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**Optimization of *Beauveria bassiana* in a spray formulation
against *Choristoneura fumiferana*.**

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Abstract

Most mycoinsecticides require a carrier of natural or synthetic oils for them to be effective when sprayed on forest defoliating caterpillars such as the eastern spruce budworm (*Choristoneura fumiferana*). Two isolates of *Beauveria bassiana* conidia were formulated in natural (vegetable) and synthetic (mineral) oils and tested against late instar larvae of the budworm. Topical application showed that the ARSEF 1850 isolate to be more effective than a commercially available strain. Additional laboratory spray trials using a hand-held spinning disc sprayer showed that ARSEF 1850 natural oil formulation significantly increased mortality when compared to controls. The use of oil formulated *B. bassiana* conidia should be considered for future budworm management under the integrated pest management of this pest in North America.

Keywords

Eastern spruce budworm, entomopathogenic fungi, *Beauveria bassiana*, formulation

38 **L'optimisation de la formulation de *Beauveria bassiana* dans**
39 **une pulvérisation contre - *Choristoneura fumiferana*.**

Résumé

La plupart des mycoinsecticides exigent l'emploi d'huiles naturelles ou synthétiques afin d'en assurer l'efficacité lorsqu'ils sont pulvérisés sur les lépidoptères forestiers défoliateurs comme la tordeuse des bourgeons de l'épinette (*Choristoneura fumiferana*). Deux isolats des conidies *Beauveria bassiana* ont été formulées dans les huiles naturelles (végétales) et synthétiques (minéraux) et sont testés contre des larves de dernier stade des tordeuses des bourgeons de l'épinette. Des applications topiques ont montré que la isolats ARSEF 1850 est plus efficace disponible commercialement. Les essais supplémentaires de laboratoire, en utilisant un pulvérisateur manuel à disques rotatifs, ont montré que la formulation d'huile naturelle (ARSEF 1850) a considérablement augmenté la mortalité lorsqu'on les compare à des témoins. L'utilisation d'une conidie *B. bassiana* à base d'huile devrait être considérée pour la gestion de l'avenir de la tordeuse des bourgeons de l'épinette sous la gestion intégrée des organismes nuisibles comme ce ravageur en Amérique du Nord.

Introduction

There are several lepidopteran species that are considered pests of trees in the Boreal Forest. Of them, the eastern spruce budworm, *Choristoneura fumiferana* (Clemens) is most notable. This species is a serious pest of conifers of North America and has a long history of outbreaks dating back several hundred years (Blais 1983; Morin et al. 1993; Krause 1997; Boulanger and Arsenault 2004)

The management of forest insect pest populations is achieved mainly by the use of all available strategies. Targeted insecticide use is a strategy that is used under the Integrated Pest Management (IPM) approach, however all attempts are made to limit their harm to other components of the ecosystem. During the past two decades there has been a concerted effort to reduce the reliance on broad-spectrum chemical insecticides in forestry and to employ other environmentally acceptable strategies. The main microbial agent that is used today against forest pest Lepidoptera is the bacterium *Bacillus thuringiensis* (Bt) which is relied upon heavily in many jurisdictions. It is within the IPM ideology that the importance of seeking alternative strategies becomes apparent. The entomopathogenic fungus, *Beauveria bassiana* (Balsama) Vuillemin (Ascomycota: Hypocreales) has been known for 170 years (Zimmerman 2007). It is a naturally occurring soil-dwelling fungus that grows as a saprophyte (Bidochka et al. 1998) but it is also a highly effective entomopathogenic fungus that causes diseases in a variety of insect species, mostly in the orders: Lepidoptera, Coleoptera and Homoptera. In fact, *B. bassiana* has been one of the most studied entomopathogenic fungi and a considerable volume of literature has accumulated on its biology, hosts, effects on non-target

organisms, and safety towards vertebrates (e.g., Ferron 1985; Goettal et al. 1990; Saik et al. 1990; Semalulu et al. 1992; Feng et al. 1994; Vestergaard et al. 2003; Zimmerman 2007).

Shortly after the turn of the 20th century, fungal species were field-tested against different forestry pests. Because the fungi have strict environmental requirements for germination that were not met at the time, the applications failed to produce significant mortality. Entomopathogenic fungi were labeled as ineffective and scientists in the western countries lost interest in these organisms (Ferron 1985). The use of fungi (*B. bassiana* in particular) has not been exploited as potential controlling agents against serious forest Lepidoptera in North America and Europe. The commercial use of *B. bassiana* in North America has been limited mainly to greenhouses and agricultural plots. This use in North America contrasts significantly with China, where *B. bassiana* was successfully applied on pine feeding Lepidoptera over a 36-year period (Feng et al. 1994; Li 2007; Wang and Feng 2014). The limiting environmental factor for fungal spore germination is their requirement for high humidity. Advances in the formulation of the fungal spores in natural and synthetic oils have practically eliminated the problem of the high humidity requirement (Bateman et al. 1993).

Initial research by Hicks (2007) involved the selection of Lepidoptera specific *B. bassiana* isolates. The research showed that an isolate originally taken from a *Choristoneura* sp. caterpillar was very effective in lab bioassays against this insect. Furthermore, it was shown that this fungus had good potential for further development

because of good long-term storage and its low impact on non-target organisms (Hicks 2007; Hicks et al. 2011).

The purpose of this research is to optimize a formulation of *B. bassiana* that could effectively be sprayed against forest caterpillars (namely the spruce budworm).

Methods and Materials

Fungus. A fungal isolate (ARSEF 1850 - Dr. R Humber, USDA Plant Protection Research Unit, Ithica, NY) that was identified earlier by Hicks *et al.* (2011) as effective against *C. fumiferana*, was grown on Sabouraud maltose + yeast extract agar in 9cm Petri dishes. Spores were harvested by suction using a Mycoharvester Mk 5 (VBS Agriculture Ltd., Beaconsfield, UK). Botanigard® (Koppert Canada Ltd, Scarborough, Ontario), a commercial *B. bassiana* product, was also tested.

Spruce budworm. Spruce budworm 2nd instar larvae, located within their overwintering hibernacula, were supplied by Insect Production Services (Canadian Forest Service, Sault Ste. Marie, Ont.). The larvae were reared in 30ml plastic cups with paper lid inserts on an artificial diet (Insect Production Services, Canadian Forest Service, Sault Ste. Marie, Ont.) in a ConViron® environmental growth chamber (Controlled Environments Ltd., Winnipeg, Manitoba) at 23°C, 75% relative humidity, and 18L:6D photoperiod.

Sprayer. A Micron “ulva+” hand-held spinning disc sprayer (Dramm Corporation Canada, Fenwick, Ontario) was used during the spray trials.

110

111 **Formulation.** Bateman *et al.* (2007) outlined some of the procedures for formulating
112 fungal spores for spraying. The spores were delivered to the spray-head in a formulation
113 consisting of oil that was either a general vegetable cooking oil containing canola and/or
114 soybean oil (Sardo Foods, Bolton, Ont.) or a mineral oil (Fischer Scientific). In both
115 cases, kerosene (Canadian Tire Corp.) was used as the solvent. Different mixtures were
116 passed through a standard B2 viscosity cup (ElectroPhysik USA, Inc, Arlington Heights,
117 Illinois, USA.) to determine their viscosities. The correct viscosity for spraying through a
118 Micron “Ulva+” sprayer is one that will flow through the viscosity cup in 45 seconds.

119

120 **Topical applications of formulations containing Botanigard®.** 30 grams of
121 Botanigard® was suspended in 300ml of the formulation to be tested. The initial tests
122 determined the efficacy of the product with topical application only. In this case, four µl
123 of the formulation was applied directly to the mid-dorsal surface of 6th instar larvae using
124 a micropipette. Ten larvae composed a replicate and three replicates were treated.
125 Controls consisted of three replicates of sterile water treated larvae and three replicates of
126 the tested carrier mixture without the fungal spores. The larvae were rear individually in
127 30ml cups supplied with ample diet. The larvae were reared in the growth chamber under
128 conditions stated above and monitored daily for mortality or signs of fungal disease until
129 pupation. Dead larvae were placed in saturated environments to see if sporulating fungi
130 would become evident.

131

132 **Spray trials of formulations containing Botanigard® and ARSEF 1850.** Calibration

of the spraying trials was accomplished using the procedures of Bateman *et al.* (2007). Field studies that employ ultra-low volume (ULV) application rates of two litres per hectare are common for mycoinsecticide spraying. Experimentation showed that, in the lab, such a rate would be accomplished if the sprayer was held (one metre high) over the insects for a three second time period. Spore formulations were made using both Botanigard[®] and ARSEF 1850. For each strain, 0.5g of spores was suspended in 100ml of the 50:50 (vegetable oil to kerosene) carrier. The sprayer was fitted with the red restrictor that limited the flow rate to 74.4 ml/min. An improved Neubauer haemocytometer was used to count the number of spores for the ARSEF formulation. It was determined to have 3.35×10^6 spores/ml. According to the number of spores per gram indicated on the Botanigard[®] label, the formulation had 2.2×10^8 spores/ml. For spraying, ten larvae (comprising one replicate) were placed onto a 1 m² metal pan lined with paper toweling with the sprayer held over the pan for three seconds. Four replicates were treated. An additional four replicates were left untreated; four with distilled water and four with the 50:50 (vegetable oil to kerosene) carrier as controls. We conducted a second experiment involving fungal spores suspended in the carrier where four replicates of larvae were sprayed for six seconds. Larvae were placed individually in their own 30ml cup containing diet and reared under the same environmental conditions as the main culture (see above). Larvae were monitored daily for mortality until pupation. Any cups with dried diet were replaced with a cup containing fresh diet.

Spray trials using Botanigard[®] and ARSEF 1850 with spray droplet size and density measurements to more accurately determine amounts applied. To determine the

amount of fungal spores reaching the larvae, we measured the mean droplet density and mean droplet volume. Mean density (drops per mm²) was measured by spraying the formulation onto five clean standard microscope slides (at 1 m high for 3 seconds) and then counting the total drops within five random 1mm² squares on each slide (25 total counted) under microscopy. To determine the mean volume, we sprayed the formulation onto spruce budworm larva silk that was suspended across the well of a depression slide. Five slides were sprayed and 27 droplets that landed on the silk threads were chosen randomly and measured. The diameters of the droplets were measured under a Leica MZ6 stereomicroscope using LAS measuring software (Leica Microsystems Canada, Toronto, Ont.)(Fig. 1). Mean volume of the droplets was calculated from the diameter measurements (see Anonymous (online) for formula calculations).

For the Botanigard[®] spray trials, 20g of Botanigard[®] spores were suspended in 200ml of the 50:50 (vegetable oil to kerosene) carrier. It was determined that the smaller yellow restrictor produced spray droplet diameters of the size required in ULV spraying. In this case, the flow rate was reduced to 31.3 ml/min. Ten larvae (comprising one replicate) were placed onto a 1m² metal pan lined with paper toweling with the sprayer held over the pan for three seconds. Five replicates were treated. An additional five replicates were sprayed with distilled water and five with the 50:50 (vegetable oil to kerosene) carrier as controls. We conducted a second experiment involving the Botanigard[®] spores suspended in the carrier where five replicates of larvae were sprayed for five seconds. Larvae were placed individually in 30ml cups containing diet and reared under the same environmental conditions as the main culture (see above). Larvae were monitored daily

for mortality until pupation.

For the ARSEF 1850 spray trials, 6.82g of ARSEF 1850 spores were suspended in 50ml of the 50:50 (vegetable oil to kerosene) carrier. As with the Botanigard[®] trial above, the smaller yellow restrictor was used and the flow rate was 31.3 ml/min. 10 larvae (comprising one replicate) were placed onto a 1m² metal pan lined with paper toweling with the sprayer held over the pan for three seconds. Four replicates were treated. An additional four replicates were unsprayed and four with the 50:50 (vegetable oil to kerosene) carrier as controls. Another four replicates were sprayed for six seconds. Larvae were placed individually in 30ml cups containing diet and reared under the same environmental conditions as the main culture (see above). Larvae were monitored daily for mortality until pupation.

Statistical analysis. Where applicable, analysis of variance (ANOVA) was used to determine the significance between treatments and controls (Minitab Version 14).

Results and Discussion

Carrier mixture selection. Upon experimentation with the viscosity cup it was determined that two mixtures of oil and kerosene would adequately flow through the cup in the required 45 second time frame. Form-1 had a 70:30 mixture of mineral oil and kerosene while form-2 had 50:50 of vegetable oil and kerosene.

Topical application onto 6th instar spruce budworm larva. The total number of

Botanigard[®] fungal spores in the four μl applied to each larva was 9.0×10^6 spores. That amount of spores topically applied to budworm larvae was detrimental to the budworm as no larvae survived no matter what oil carrier was used (Table 1). The 70:30 (mineral oil to kerosene) carrier alone negatively impacted the larvae with only 13.3% surviving to pupation. That is considerably lower than the water treated controls (90% surviving). The 50:50 (vegetable oil to kerosene) carrier had 46.7% surviving. It was concluded that the 50:50 (vegetable oil to kerosene) carrier would be best to explore further. An additional conclusion here was that Botanigard[®] appeared to be effective in killing spruce budworm larvae.

Spray trials of formulations containing Botanigard[®] and ARSEF 1850. It is difficult to determine how many spores actually land on the caterpillars when spraying with a suspension. The red restrictor on the spray-head limited the flow rate to 74.4 ml/min. For the ARSEF formulation, 3.3×10^5 spores/ml were counted using the improved Neubauer haemocytometer. Therefore 1.2×10^6 spores were released from the spray-head over the 3 seconds that the sprayer was held in place. If we assume that the plume of the spray covered 3m^2 and that the mean surface area of a 6th instar larvae is about 70.69mm^2 , then it is estimated that approximately 40 spores may have landed on each larva. This amount is very low compared to Hicks (2007) who showed the LD_{50} for this fungal strain on 6th instar larvae was 27,000 spores per larva. The data for the present study showed 82.5% survival of larvae sprayed for 3 seconds (Table 2). When we sprayed the larvae for double time (6 seconds = 80 spores/larva) the percent surviving decreased to 60%. This was significantly lower than the untreated and water treated

controls, but failed (marginally) to reach statistical significance with the formulation control ($P = 0.058$).

For the Botanigard[®] spray, the red restrictor allowed 8.18×10^8 spores to be released from the spray-head. Again, if we assume that the plume covered three m^2 and that the surface area of a typical 6th instar larva is 70.69mm^2 , then 27,648 spores may have landed on each sprayed larva. While this is comparable to the LD_{50} indicated by Hicks (2007) it must be noted that this is a different strain and is thought not to be as effective as one isolated from a spruce budworm (Hicks 2007). The present data suggests that the ARSEF strain is more effective because, while there were a significantly smaller number of ARSEF spores sprayed onto larvae, the percent surviving was the same as Botanigard[®].

Spray trials using Botanigard[®] and ARSEF 1850 with spray droplet size and density measurements to more accurately determine amounts applied. For this trial, the yellow restrictor was used on the spray head and this reduced the flow rate to 31.3 ml/min. At this flow rate, 1.57 ml was sprayed during the three seconds that the sprayer was held into position. The measurement of mean droplet density showed 1.84 drops/ mm^2 . The mean droplet diameter was used to calculate mean droplet volume of 7.54×10^{-6} ml. The surface area of a generalized 6th instar budworm larva is about 70.69mm^2 . Each larva thus received about 130.1 droplets ($70.69 \text{mm}^2 \times 1.84 \text{ droplets/mm}^2$). The volume applied to a budworm larva was 1.95×10^{-3} ml/larva ($130.1 \text{ droplets} \times 7.54 \times 10^{-6} \text{ ml}$). With 20g of Botanigard[®] spores mixed in 200ml of carrier,

there was 4.4×10^9 spores/ml (determined by the information indicated on the Botanigard[®] label). The number of Botanigard[®] spores sprayed on a larva over three seconds was 8.58×10^6 spores/larva. For a five second spray, there were 1.43×10^7 spores sprayed on each larva. The application of Botanigard[®] spores to spruce budworm larvae showed a typical dose-response (Table 3). As the amount of Botanigard[®] applied increased, the percent of surviving larvae was significantly reduced. The amounts of Botanigard[®] sprayed that caused mortality was considerably higher than the amount of the *B. bassiana* strains used by Hicks (2007). This is not surprising as the strain of *B. bassiana* occurring in Botanigard[®] was initially isolated from a grasshopper (Orthoptera) and would likely not be as effective across different insect orders. Xu (1988) indicated that isolates of *B. bassiana* tend to have higher virulence to their original hosts, or species closely related to the original hosts. Alternatively, we cannot rule out the possibility that the Botanigard[®] was negatively affected by the oil carrier.

For the ARSEF 1850 trials, the mean droplet volume was 7.54×10^{-6} ml, each larva received 130.1 droplets and the volume applied was 1.95×10^{-3} ml/larva. With 6.82g of ARSEF 1850 spores suspended in 50ml of the 50:50 (vegetable oil to kerosene) carrier, there were 1.4×10^6 spores/ml (determined using an improved Neubauer haemocytometer). The number of spores sprayed on each larva for 3 seconds was 8190 spores/larva while 16380 spores/larva were sprayed onto each larva over six seconds. Table 4 shows that spraying with ARSEF 1850 was very effective against 6th instar larvae of the spruce budworm. The application of 8190 spores/larva caused 42.5% surviving larvae while 16380 spores/larva resulted in only 15% surviving. Hicks (2007) showed

the LD₅₀ for this fungal isolate suspended in water and topically applied to 6th instar larvae was 27,000 spores per larva. The present data indicates that formulating the spores in 50:50 (vegetable oil to kerosene) increased the virulence of this strain. Prior et al. (1988) showed that oil formulations of *B. bassiana* were 30 times more effective at controlling *Pantorhytes plutus* (Coleoptera: Curculionidae) than water formulated conidia. Similarly, Moslin et al. (2004) showed increased infectivity of *B. bassiana* toward larvae of the oil palm bagworm, *Metisa plana* (Lepidoptera: Psychidae) when formulated in either natural (Soybean) or synthetic (Paraffin = mineral) oils. Ummidi and Vadlamani (2014) bioassayed *B. bassiana* conidia formulated in several natural oils against *Spodoptera litura* (Lepidoptera: Noctuidae) and showed that all formulations displayed higher mortalities in the targeted pest than compared to unformulated conidia. Kirubakaran et al. (2014) showed that canola oil-formulated *B. bassiana* conidia was more virulent to all stages of the rice leafroller, *Cnaphalocrocis medinalis* (Lepidoptera: Pyralidae). Finally, similar results have been shown for *Metarhizium flavoviride* (Bateman et al. 1993; Ummidi and Vadlamani 2014; Kirubakaran et al. 2014), *Nomuraea rileyi*, and *Isaria tenuipes* (Vega-Aquino et al. 2010).

Conclusions

Entomopathogenic fungi are affected by many environmental factors, including low humidity, UV radiation, and temperatures. In the past, failure of entomopathogens in field experiments occurred because these limiting factors were not taken into consideration with the consequence of labelling entomopathogens ineffective for use in forestry. This is not the case now as several studies have shown that formulating fungal

spores in natural and synthetic oils minimize the limiting factors and have opened the doors to their use in forest ecosystems under an IPM approach. Delgado et al. (1999) showed that *B. bassiana* conidia formulated in synthetic oil combined with concentrations of Dimilin[®] (Diflubenzuron) had synergistic effects on grasshoppers in the Sahel of Africa. If *B. bassiana* can be formulated to be effective under low humidity conditions of the Sahel, then its use in the Boreal Forest is achievable. The present study has shown that formulation of *Beauveria bassiana* conidia in a natural oil (i.e. vegetable oil) carrier is effective in controlling spruce budworm larvae when sprayed with ultra-low volume regimes. While some caution should be exercised when interpreting the values calculated as several assumptions were made that may have affected the true values, the general results showed that this fungus has considerable promise in IPM of the Eastern Spruce Budworm. Future trials should involve more complex greenhouse and/or field experiments.

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Draft

Figure 1. A spray droplet that settled on a thread of spruce budworm silk that was suspended across the well of a depression slide. This allowed for a more accurate measure of the diameter of the droplet sphere.

Table 1. Topical treatments of 6th instar spruce budworm larvae with mixtures of natural and synthetic oils in kerosene and formulations of spores of the commercial product Botanigard[®].

Treatment	Reps. (total insects treated)	% surviving (SE)
Distilled water	2(20)	90.0 (1.0)
Form-1 [†]	3(30)	13.3 (0.67)a*
Form-2 [‡]	3(30)	46.7 (0.33)
Botanigard+Form-1	3(30)	0 (0)a
Botanigard+Form-2	3(30)	0 (0)a

[†] Form-1 consisted of 70:30 (mineral oil to kerosene)

[‡] Form-2 consisted of 50:50 (general cooking vegetable oil to kerosene)

* Values followed by the same letter indicates no significant difference P = 0.05

Table 2. Spray tests for test formulations of *Beauveria bassiana* strains against spruce budworm larvae.

Treatment	# spores applied	Reps (total larvae treated)	% surviving (SE)
untreated	-	4(40)	100 (0)a*
Distilled water	-	4(40)	100 (0)a
Form-2 [†]	-	4(40)	95 (0.29)ab
Botanigard [®] + Form2 - 3secs	27,648	4(40)	82.5 (1.03)ab
ARSEF 1850 + Form2 - 3 secs	40	4(40)	82.5 (1.03)ab
ARSEF 1850 + Form2 - 6 secs	80	4(40)	60 (1.47)b

[†] Form-2 consisted of 50:50 (general cooking vegetable oil to kerosene)

* Values followed by the same letter indicates no significant difference P = 0.05

Table 3. Spray trials using a formulation of Botanigard[®] against spruce budworm larvae where the droplet density and diameter were determined.

Treatment	# spores applied	Reps (total larvae treated)	% surviving (SE)
Distilled water	-	5(50)	96 (0.40)a [*]
Form-2 [†]	-	5(50)	94 (0.25)ab
Botanigard [®] + Form2 -3 secs	8.58x10 ⁶	5(50)	66 (1.21)b
Botanigard [®] + Form2 -5 secs	1.43x10 ⁷	5(50)	26 (0.60)

[†] Form-2 consisted of 50:50 (general cooking vegetable oil to kerosene)

^{*} Values followed by the same letter indicates no significant difference P = 0.05

Table 4. Spray trials using a formulation of ARSEF 1850 against spruce budworm larvae where the droplet density and diameter were determined.

Treatment	# spores applied	Reps (total larvae treated)	% surviving (SE)
untreated	-	4(40)	97.5 (0.25)a [*]
Form-2 [†]	-	4(40)	92.5 (0.50)a
ARSEF 1850 + Form2 -3 secs	8190	4(40)	42.5 (1.03)
ARSEF 1850 + Form2 -6 secs	16380	4(40)	15.0 (0.29)

[†] Form-2 consisted of 50:50 (general cooking vegetable oil to kerosene)

^{*} Values followed by the same letter indicates no significant difference P = 0.05