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Spinosad and Mixtures of an Entomopathogenic Fungus and Pyrethrins for Control of *Sitona lineatus* (Coleoptera: Curculionidae) in Field Peas

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Abstract

The pea leaf weevil, *Sitona lineatus* L., is an important pest of field peas and faba beans in most temperate regions. As no information is currently available on efficacy of biopesticides for *S. lineatus* control, laboratory bioassays were performed to evaluate the impact of biopesticides (spinosad, *Beauveria bassiana* strain GHA, pyrethrins, *B. bassiana* GHA + pyrethrins, and *B. bassiana* GHA + azadirachtin) against adults of this pest. The concentrations used in this bioassay were 0.1, 0.5, 1.0, and 2.0 times the lowest labeled application rate of each product. Results were further verified in cage experiments by assessing biopesticide effects on adult mortality and feeding damage in pea plants. The impact of biopesticides on mortality of larvae of two beneficial species, *Chrysoperla carnea* and *Adalia bipunctata*, was also tested in laboratory conditions. We found spinosad to be the most promising candidate, causing 100% adult mortality at high and medium concentrations. *Beauveria bassiana* and its combination with pyrethrins caused 60–62% adult mortality, but only at the highest concentration. In contrast, *B. bassiana* + azadirachtin and pyrethrins treatments caused only minimal adult mortality at all concentrations. In cage experiments, spinosad and *B. bassiana* + pyrethrins had significant effects on adult mortality and provided foliage protection from adult feeding. Conversely, the fungus treatment alone showed inconsistent performance. *Beauveria bassiana* and spinosad were generally harmless to *C. carnea* and *A. bipunctata* larvae, but *B. bassiana* + pyrethrins was toxic toward beneficial species. These results could help to improve integrated pest management programs intended to control *S. lineatus*.

Key words: pea leaf weevil, pulse crop, biological control, biopesticide, natural enemy

The pea leaf weevil, *Sitona lineatus* L., native to Europe and North Africa, is an economically important pest of field peas, *Pisum sativum* L. and faba beans, *Vicia faba* L. (Fabales: Fabaceae) in most temperate regions of the world (Baliddawa 1984, Landon et al. 1995, Cárcamo et al. 2018). Although adult weevils feed on several legume species, they prefer to feed on field peas and faba beans, both of which support population growth and larval development (Baliddawa 1984, Schotzko and O'Keeffe 1988, Landon et al. 1995, Cárcamo et al. 2018). In North America, *S. lineatus* causes economic losses from larval feeding on root nodules in the late spring and summer and, to a lesser degree, from adult feeding on foliage in spring (Wanner 2016).

The most common means of controlling *S. lineatus* is the use of seeds treated with imidacloprid or thiamethoxam, combined with foliar insecticide applications when adults damage 30% of seedlings during the second to sixth node stages of field pea plants (Cárcamo et al. 2012, 2015). However, sole reliance on insecticide-based pest management may increase the risk of resistance development in *S. lineatus* populations, as well as harm the environment and nontarget organisms. For instance, Briggs (2015) reported a failure to control pea leaf weevil in England with pyrethroids in 2014 and showed that the weevils were resistant to this insecticide. Hence, there is a need for alternative pest management strategies that can complement or partially replace current insecticide-based pea leaf weevil management practices.

As part of an alternative pest management strategy, several biological control agents, including parasitoids, predators and insect pathogens, have been studied for this pest. Four European

endoparasitoids, *Microctonus aethiops* Nees, *Perilitus rutilus* Nees, *Pygostolus falcatus* Nees (Hymenoptera: Braconidae), and *Campogaster exigua* Meigen (Diptera: Tachinidae), are important parasitoids of *Sitona* species adults (Loan and Holdaway 1961). All four parasitoids were released in Canada during the 1950s (Cárcamo and Vankosky 2011) and two species (*M. aethiops* and *C. exigua*) in the United States during 1948 (Clausen 1956) for control of sweet clover weevil, *S. cylindricollis* Fahr. However, there are no reports of these parasitoids becoming established in either country. Another European egg parasitoid species, *Patasson lameerei* Debauche (Hymenoptera: Mymaridae), appears to be a *Sitona* specialist parasitoid and parasitizes eggs of this pest in Europe (Aeschlimann 1986). Nevertheless, no releases have been made to control *Sitona* species in United States and Canada. Vankosky et al. (2011) reported the generalist ground beetle *Bembidion quadrimaculatum* L. (Coleoptera: Carabidae) as a voracious predator of *S. lineatus* eggs under laboratory conditions, but its potential for controlling weevils under field conditions needs to be verified.

Several previous studies, primarily in Europe during the mid-1980s to early 1990s, assessed the virulence of entomopathogenic fungi (Müller-Kögler and Stein 1970, Poprawski et al. 1985, Verkleij et al. 1992, Jaronski 2018) and nematodes (Jaworska and Wiech 1988, Wiech and Jaworska 1990, Jaworska and Ropek 1994, Hokkanen and Menzler-Hokkanen 2018) against *S. lineatus* populations. Specifically, strains of *Metarhizium flavoviride* (Gams & Rozsypal) and *Beauveria bassiana* (Bals-Criv.) Vuill. (Ascomycota: Hypocreales) were shown to be effective against eggs and larvae of *S. lineatus* (Müller-Kögler and Stein 1970, Poprawski et al. 1985). Three nematode species, *Steinernema feltiae* Weiser, *S. bibionis* (Rhabditida: Steinernematidae), and *Heterorhabditis bacteriophora* Poinar (Rhabditida: Heterorhabditidae) caused 100% mortality of *S. lineatus* adults by 14 d after application under laboratory conditions (Jaworska and Wiech 1988), but no reports exist of their efficacy under field conditions.

Several commercially available biopesticides have been tested or used against a variety of insect pests, including aphids, thrips, and beetles. The important biopesticides that have been explored for their role on controlling agricultural crop insect pests include the use of soil microbes (spinosyns), compounds derived from plants (e.g., azadirachtin and pyrethrins), and insect pathogenic fungi (*B. bassiana*). In recent years, the use of combinations of different biopesticides has also been an area of research in insect pest control programs.

Spinosad biopesticide is a mixture of two organic chemicals (spinosyn A and spinosyn D), both produced by *Saccharopolyspora spinosa* Mertz and Yao (Actinomycetales: Pseudonocardiaceae) and isolated from soil (Mertz and Yao 1990). Spinosad acts as a neurotoxin in susceptible insect species, causing insect muscles to flex uncontrollably, paralyzing the insect body, and eventually leading to insect death over a period of 1–3 d. It provides effective control of pests in the insect orders Lepidoptera, Thysanoptera, and Diptera but is generally ineffective in the control of sucking insect pests and mites (Thompson et al. 2000). Also, spinosad has been reported as toxic to some foliage-feeding beetles including 1) Colorado potato beetle *Leptinotarsa decemlineata* Say larvae and adults (Igrc et al. 1999), 2) crucifer flea beetle *Phyllotreta cruciferae* Goeze adults (Elliot et al. 2007), 3) cereal leaf beetle *Oulema melanoplus* L. (Coleoptera: Chrysomelidae) adults (Buntin et al. 2004), and 4) alfalfa weevil *Hypera postica* Gyllenahl (Coleoptera: Curculionidae) larvae (Reddy et al. 2016). In addition, spinosad has shown high toxicity to several stored product beetle species (Fang and Subramanyam 2003, Nayak et al. 2005, Daglish and Nayak 2006). However, there are several

beetles, including stored product beetles (Nayak et al. 2005) and a seedpod-feeding beetle (Cárcamo et al. 2005), for which spinosad was less effective. Elliot et al. (2007) reported that multiple factors, such as mode of exposure, exposure time, and temperature, could affect the susceptibility of flea beetle adults to spinosad.

Botanical biopesticides extracted from plant species of Asteraceae, Rutaceae, and Meliaceae are toxic and/or repellent to insect pests and have potential to improve the management of a variety of agricultural crop insect pests (Isman 2006). Presently, azadirachtin and pyrethrins are two prominent botanical biopesticide products and represent alternatives to synthetic insecticides. Azadirachtin and pyrethrins contain active ingredients of a complex tetranortriterpenoid limonoid and pyrethrins, respectively. Azadirachtin is extracted from neem *Azadirachta indica* A. Juss (Sapindales: Meliaceae) tree seeds, whereas pyrethrin is from dried flowers of *Chrysanthemum cinerariaefolium* L. (Asterales: Asteraceae). Both biopesticides display a range of effects on insect pests, including antifeedancy, growth reduction, molting inhibition, anatomical abnormalities, and mortality (Isman 2006). The entomopathogenic fungus, *B. bassiana*, is pathogenic to many pest species, and the strain GHA, commercially available as Mycotrol (Lam International, Butte, MT), has been reported as an effective biopesticide for a variety of insect pests. This fungus infects insect hosts by direct penetration of host cuticles to invade hemocoel and eventually kill the hosts within 6–7 d (Vega et al. 2012).

In addition to applications of individual biopesticides, several studies have examined whether the combined application of *B. bassiana* with azadirachtin (Akbar et al. 2005, Mohan et al. 2007, Islam et al. 2010, Hernández et al. 2012, Aristizábal et al. 2017) or with pyrethrins (Reddy et al. 2016, Shrestha and Reddy 2019) can improve insect pest control. For instance, *B. bassiana* + azadirachtin combinations had an additive effect on insect pest mortality for nymphs of two-spotted spider mite, *Tetranychus urticae* Koch (Acari: Tetranychidae) (Hernández et al. 2012), and sweet potato whitefly, *Bemisia tabaci* Gennadius (Hemiptera: Aleyrodidae) (Islam et al. 2010). In contrast, Akbar et al. (2005) found antagonistic effects on adult mortality of red flour beetle, *Tribolium castaneum* Herbst (Coleoptera: Tenebrionidae). Therefore, it is evident that effect of biopesticide product combinations differs with insect pest species.

No previous attempts have been made to study the effects of commercially available biopesticides against pea leaf weevil. Therefore, this study represents a first attempt to identify appropriate biopesticide products to manage *S. lineatus* adults to prevent larval development, as larvae are generally the most damaging stage (Wanner 2016). The commercially available biopesticides selected for this study were as follows: 1) *B. bassiana* GHA, 2) azadirachtin, 3) *B. bassiana* GHA + pyrethrins, 4) *B. bassiana* GHA + azadirachtin, and 5) spinosad. These biopesticides were considered for study as they have potential for control of other important agricultural pests of Montana (Reddy et al. 2016, Briar et al. 2018, Shrestha and Reddy 2019).

This study aimed to investigate 1) the effect of biopesticide treatments on mortality of adults at different concentrations (0.1, 0.5, 1.0, and 2.0 times the lowest labeled application rate) of each product, 2) the influence of biopesticides (based on the first study) on defoliation and adult mortality, and 3) the toxicity of biopesticides against larvae of green lacewing, *Chrysoperla carnea* Stephens (Neuroptera: Chrysopidae) and the two-spotted lady beetle, *Adalia bipunctata* L. (Coleoptera: Coccinellidae). Both beneficial species are common natural enemies of pea aphids, *Acyrthosiphon pisum* Harris (Hemiptera: Aphididae) in Montana field peas, and they are likely to

co-occur with pea weevil. Once we verified that biopesticides possess the ability to infect and kill weevil adults and natural enemies in the laboratory, then we tested the hypothesis that treating pea seedlings with biopesticides will result in higher adult mortality and higher foliage protection compared with control treatment in cage experiments.

Materials and Methods

Plants

Green pea, *Pisum sativum* L. variety 'Banner', was used as a source of plant material for the cage experiments. Seeds were sown in orange square pots (13 × 13 × 13.5 cm) at a density of six seeds per pot. Each pot contained 1.62 kg of prepared soil mixture, which was a 1:1:2 mixture of sand, potting mix, and clay loam soil by weight. Pots were placed inside nylon-netted insect cages (68 × 75 × 82 cm). After plant emergence, plant density was reduced to four per pot. Plants were held in outdoor conditions at the Western Triangle Agricultural Research Center (WTARC) of Montana State University in Conrad, MT, until used for experiments. Plants were 24 d old and had three pairs of unfolded leaves at the start of experiments.

Insects

Pea Leaf Weevil

Overwintered field-collected pea leaf weevil adults were used for all laboratory and cage experiments. Weevils were collected either by using pitfall traps baited with sex pheromone lures (4-methyl-3,5-heptanedione; Reddy et al. 2018) or sweep netting (diameter = 38 cm; BioQuip Products, Rancho Dominguez, CA) from pea and alfalfa fields. Both approaches were used in April-May of 2016 and 2018, in Pondera County, Montana. Field-collected adults (50 individuals per container) were immediately transferred into round deli plastic cup containers (diameter = 12 cm, 473 ml). Insects were fed alfalfa leaves and maintained in a climate cabinet at 12°C for 2–3 d until a sufficient number of adults were collected for the experiments.

Beneficial Insects

Larvae of green lacewing (*C. carnea*) and two-spotted ladybeetle (*A. bipunctata*), both 1 and 2 d old, were obtained from Biobest Canada Ltd. (www.biobestgroup.com). Flour moth, *Ephestia kuehniella* Zeller (Lepidoptera: Pyralidae) eggs (Beneficial Insectary, California; www.insectary.com), were used as a food source for the beneficial insect larvae. Larvae were held in Petri dishes and fed *E. kuehniella* eggs as needed.

Biopesticide Source and Rate

Biopesticides were commercially available formulations (Table 1). They were stored at 4–5°C until diluted to the desired concentration for use. Three biopesticides, *B. bassiana* GHA, *B. bassiana* GHA + pyrethrins, and *B. bassiana* GHA + azadirachtin were obtained from Lam International. Spinosad was obtained from Dow Agro Sciences (Indianapolis, IN), and the pyrethrins was from McLaughlin Gormley King (Minneapolis, MN). The trade names and general application rates of each biopesticide are given in Table 1. Concentrations for each biopesticide were based on the lowest labeled rate in a solution to be applied at 935 liters/ha (10 gal/ac), except a high rate for *B. bassiana* GHA + pyrethrins. Serial dilutions were made to create concentrations that were 2.0, 1.0, 0.5, and 0.1 times the base concentration (Table 1).

Experiment #1: Laboratory Bioassay on Pea Leaf Weevil Adult Mortality

The laboratory bioassay experiment was performed to determine the susceptibility of *S. lineatus* adults to four concentrations of each product and a control (water; Table 1). Stock solutions of each product were prepared by dissolving its concentrates in tap water, and reduced concentrations were prepared by serial dilution (Table 1). Before conducting this experiment, two application methods, immersion (i.e., dipping adult weevils in solution for 5 s) and topical application (i.e., spraying solution [1 ml] directly on the weevil's body surface using 750-ml hand-held and perfume sprayers), were evaluated for their suitability. We chose the perfume sprayer for this experiment because other spraying methods caused high levels of mortality both in the treatment and water control groups (G. Shrestha, personal observation, 2016).

Pea leaf weevil adults of similar size (length = 5.0–5.5 mm) were placed in groups of seven in Petri dishes lined with filter paper (diameter = 9 cm) with the help of a fine camel hair paintbrush (Creative Addictions, Conrad, MT). Petri dishes were then held in a growth chamber (Versatile Environmental Test Chamber, Acton, MA) for 1 h at 5°C to minimize adult movement that allowed easy handling of insects while spraying biopesticides. The adults were topically treated with 1 ml of the appropriate biopesticide product suspension using a perfume sprayer. Controls were treated with 1.0 ml of tap water. Thirty minutes after weevils were treated, one fresh alfalfa, *Medicago sativa* L. (Fabales: Fabaceae) stem (5 cm long with 9–12 unfolded leaves) was placed as a food source in each Petri dish near the treated weevils. Alfalfa stems were replaced every 2–3 d. Petri dishes were held in a climate cabinet at 22 ± 1°C, 16:8 (L:D) h, and 75% RH. The whole experiment (all treatments) was conducted twice on different dates with five and three replicates per run. Each

Table 1. Biopesticide treatments and concentrations used for the laboratory bioassays against pea leaf weevil adults

Treatments	Trade name	General application rate (387.54-liter water/4,046.86 m²)	Concentrations (ml product/liter water)				
			High (2×)	Medium (1×)	Low (0.5×)	Very low (0.1×)	Control (0×)
Spinosad (*Saccharopolyspora spinosa*) (80% a.i.)	Entrust WP	28.35–56.70 g (CDMS 2019)	0.182	0.091	0.0455	0.0091	0.00
Beauveria bassiana GHA (11.30% a.i.)	Mycotrol ESO	473.176–946.35 ml (BioWorks 2019)	1.44	0.72	0.36	0.072	0.00
B. bassiana GHA (0.06% a.i.) + pyrethrins (0.75% a.i.)	Xpectro OD	473.176–1,892.71 ml (Lam International 2019a)	5.00	2.50	1.25	0.25	0.00
B. bassiana GHA (0.06% a.i.) + azadirachtin (10.00% a.i.)	Xpulse OD	473.176–1,892.71 ml (Lam International 2019b)	1.44	0.72	0.36	0.072	0.00
Pyrethrins (10.00% a.i.)	PyGanic EC	946.35–1,892.71 ml (Valent 2019)	2.88	1.44	0.72	0.072	0.00

Petri dish represented a replicate, and a total of eight replicates per treatment were completed.

Starting 1 d after the treatment, adult mortality of all weevils was checked daily for 9 d. The monitoring of adult mortality until 9 d was mainly because of selected biopesticides mode of actions. Spinosad can kill insects within 3–6 d and other biopesticides within 5–7 d. Because *S. lineatus* adults have a defensive behavior in which they feign death through immobility following even minimal disturbance (Jackson and Macdougall 1920), mortality was determined by gently prodding each adult with a camel hair paintbrush, and only individuals that failed to move were considered dead. Dead weevils collected from *B. bassiana* GHA, *B. bassiana* GHA + pyrethrins, and *B. bassiana* GHA + azadirachtin treatment groups were placed separately on moist filter paper lined in a Petri dish to confirm mortality via checking fungus sporulation. However, fungal development on dead adults was only observed in *B. bassiana* GHA, *B. bassiana* GHA + pyrethrins treatments (Fig. 1). Toxicity symptoms such as twitching of legs, paralysis, and spreading of wings (Fig. 1) in dead weevils caused by spinosad were also recorded over the course of the experiments.

Experiment #2: Cage Experiment on Pea Leaf Weevil Adult Mortality and Foliage Damage

The cage experiment was performed to determine the impact of *B. bassiana* GHA (1.44 ml/liter), *B. bassiana* GHA + pyrethrins (5 ml/liter), and spinosad (0.182 ml/liter) biopesticide products on foliage damage and pea leaf weevil adult mortality. Only these biopesticides were chosen for this experiment since the other formulations failed to cause sufficient *S. lineatus* adult mortality in the previous laboratory experiment.

Potted pea plants established outdoors were transported to a lab and placed in small nylon net cages (12 × 10 × 10 cm) with three pots per cage, containing four plants in each pot. Each potted plant was individually marked and infested with 25 *S. lineatus* adults per cage (eight or nine per plant). To apply biopesticides, pea plants (already infested with adults) were moved to a spraying room. For each cage (containing 12 plants with 4 plants per pot), we used a 750-ml handheld sprayer to apply 10 ml of spray solution. Control plants were treated with tap water using the duplicate device. Each cage represented an experimental unit and the number of replicates per treatment was three to five. The entire experiment was run twice on two different dates.

Adult mortality and foliage damage levels caused by adult feeding were assessed at 1, 3, 7, and 9 d after treatment. The number of U-shaped feeding notches on the leaf margins (see Wanner 2016 for description of weevil feeding) were counted to determine the adult feeding damage level in pea plants.

Experiment #3: Laboratory Bioassay on Beneficial Insects

A bioassay was performed to determine the toxicity of *B. bassiana* GHA (1.44 ml/liter), *B. bassiana* GHA + pyrethrins (5 ml/liter), and spinosad (0.182 ml/liter) to first instars of *C. carnea* and *A. bipunctata*. Larvae of *C. carnea* or *A. bipunctata* were placed in groups of 10 per Petri dish (diameter = 9 cm) with a fine camel hair paintbrush. The appropriate biopesticide was sprayed topically onto test individuals using a 750-ml hand-held sprayer, applying 1 ml of spray solution per Petri dish (distributed over all 10 insects). A control treatment was sprayed in a similar fashion with 1.0 ml of tap water. Immediately after spraying, treated larvae were transferred individually into 60-ml plastic deli cup containers capped with a lid having three to four small holes poked with an insect dissecting needle. The beneficial larvae were reared separately due to their cannibalistic nature (Yasuda and Ohnuma 1999, Canard 2001). Dishes were held in a climate cabinet at 22 ± 1°C, 16:8 (L:D) h photoperiod, and 75% RH. Larval mortality was recorded at 1- to 3-d intervals for 7 d. Treatments were replicated three times in each run of the experiment. The whole experiment was run twice for *C. carnea* and three times for *A. bipunctata* on separate dates.

Statistical Analysis

Statistical analyses were carried out using R software 3.4.4 for analysis of variance (ANOVA; R Development Core Team 2012), PROC GLM procedure for MANOVA (SAS Institute Inc. 2015) and survival analyses in Sigma Plot 12.3. For all data, a normal quantile–quantile plot was first used to verify the normality of residuals and the equality of residual variances. Angular transformation was used to normalize mortality data before the application of statistical tests. Tukey's contrast for pairwise multiple comparisons was used to compare significant differences (alpha = 0.05) among means, unless stated otherwise.

Laboratory Bioassay on Pea Leaf Weevil Adult Mortality

Data on cumulative mortality were corrected for control mortality (Abbott 1925). Three-way ANOVA was used to determine the
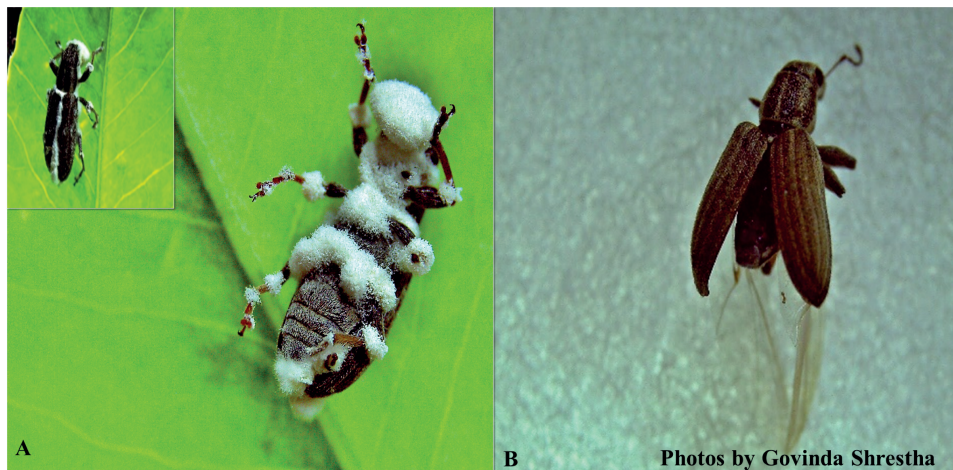

Fig. 1. Pea leaf weevil adults killed by biopesticides: (A) *Beauveria bassiana* GHA alone or in combination with pyrethrins (mycosis on adults caused by fungus), (B) Spinosad (a toxic symptom, spreading of wings on adult).

influence of biopesticide, concentration, and experiment date on adult mortality. One-way ANOVA was then used to evaluate 1) the effect on mortality across different biopesticides and 2) the effect on mortality across biopesticide concentrations. Log-rank Kaplan–Meier survival analyses were performed to compare survival at different concentrations of each biopesticide product. Pairwise multiple comparison procedures were followed to compare survival curves, using the Holm–Sidak method.

Cage Experiment on Pea Leaf Weevil Adult Mortality and Foliage Damage

Two-way analysis of variance was carried out to determine the effect of biopesticide and experiment date on adult mortality in the cage experiment. Multivariate analysis of variance (MANOVA) was used to determine the impact of biopesticide and time on adult feeding damage in plants for each cage experiment, and this analysis was chosen to account for data measured on the same set of plants over time. A significant MANOVA was followed by a univariate analysis (ANOVA) to determine the effect on adults feeding damage across biopesticide treatment levels at each sampling time.

Laboratory Bioassay on Beneficial Insects

Two-way analysis of variance was carried out to determine the effect of biopesticide and experiment date on *C. carnea* and *A. bipunctata* larval mortality.

Results

Laboratory Assessment

Pea Leaf Weevil Adult Mortality

The average control mortality ($\pm$ SE) across treatments was $4.32 \pm 2.40\%$ at 9 d post-inoculation. Overall, we found significant effects of the concentration level ($F = 112.60$; df = 3, 120; $P < 0.0001$) and biopesticide type ($F = 147.92$; df = 4, 120; $P < 0.0001$) on *S. lineatus* adult mortality. The experiment dates had no effect ($F = 0.54$; df = 1, 120; $P = 0.46$) on adult mortality, so data from the first and second experiments were combined. There was an interaction between concentration level and biopesticide type ($F = 5.78$; df = 12, 140; $P < 0.0001$).

Across the types of biopesticides, significant differences in weevil adult mortality occurred at concentration levels: high ($F = 69.98$; df = 4, 35; $P < 0.0001$), medium ($F = 72.65$; df = 4, 35; $P < 0.0001$), low ($F = 22.98$; df = 4, 35; $P < 0.0001$), or very low ($F = 15.38$; df = 4, 35; $P < 0.0001$; Table 2). Spinosad consistently caused higher adult mortality than other biopesticide treatments, irrespective of concentration level (Table 2). Among the other treatments, *B. bassiana* GHA caused greater mortality alone than it did when combined with azadirachtin or pyrethrins, but only at the high and medium concentration levels (Table 2). On the other hand, the combined formulation of *B. bassiana* + pyrethrins caused higher adult mortality than either *B. bassiana* + azadirachtin or pyrethrins alone, at the high and medium concentration levels (Table 2). However, *B. bassiana* + pyrethrins did not cause higher adult mortality than *B. bassiana* GHA, regardless of concentration level (Table 2).

Within each biopesticide product, the concentration level affected adult mortality: spinosad ($F = 204.10$; df = 3, 28; $P < 0.0001$), *B. bassiana* GHA + pyrethrins ($F = 21.99$; df = 3, 28; $P < 0.0001$), *B. bassiana* GHA ($F = 28.67$; df = 3, 28; $P < 0.0001$), *B. bassiana* GHA + azadirachtin ($F = 11.48$; df = 3, 28; $P < 0.0001$), and pyrethrins ($F = 4.58$; df = 3, 28; $P < 0.01$). The mortality caused by each product was generally concentration dependent, with the highest

mortality recorded at the highest concentration, except that for spinosad, there was no difference in the mortality of adult weevils between high and medium concentrations of spinosad (Table 2). Mean mortality in biopesticide treated groups varied from 36 to 100% for spinosad, from 6 to 62% for *B. bassiana* GHA, from 6 to 60% for *B. bassiana* GHA + pyrethrins, from 5 to 32% for *B. bassiana* GHA + azadirachtin, and from 3 to 17% for pyrethrins (very low concentration to high label rate, respectively; Table 2).

Survival Time of Adult Weevils

Mean survival time of adult weevils differed between concentration levels of the four products: spinosad (log-rank, df = 4; $\chi^2 = 332.94$; $P < 0.001$), *B. bassiana* GHA + pyrethrins (log-rank, df = 4; $\chi^2 = 75.68$; $P < 0.001$), *B. bassiana* GHA (log-rank, df = 4; $\chi^2 = 91.59$; $P < 0.001$), and *B. bassiana* GHA + azadirachtin (log-rank, df = 4; $\chi^2 = 29.14$; $P < 0.001$; Fig. 2A–D). The pyrethrin treatment was the exception with no effect (log-rank, df = 4; $\chi^2 = 7.20$; $P > 0.05$) on adult survival time (Fig. 2E).

In the spinosad treatment, pea leaf weevil adults had shorter mean survival times ($\pm$ SE) when adults were treated with high (1.98 ± 0.13 d), medium (2.95 ± 0.12 d), low (6.41 ± 0.37 d), and very low (7.21 ± 0.38 d) concentrations compared with control adults (8.86 ± 0.19 d; Fig. 2A). In contrast, with other biopesticides, mean adult survival was affected with high and medium concentration levels of either *B. bassiana* GHA + pyrethrins or *B. bassiana* GHA alone, and only with a high concentration level of *B. bassiana* GHA + azadirachtin, when compared with control-treated adults (Fig. 2B–D).

Cage Experiments

Leaf Damage Assessment

The overall mean number ($\pm$ SE) of adult-damaged leaf notches per plant for control treatment group in cage experiment #1 was as follows: 5.20 ± 0.66, 16.13 ± 2.03, 34.46 ± 4.17, and 40.93 ± 4.78 at the 1, 3, 7, and 9 d, respectively, post-application. The corresponding values for cage experiment #2 were as follows: 2.86 ± 1.12, 8.58 ± 2.76, 17.83 ± 4.67, and 22.44 ± 5.01, respectively. Based on the two-way repeated-measures MANOVA analysis, time played a significant (cage experiment #1: Wilks's $\lambda = 0.09$; $F = 44.65$; df = 3, 14; $P < 0.0001$; cage experiment # 2: Wilks's $\lambda = 0.21$; $F = 9.76$; df = 3, 8; $P = 0.005$) role for showing impacts of biopesticide treatments on adult feeding damage on pea plants. There were also interactions between time and biopesticide treatment (cage experiment #1: Wilks's $\lambda = 0.13$; $F = 5.08$; df = 9, 34; $P = 0.0002$; cage experiment # 2: Wilks's $\lambda = 0.11$; $F = 3.27$; df = 9, 20; $P = 0.01$). The model indicated that biopesticide treatments had an effect (cage experiment #1: $F = 17.70$; df = 3, 16; $P < 0.0001$; cage experiment #2: $F = 7.17$; df = 3, 10; $P = 0.007$) on adult feeding damage on plants. Consequently, biopesticide treatment data of each sampling time were analyzed separately using one-way ANOVA.

Across the treatment levels, differences in adult feeding damage per plant occurred at 1 d (cage experiment #1: $F = 37.48$; df = 3, 56; $P = 0.0001$; cage experiment #2: $F = 7.36$; df = 3, 38; $P = 0.0001$), 3 d (cage experiment #1: $F = 31.43$; df = 3, 56; $P = 0.0001$; cage experiment #2: $F = 9.53$; df = 3, 38; $P = 0.0001$), 7 d (cage experiment #1: $F = 29.34$; df = 3, 56; $P = 0.0001$; cage experiment #2: $F = 13.71$; df = 3, 38; $P = 0.0001$), and 9 d (cage experiment #1: $F = 31.25$; df = 3, 56; $P = 0.0001$; cage experiment #2: $F = 18.7$; df = 3, 38; $P = 0.0001$).

Irrespective of sampling time, spinosad provided the most foliage protection against adult feeding damage, with <2 notches per plant throughout the sampling period (9 d; Fig. 3). The combined formulation of *B. bassiana* GHA + pyrethrins was also effective in reducing

Table 2. Susceptibility of pea leaf weevil adults to five commercially available biopesticide products

	Concentration levels			
Treatments	High (2×)	Medium (1×)	Low (0.5×)	Very low (0.1×)
Spinosad	100.00 ± 0.00Aa	100.00 ± 0.00Aa	62.50 ± 4.91Ab	36.25 ± 3.75Ac
Beauveria bassiana GHA	62.50 ± 4.91Ba	38.12 ± 5.97Bb	11.25 ± 3.37Bbc	6.24 ± 1.83Bc
B. bassiana GHA + pyrethrins	60.62 ± 6.30Ba	32.50 ± 6.34Bb	19.37 ± 3.95Bb	6.25 ± 1.82Bc
B. bassiana GHA + azadirachtin	32.50 ± 5.67Ca	21.25 ± 3.75BCab	8.12 ± 2.98Bbc	4.99 ± 1.89Bc
Pyrethrins	17.49 ± 4.00Ca	11.25 ± 3.37Cab	5.62 ± 3.19Bbc	3.74 ± 1.83Bc

Mean percentage of corrected mortality (± SE) 9 d post-inoculation. Mean values within columns bearing the same upper case letter and within rows bearing the same lower case letters are not significantly different (Tukey test, $P > 0.05$).
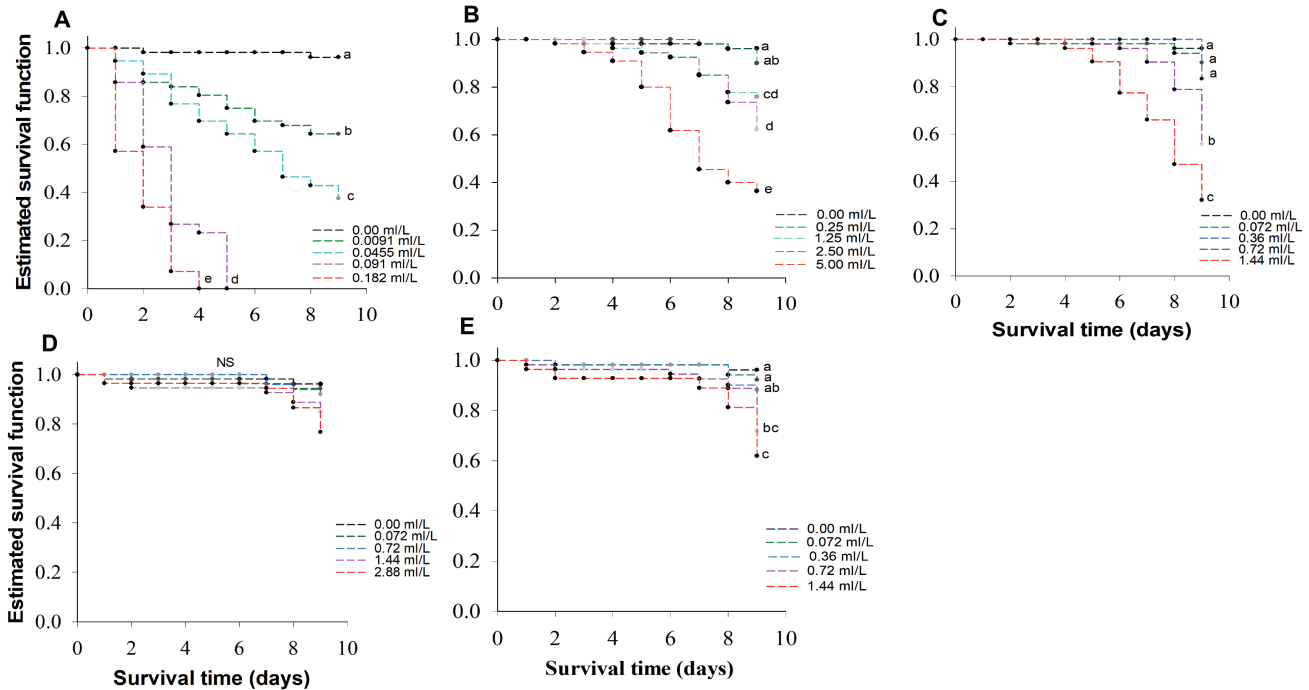

Fig. 2. Survivorship of pea leaf weevil adults treated with different concentrations of biopesticides: (A) Spinosad, (B) *Beauveria bassiana* GHA + pyrethrins, (C) *B. bassiana* GHA, (D) *B. bassiana* GHA + azadirachtin, and (E) Pyrethrins. Survival curves of different colors different by Holm–Sidaḱs test ($P > 0.05$). Color figure is only available in online version.

feeding damage with fewer than 15 cumulative notches over 9 d. In contrast, *B. bassiana* GHA was unable to protect foliage from adult feeding damage in cage experiment #1, but it provided protection in cage experiment #2 (Fig. 3).

Pea Leaf Weevil Adult Mortality

Overall, the study showed that biopesticide type ($F = 61.45$; df = 3, 26; $P < 0.0001$) and experiment date ($F = 15.92$; df = 1, 26; $P < 0.001$) had effects on adult mortality (Fig. 4). Therefore, the data from first and second experiments are presented separately. No interaction ($F = 2.77$; df = 3, 26; $P = 0.06$) was evident between biopesticide type and experiment date.

Regardless of the experiment date, treatments had impacts on adult mortality in both cage experiment #1 ($F = 31.80$; df = 3, 16; $P < 0.0001$) and cage experiment #2 ($F = 37.58$; df = 3, 16; $P < 0.0001$; Fig. 4). Among the three biopesticides, spinosad was found to be the most effective treatment, killing 83–92% of adults on pea plants (Fig. 4). The combined treatment of *B. bassiana* GHA + pyrethrins provided a similar level of adults control in cage experiment #2 (mean ± SE: 70.68 ± 6.41), but not in cage experiment #1 (Fig. 4).

Conversely, *B. bassiana* GHA was found to be the least effective treatment, causing only 20–40% adult mortality, which was not different from the untreated control in cage experiment #1 (Fig. 4).

Biopesticide Product Toxicity to Beneficial Insects

This study demonstrated that treatments had effects on mortality of larvae of two beneficial species, but experiment date were not significant: 1) *C. carnea* (treatment: $F = 10.03$; df = 3, 24; $P < 0.001$; experiment date: $F = 0.98$; df = 2, 24; $P = 0.39$) and 2) *A. bipunctata* (treatment: $F = 40.05$; df = 3, 16; $P < 0.0001$; experiment date: $F = 4.50$; df = 1, 16; $P = 0.06$; Fig. 5). The data from lab bioassays of each beneficial species over experiment dates were subsequently combined.

Generally, *C. carnea* larvae were found less susceptible to biopesticide treatments than *A. bipunctata* larvae. Among the three biopesticides, *B. bassiana* GHA was found harmless to both beneficial insects because the mortality caused by fungus was not different from the control treatment (Fig. 5). Spinosad had no effect on mortality of *A. bipunctata* but did effect *C. carnea* larvae, when compared with their control treatments (Fig. 5). Conversely, the combination of *B. bassiana* GHA + pyrethrins was toxic to both
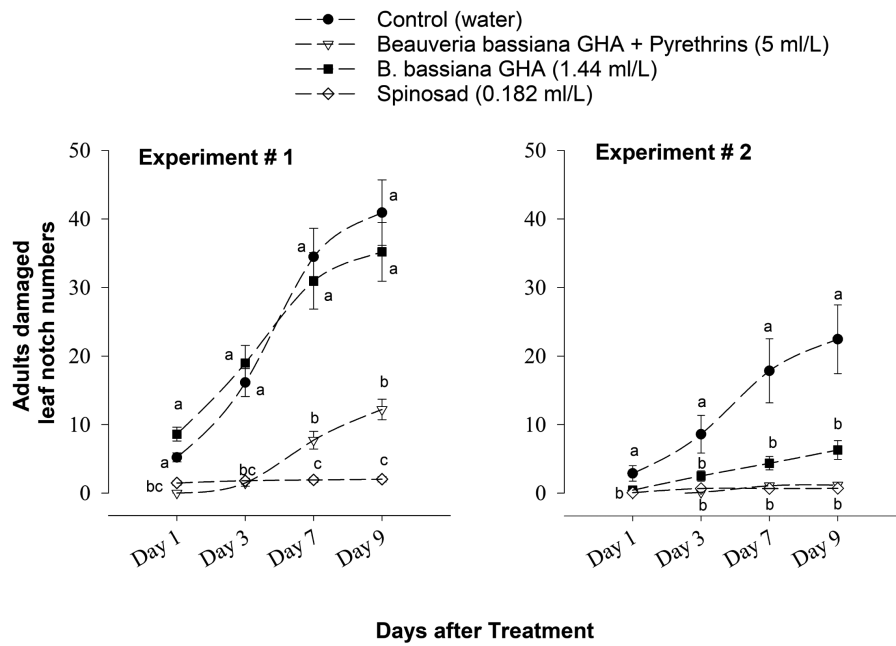
Fig. 3. The effect of high concentration levels of biopesticide treatments on pea leaf weevil adult feeding damage levels (mean ± SE) on pea plants, over 1, 3, 7, and 9 d post-inoculation. Lines bearing the same letters within each date are not significantly different (Tukey's test, $P > 0.05$).
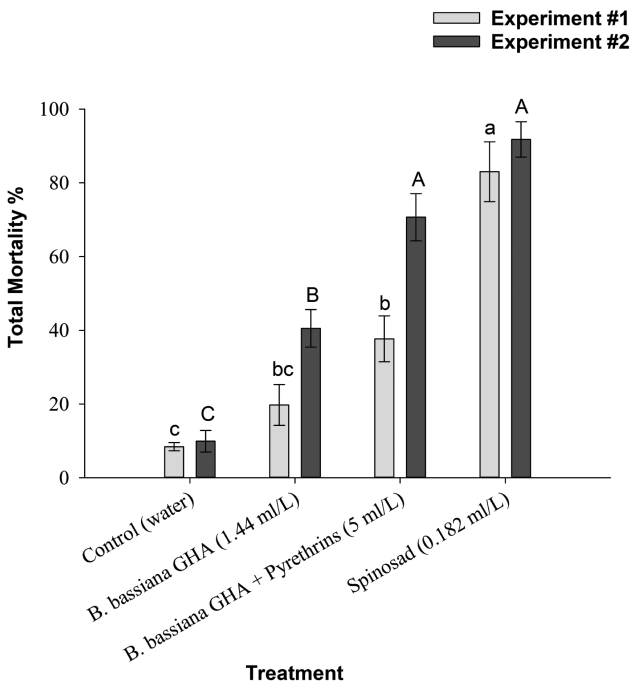

Fig. 4. Total mortality (mean ± SE) of pea leaf weevil adults treated with high concentration level of each biopesticide treatment in cage experiments, over 9 d post-inoculation. Bars bearing the same upper case or lower case letters are not significantly different (Tukey's test, $P > 0.05$).

beneficial species, causing 98 and 38% mortality in *A. bipunctata* and *C. carnea* larvae, respectively (Fig. 5).

Discussion

The results of laboratory and cage experiments indicated that spinosad and the combination of *B. bassiana* GHA + pyrethrins can
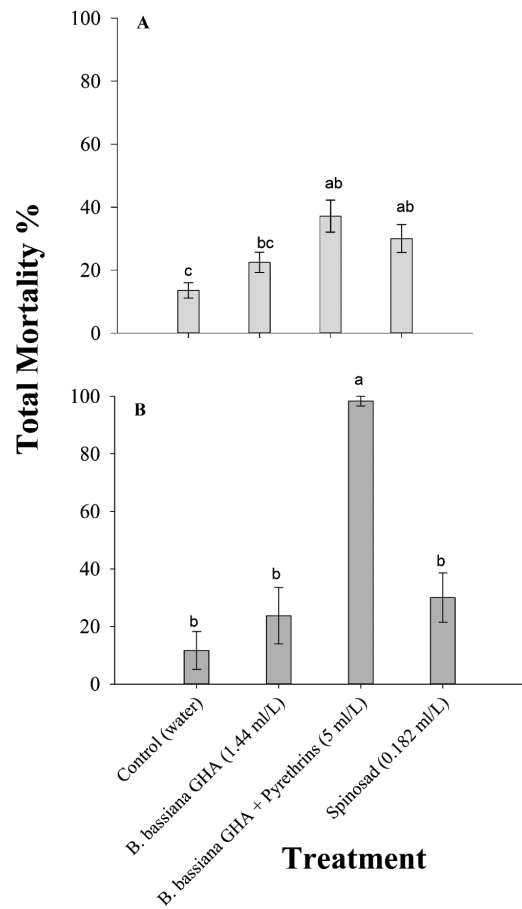

Fig. 5. Total mortality (mean ± SE) of *Chrysoperla carnea* (A) and *Adalia bipunctata* (B) larvae treated with high concentration level of each biopesticide treatment in lab experiments, over 7 d post-inoculation. Both beneficial larvae were at first larval instar at the time of biopesticide treatment. Bars bearing the same letters are not significantly different (Tukey's test, $P > 0.05$).

be used as alternatives or as a complement to synthetic insecticides for *S. lineatus* adult control. No previous information exists regarding the susceptibility of *S. lineatus* adults to spinosad. However, our results are consistent with earlier findings from foliage-feeding beetles (Igrc et al. 1999, Buntin et al. 2004, Elliot et al. 2007) and demonstrate that pea leaf adults are highly susceptible to spinosad, with 80–100% mortality at high and medium concentrations. Our results suggested that spinosad has a high toxicity to *S. lineatus* adults by topical contact (Sparks et al. 1998, Toews et al. 2003). In our study, spinosad exposure produced several toxic symptoms including twitching of legs, paralysis, and spreading of wings (Fig. 1) in *S. lineatus* adults.

Mean survival time of adults (2–3 d) at high and medium concentrations and reduced feeding damage (<5%) from spinosad treatments resemble previous studies where spinosad killed >80% of the eggplant flea beetle, *Epitrix fuscula* Crotch (Coleoptera: Chrysomelidae) (McLeod et al. 2002) and *P. cruciferae* (Elliot et al. 2007) adult populations within 3–6 d. Spinosad also reduced feeding damage on eggplant, *Solanum melongena* L. (Solanales: Solanaceae) and canola, *Brassica napus* L. (Brassicales: Brassicaceae).

Our laboratory bioassays indicate that *B. bassiana* GHA can cause pea leaf weevil adult mortality, particularly at high concentration, similar to the findings of several previous studies from Europe (Müller-Kögler and Stein 1970, Poprawski et al. 1985, Verkleij et al. 1992). However, in the cage studies, *B. bassiana* caused little or no adult mortality, and feeding damage was not different from the untreated control groups in one of the two experiments. The differences in feeding damage between the two experiments were possibly due to human handling errors while spraying biopesticides and/or differences in pea plant growth. Plants were smaller in size in the first experimental run compared with the second run. The minimal impact of fungus on adult mortality might have been due to a number of factors that occurred in our cage studies: 1) low relative humidity (~55%), 2) limited direct contact of adults with fungal spores, and 3) inadequate spore concentration due to pea leaf expansion. Increased relative humidity (80–90%; Jaronski 2010), maximum direct contact of adults with fungal spores (Wright et al. 2004), and higher spore concentrations on the treated leaf surface (Inyang et al. 1998, Shrestha et al. 2015) enhance fungal efficacy against target insects.

In our laboratory test, pyrethrins had no effect on mortality. Pyrethrins usually paralyze the nervous system of susceptible insects, but it often does not kill the insect and can be detoxified by enzymes within the insect (Buss and Park-Brown 2002). We speculate that *S. lineatus* adults metabolized the toxins, allowing the adults to recover from exposure to this biopesticide.

The effects of *B. bassiana* + pyrethrins were different. When the fungus was applied with pyrethrins in the laboratory bioassay, adult mortality (60%) was not different from the fungus treatment alone (62%) at the high concentration. However, in the cage study, the same combined treatment caused numerically or significantly higher adult mortality compared with the fungus treatment alone. This may result due to antagonism in the lab study or due to additive or synergism in the cage study. It is further supported via lower feeding damage in the combined treatment compared with fungus alone. These results are in agreement with Shrestha and Reddy (2019), who demonstrated a reduction in wheat midge, *Sitodiplosis mosellena* Géhin (Diptera: Cecidomyiidae) and wheat kernel damage in spring wheat fields sprayed with *B. bassiana* + pyrethrins compared with the *B. bassiana* GHA alone and untreated plots. Together, these studies illustrate that *B. bassiana* + pyrethrins can cause insect mortality and act as an antifeedant for improving insect pest control.

In contrast, treatment with *B. bassiana* + azadirachtin significantly reduced adult mortality (62%) compared with when fungus was applied alone (32%) at the high concentration. No previous reports are available on impact of *B. bassiana* + pyrethrins and fungus treatments on weevil adult mortality, except a lab study by Smart et al. (1994) that demonstrated azadirachtin had no effect on *S. lineatus* adults. Depending on the insect species, *B. bassiana* + azadirachtin treatment can cause additive (Islam et al. 2010, Hernández et al. 2012) or antagonistic (Akbar et al. 2005, Mohan et al. 2007) effects on insect mortality. The effect of fungus and azadirachtin combinations can also depend on the fungal isolate as shown by Mohan et al. (2007), who demonstrated additive effects on larvae of tobacco cutworm, *Spodoptera litura* Fab. (Lepidoptera: Noctuidae) with only two out of three tested isolates of *B. bassiana*. Also, azadirachtin has been reported to reduce germination and viability of *B. bassiana* spores (Akbar et al. 2005, Depieri et al. 2005). Our finding suggests that *B. bassiana* + azadirachtin treatment may have an antagonist effect on weevil adult mortality. Hence, this study, along with two previous studies (Akbar et al. 2005, Mohan et al. 2007), shows that the effect of *B. bassiana* + azadirachtin on insect mortality level can differ between insect species, possibly in combination with *B. bassiana* isolate.

The toxicity of *B. bassiana* GHA and spinosad against the two natural enemies confirms previous studies, showing that both products have a minimal impact on the larvae of *C. carnea* (Cisneros et al. 2002, Mandour 2009) and *A. bipunctata* (Jalali et al. 2009). Conversely, we found that *B. bassiana* + pyrethrins was highly (98%) and moderately toxic (38%) to *A. bipunctata* and *C. carnea* larvae, respectively. Although no information is available on toxicity of *B. bassiana* + pyrethrins against natural enemies, the application of pyrethrins in combination with other chemicals had been previously found highly toxic to several natural enemies including *A. bipunctata* (Jansen et al. 2010). For instance, application of pyrethrins in conjunction with rapeseed oil caused 100% mortality of first instar larvae of *A. bipunctata*. However, the impact of *B. bassiana* + pyrethrins on beneficial insects remains to be confirmed in the field.

In conclusion, spinosad and the combination of *B. bassiana* and pyrethrins caused the greatest mortality to adults of pea leaf weevil and reduced damage to plants. On the other hand, *B. bassiana* GHA caused significant adult mortality only under laboratory conditions and not on pea plants in cage studies. Field studies are necessary to further test the efficacy of spinosad and *B. bassiana* + pyrethrins against larval stages of pea leaf weevil, nodulation, and their ability to improve seed yield. In addition, cost/benefit analysis has to determine whether the application of these two biopesticides are economical for Montana field pea producers. Overall, spinosad could be an option for managing pea leaf weevil population, especially for organic field pea producers, and could be easily incorporated into an integrated pest management program.

Acknowledgments

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