineata* (Say) (Coleoptera: Chrysomelidae). *Canadian Entomologist* **128**, 1087-1099
- Danks HV (1987) Insect dormancy: An ecological approach. *Biological Survey of Canada Monograph Series No 1*, Ottawa, Canada, 439 pp
- Danks HV (2000) Dehydration in dormant insects. *Journal of Insect Physiology* **46**, 837-852
- Denlinger D (2002) Regulation of diapause. *Annual Review of Entomology* **47**, 93-122
- De Wilde J (1954) Aspects of diapause in adult insects with special regard to the Colorado potato beetle *Leptinotarsa decemlineata* (Say). *Archives Néerlandaises de Zoologie* **10**, 375-385
- Dortland JF, de Kort CAD (1978) Protein synthesis and storage in the fat body of the Colorado potato beetle. *Insect Biochemistry* **8**, 93-98
- Gaugler R, Costa SD, Lashomb J (1989) Stability and efficacy of *Beauveria bassiana* soil inoculations. *Environmental Entomology* **18**, 412-417
- Goettel MS, Inglis GD (1997) Fungi: Hyphomycetes. In: Lacey L (Ed) *Manual of Techniques in Insect Pathology*, Academic Press, San Diego, pp 213-249
- Goettel MS, St. Leger RJ, Rizzo NM, Staples RC, Roberts DW (1989) Ultrastructural localization of the cuticle-degrading protease produced by entomopathogenic fungus *Metarhizium anisopliae* during penetration of host (*Manduca sexta*) cuticle. *Journal of General Microbiology* **135**, 2233-2239
- Gupta SC, Leathers TD, El-Sayed GN, Ignoffo CM (1992) Insect cuticle-degrading enzymes from the entomogenous fungus *Beauveria bassiana*. *Experimental Mycology* **16**, 132-137
- Hajek AE, Soper RS, Roberts DW, Anderson TE, Biever KD, Ferro DN, LeBrun RA, Storch RH (1987) Foliar applications of *Beauveria bassiana* (Balsamo) Vuillemin for control of the Colorado potato beetle, *Leptinotarsa decemlineata* (Say) (Coleoptera: Chrysomelidae): An overview of pilot test results from the northern United States. *Canadian Entomologist* **119**, 959-974
- Hare J D (1990) Ecology and management of the Colorado potato beetle. *Annual Review of Entomology* **35**, 81-100
- Inglis GD, Duke GM, Kanagaratnam P, Johnson DL, Goettel MS (1997) Persistence of *Beauveria bassiana* in soil following application of conidia through crop canopies. In: Goettel MS, Johnson DL (Eds) *Microbial Control of Grasshoppers and Locusts. Memoirs of the Entomological Society of Canada* **171**, pp 253-263
- Inglis GD, Ivie TJ, Duke GM, Goettel MS (2000) Influence of rain and conidial formulation on persistence of *Beauveria bassiana* on potato leaves and Colorado potato beetle larvae. *Biological Control* **18**, 55-64
- Lefevre KS, Koopmanschap AB, de Kort CAD (1989) Changes in the concentration of metabolites in hemolymph during and after diapause in female Colorado potato beetle *Leptinotarsa decemlineata*. *Journal of Insect Physiology* **35**, 121-128
- Ling AJ, Donaldson MD (1981) Biotic and abiotic factors affecting stability of *Beauveria bassiana* conidia in soil. *Journal of Invertebrate Pathology* **38**, 191-200
- MacDougall JI, Veer C, Wilson F (1988) Soils of Prince Edward Island. *Agriculture Canada Publication No. 8354*, 210 pp
- Mietkiewski R, Sapięha A, Tkaczuk C (1996) The effects of soil-borne entomogenous fungi on the mycoses of the Colorado potato beetle during hibernation period. In: *Insect Pathogens and Insect Parasitic Nematodes. Bulletin of the Proceedings of the First Joint Meeting of the IOBC/WPRS and IOBC/EPRS*, 27 August-1 September 1995, Poznan, Poland, **19**, 162-165
- Minder IF (1966) Hibernation conditions and survival rates of the Colorado potato beetle in different types of soils. In: Arnoldi KV (Ed) *Ecology and Physiology of Diapause in the Colorado Beetle*. Indian National Scientific Documentation Center, New Delhi (reprinted by the USDA), pp 28-58
- Morita A, Soga K, Hoson T, Kamisaka S, Numata H (1999) Changes in mechanical properties of the cuticle and lipid accumulation in relation to adult diapause in the bean bug, *Riptortus clavatus*. *Journal of Insect Physiology* **45**,

241-247

- Noronha C, Cloutier C** (1998) Effect of soil conditions and body size on digging by prediapause Colorado potato beetles (Coleoptera: Chrysomelidae). *Canadian Journal of Zoology* **76**, 1705-1713
- Noronha C, Cloutier C** (1999) Ground and aerial movement of adult Colorado potato beetle (Coleoptera: Chrysomelidae) in a univoltine population. *Canadian Entomologist* **131**, 521-538
- Noronha C, Duke GM, Chinn JM, Goettel MS** (2001) Differential susceptibility to insecticides by *Leptinotarsa decemlineata* (Coleoptera: Chrysomelidae) populations from western Canada. *Phytoprotection* **82**, 113-121
- Noronha C, Duke GM, Goettel MS** (2002) Damage potential and phenology of the Colorado potato beetle (Coleoptera: Chrysomelidae) on potato in southern Alberta. *Phytoprotection* **83**, 89-98
- Noronha C, Cloutier C** (2006) Effects of potato foliage and temperature regime on prediapause Colorado potato beetle *Leptinotarsa decemlineata* (Coleoptera: Chrysomelidae). *Environmental Entomology* **35**, 590-599
- Noronha C, Goettel MS** (2009) The effect of digging by the Colorado potato beetle (*Leptinotarsa decemlineata*) (Coleoptera: Chrysomelidae) on the acquisition and retention of *Beauveria bassiana* conidia. In: Tennant P, Benkeblia N (Eds) Potato II. *Fruit, Vegetable and Cereal Science and Biotechnology* **3** (Special Issue 1), 59-64
- Poprawski TJ, Carruthers IR, Speese J III, Vacek DC, Wendel LE** (1997) Early-season applications of the fungus *Beauveria bassiana* and introduction of the hemipteran predator *Perillus bioculatus* for control of Colorado potato beetle. *Biological Control* **10**, 48-57
- Roush RT, Hoy CW, Ferro DN, Tingey WM** (1990) Insecticide resistance in the Colorado potato beetle (Coleoptera: Chrysomelidae): Influence of crop rotation and insecticide use. *Journal of Economic Entomology* **83**, 315-319
- Saito T, Aoki J** (1983) Toxicity of free fatty acids on the larval surfaces of two lepidopterous insects towards *Beauveria bassiana* (Bals.) Vuill. and *Paecilomyces fumoso-roseus* (Wize) (Deuteromycetes: Moniliales). *Applied Entomology and Zoology* **18**, 225-233
- SAS** (2005) SAS OnlineDoc® 9.1.3. SAS Institute Inc., Cary, NC
- Smith RJ, Grula EA** (1982) Toxic components on the larval surface of the corn earworm (*Heliothis zea*) and their effects on germination and growth of *Beauveria bassiana*. *Journal of Invertebrate Pathology* **39**, 15-22
- Ushatinskaya RS** (1978) Seasonal migration of adult *Leptinotarsa decemlineata* (Insecta, Coleoptera) in different types of soil and physiological variations of individuals in hibernating population. *Pedobiology* **18**, 120-126
- Voss RH, Ferro DN** (1990) Phenology of flight and walking by Colorado potato beetle (Coleoptera: Chrysomelidae) adults in western Massachusetts. *Environmental Entomology* **19**, 117-122
- Wraight SP, Ramos ME** (2002) Application parameters affecting field efficacy of *Beauveria bassiana* foliar treatments against Colorado potato beetle *Leptinotarsa decemlineata*. *Biological Control* **23**, 164-178
- Wraight SP, Lacey LA, Kabaluk RT, Goettel MS** (2009) Potential for microbial biological control of Coleopteran and Hemipteran pests of potato. In: Tennant P, Benkeblia N (Eds) Potato II. *Fruit, Vegetable and Cereal Science and Biotechnology* **3** (Special Issue 1), 25-38