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Optimal concentration of *Beauveria bassiana* vectored by bumble bees in relation to pest and bee mortality in greenhouse tomato and sweet pepper

Jean Pierre Kapongo · Les Shipp · Peter Kevan · Bruce Broadbent

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Abstract Greenhouse cage trials were conducted to determine the optimal concentration of *Beauveria bassiana* (Balsamo) Vuillemin (Ascomycota: Hypocreales) (BotaniGard 22WP® formulation) as vectored by the bumble bee, *Bombus impatiens* (Cresson) (Hymenoptera: Apidae) pollinator for control of greenhouse whitefly, *Trialeurodes vaporariorum* (Westwood) (Hemiptera: Aleyrodidae) on greenhouse tomato, tarnished plant bug, *Lygus lineolaris* (Palisot de Beauvois) (Hemiptera: Miridae) and green peach aphid, *Myzus persicae* (Sulzer) (Hemiptera: Aphididae) on greenhouse sweet pepper. Three inoculum concentrations of *B. bassiana*: low, 9×10^9; middle, 6.24×10^{10}; and high, 2×10^{11} conidia g^{-1} of inoculum and two controls (one with bees and heat-inactivated inoculum, and the other which contained only the host plants and pest species) were tested in a completely randomized block design. *Beauveria bassiana* killed 18, 54 and 56% of the adult *T. vaporariorum* and 33, 70 and 67% of the adult *L. lineolaris*, respectively, at the low, middle and high concentrations; but no infection from *B. bassiana* occurred in each of the control treatments. Internal infection rates after surface sterilization of the pest insects were 11, 34 and 35% for adult *T. vaporariorum*, 29, 54 and 58% for adult *L. lineolaris*, 22, 34 and 30% for nymphal *M. persicae* and 17, 29 and 32% for nymphal *T. vaporariorum*, respectively, at the low, middle and high concentrations. Significantly more bumble bees died at the high concentration of *B. bassiana* (42–45%) than at the other concentrations

J. P. Kapongo · P. Kevan
Department of Environmental Biology, University of Guelph, Guelph, ON, Canada N1G 2W1
e-mail: tkapongo@uoguelph.ca

P. Kevan
e-mail: pkevan@uoguelph.ca

L. Shipp (✉)
Agriculture and Agri-Food Canada, Greenhouse and Processing Crops Research Centre, Harrow, ON, Canada N0R 1G0
e-mail: shippl@agr.gc.ca

B. Broadbent
Agriculture and Agri-Food Canada, Southern Crop Protection and Food Research Centre, London, ON, Canada N5V 4T3
e-mail: broadbentb@agr.gc.ca

(9–15%) and the controls (5–7%). Spores of *B. bassiana* were collected throughout the plant canopy with the greatest numbers sampled from the top third of the canopy [ca. 1,200 colony forming units (CFU) per cm^{-2}]. The middle concentration was selected as the optimal concentration because it provided the best pest control with the least impact on the bees.

Keywords *Beauveria bassiana* · Biological control · *Bombus impatiens* · Greenhouse tomatoes and sweet peppers · *Lygus lineolaris* · *Myzus persicae* · *Trialeurodes vaporariorum*

Introduction

Pests (arthropods and diseases), both native and exotic, are important problems for integrated pest management (IPM) of greenhouse crops. Over the last two decades in Canada, more than one new pest per year has invaded greenhouse vegetable crops (Ferguson and Shipp 2002; Gillespie 2002). The presence of these new pests necessitates implementation of control programs that are compatible with existing IPM strategies. Parasitoids and predatory arthropods are usually the main control measures in greenhouse vegetable crops (Albajes et al. 1999; Heinz et al. 2004). Among microbial control agents, *Bacillus thuringiensis* (Berliner) (Bacillales: Bacillaceae) has been the most popular control agent (Osborne et al. 2004).

Many entomopathogenic fungi also represent promising control measures for insect pests (Faria and Wraight 2001). The fungi invade their host insects through the external cuticle. Spores of the fungi attach to the cuticle, germinate and penetrate the host. They then proliferate in the host haemocoel as walled hyphal bodies or wall-less amoeboid protoplasts. The host insects die as a result of several modes of action, including depletion of nutrients, physical obstruction or invasion of organs, and toxinosis (Hajek 1997; Butt and Goettel 2000).

Beauveria bassiana (Balsamo) Vuillemin (Ascomycota: Hypocreales) is a potentially effective control agent for several greenhouse pests including whiteflies, *Lygus*, thrips and aphids (Gindin et al. 1996; Liu et al. 2003; Shipp et al. 2003; Al-mazra'awi et al. 2006a; Quesada-Moraga et al. 2006). Fortunately, *B. bassiana* can be used effectively in association with natural enemies, others pathogens, different types of pesticides and pollinators (Goettel et al. 1990; Steinkraus and Tugwell 1997; Jacobson et al. 2001; Shipp et al. 2003; Kouassi et al. 2003). Goettel and Jaronski (1997) found that <1% of the honey bee workers became infected and no infection was detected in the brood when treated with the equivalent of 5×10^{13} conidia of *B. bassiana* per hectare of canola three times at 5-day intervals. However, the bumble bees, *Bombus pratorum* (L.) and *B. terrestris* (L.) (Hymenoptera: Apidae) have been reported infected by *B. bassiana* (Goettel et al. 1990) and *B. bassiana* will infect *B. impatiens* without epizootics in the hive (Al-mazra'awi et al. 2006a). This latter relationship needs to be quantified.

Al-mazra'awi et al. (2006a) demonstrated for the first time that bumble bees can be used to vector *B. bassiana* for control of *Lygus lineolaris* (Palisot de Beauvois) (Hemiptera: Miridae) and *Frankliniella occidentalis* (Pergande) (Thysanoptera: Thripidae) in greenhouse sweet pepper. Mean infection mortalities for *L. lineolaris* ranged from 34–45% compared to 9–15% in the control. Mean infection rates for *F. occidentalis* ranged from 34–40% compared to 3% in the control. For outdoor crops, honey bees have been shown capable to deliver *Metarhizium anisopliae* (Metsch.) Sorokin for control of pollen beetle,

Meligethes aeneus (F.) (Coleoptera: Nitidulidae) (>70% infection levels) (Butt et al. 1998; Carreck et al. 2007) and *B. bassiana* for *L. lineolaris* (22–56% infection levels) (Al-mazra'awi et al. 2006b) on canola. None of these studies investigated the optimal concentration of bee-vector entomopathogenic fungi for the pest control; distribution of the fungal control agent by the bee in the plant canopy; or quantified the effects of this fungus on bees.

Bumble bees are the main pollinator of greenhouse tomatoes and peppers worldwide (Kevan et al. 1991; Shipp et al. 1994; Velthuis and Van Doorn 2006). Combining the Bee Vector Technology and its use as a crop pollinator provides a new value added management strategy for pest management and crop production improvement in greenhouse tomato and sweet pepper. This technology has multiple potential benefits to growers such as: the delivery of the biological control agent (inoculum) to flowers, leaves and developing fruit; continuous dissemination of the inoculum to the crop 7 days a week; no involvement of manual application costs (labour) for the biological control agents; the combination of pollination and distribution of biological control agents; and the reduced negative impact on the environment as most of the inoculum lands on the plant and not in the soil or air (Kevan et al. 2007).

Thus, the objectives of the current studies were: (1) to quantify relationships among inoculum densities of *B. bassiana* as vectored by bumble bees on arthropod pest mortality [*Trialeurodes vaporariorum* (Westwood) (Hemiptera: Aleyrodidae), *Myzus persicae* (Sulzer) (Hemiptera: Aphididae) and *L. lineolaris*]; (2) to assess mortality of bumble bee vectors exposed to three inoculum densities of *B. bassiana*; and (3) to investigate the distribution of bee-vectored *B. bassiana* in the plant canopy.

Materials and methods

Plants

Tomato, *Lycopersicon esculentum* Mill. (Solanaceae) (cv Rapsodie), and sweet pepper, *Capsicum annuum* L. (Solanaceae) (cv Edison), plants were grown in a mixture of 1:1 sand and peat moss in 20 cm pots. The plants were maintained in a separate greenhouse compartment until they produced two sets of flowers. They were then transferred to fine-meshed experimental cages (520 × 240 × 220 cm). An Argus Computer Control System (Argus Control Systems Ltd., White Rock, BC, Canada) was used to maintain climatic conditions of 21–23°C and 80–85% RH for both crops. Plants were watered and fertilized with the Harrow Fertigation Manager (Labbate Climate Control Systems, Leamington, ON, Canada) following standard commercial practices (Ontario Ministry of Agriculture and Food 2005).

Insects

Adult *T. vaporariorum* and *M. persicae* used in the trials were collected from greenhouse colonies that were maintained on tomato and sweet pepper plants at the Agriculture and Agri-Food Canada (AAFC), Greenhouse and Processing Crops Research Centre (GPCRC), Harrow, ON, Canada. Second instar nymphs of *T. vaporariorum* for the cage trials were obtained by artificially infesting one top canopy leaf per plant on 12 randomly-selected tomato plants per treatment with 80 adult whiteflies inside an organdy sleeve cage

(300 μm mesh) 2 weeks before each sampling date. The adults were left on the leaves for 3 days to allow for production of at least 50–100 eggs leaf^{-1}. The late instar nymphs of *M. persicae* that were sampled in the trials were obtained from progeny of *M. persicae* that were released at the beginning of the trial.

Newly emerged adults of *L. lineolaris* (24–48-h old) were obtained from a laboratory culture maintained at AAFC, Southern Crop Protection and Food Research Centre (SCPFRC), London, ON, Canada. The colony of *L. lineolaris* was established from field-collected adult insects on alfalfa at the SCPFRC Research Farm. The laboratory colony of *L. lineolaris* was maintained on organic lettuce as a food source at 24°C, 60% RH and 16-h photoperiod.

Bumble bee colonies (*B. impatiens*), provided by Biobest Canada Ltd., Leamington, ON, Canada, were used in all trials. Each colony consisted of 1 queen and 50 workers. The colonies were anaesthetized by CO_2 and transferred (bees + cells) from the commercial box to a wooden hive box (32 × 23 × 24 cm) fitted with an inoculum dispenser (Fig. 1). The bees were kept in the wooden boxes for 2 days and fed sugar solution (syrup) before using them in the cage trials to allow the bees to become familiar with the new hive box. The dispenser used was the same as Yu and Sutton (1997)'s model but as modified by Al-mazra'awi (2004).

Biological control agent

The commercial formulation of *B. bassiana*, GHA strain (BotaniGard 22WP®, Laverlam International Corporation, Butte, MT, USA) was used for all trials. The initial concentration of BotaniGard 22WP® was 2 × 10^{11} conidia of *B. bassiana* per g of product. Viability of the *B. bassiana* was determined before preparing the inoculum for each trial. This allowed for readjustment of the amount of product used to make the appropriate concentration for each treatment. To determine viability of the conidia, six 0.01 g samples of Botanigard 22WP® were suspended each in flask containing 100 ml distilled water and 0.1% Tween 80. Then 200 μl of the conidial suspension were added to 1 ml of Sabouraud's dextrose broth with 1% yeast extract in a sterile test tube. The suspension was

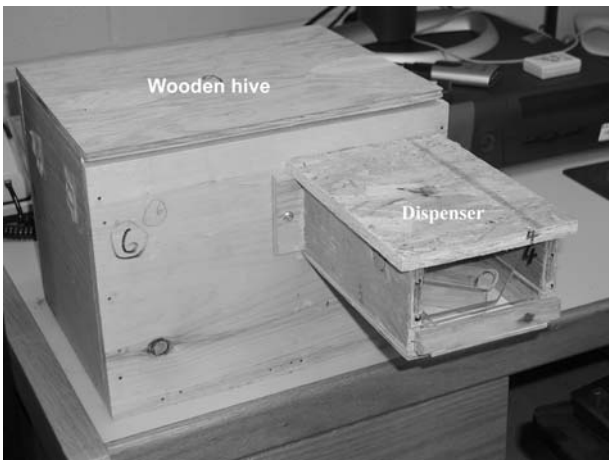

Fig. 1 Wooden dispenser (21 × 12 × 8.5 cm) affixed to wooden hive (32 × 23 × 24 cm)

incubated at 24 ± 1°C for 24 h. After this, four groups each consisting of 200 conidia were examined for percentage germination using a hemacytometer and a compound microscope. Viability of the *B. bassiana* ranged from 95 to 99% for each of the trials. Three active concentrations of *B. bassiana* were prepared by mixing autoclaved corn flour, the diluent/carrier found to be most efficacious with the BotaniGard 22WP® (Al-mazra'awi et al. 2007). These concentrations were: 9×10^9 (low concentration), 6.24×10^{10} (middle concentration) and 2×10^{11} (high concentration) conidia of *B. bassiana* per g of the product.

Experimental design and procedures

The cage trials were conducted in greenhouse compartments (13 × 8 m) containing two or three cages per greenhouse at the GPCRC. Fine mesh screening was used to contain the bees as well as the target pests. Each cage contained 64 potted sweet pepper (spring and summer 2004) or tomato plants (spring and summer 2005). The plants were arranged in two double rows of 16 plants per row in each cage. The environment within the cages was maintained at 21–23°C and 80–85% RH throughout the trials. A completely randomized block design was used for each of the five treatments with four replicates (sweet pepper) and five replicates (tomatoes) over time. The treatments included three inoculum densities: 9×10^9 conidia g^{-1}, 6.24×10^{10} conidia g^{-1} and 2×10^{11} conidia of *Beauveria* g^{-1} (label rate for commercial formulation), heat inactivated *B. bassiana* treatment and a control treatment in which the inoculum was absent. One hive of 50 bumble bee workers (*B. impatiens*) was introduced into each treatment, except for the control treatment which had no bees.

Each colony of bumble bees was introduced 1 day after the plants were placed in the cages to allow acclimation to their new hive and the cage. On day 2, 256 *L. lineolaris* and 960 green peach aphids, *M. persicae* were released per treatment for the pepper trials and 1,000 *T. vaporariorum* in the tomato trials for each replicate. Wooden dispensers (21 × 12 × 8.5 cm) filled with 25 g of the appropriate inoculum concentration (Botani-Gard 22WP® and corn flour mixture) were placed at the exit/entry opening of the hives on day 3, after which the bees were allowed to forage and disseminate the inoculum. The first samples (50 *L. lineolaris* and *T. vaporariorum* adults, and 48 nymphal *T. vaporariorum* or *M. persicae*) were collected on day 6 after which the dispensers were removed. The insect pests were collected to determine the percentage of mortality caused by *B. bassiana*, the number of *B. bassiana* propagules per *Lygus* or groups of five adult *T. vaporariorum* and groups of six immature *T. vaporariorum* and *M. persicae*, and the internal infection level of surface sterilized insect pests by *B. bassiana*. The hives were then closed and the bees (*B. impatiens*) fed pollen patties (pollen grains mixed with 50% wt./wt. sugar solution) until the dispensers were replaced. The dispensers were refilled and replaced on day 10 and a second set of samples of pest insects was collected 3 days later. After each sampling period, the remaining inoculum in the dispenser was dried and weighed using a Mettler AT250's scale (Mettler-Toledo, Inc., USA) to determine the amount of inoculum that the bumble bees vectored during the sampling period. At each sampling date, five bees were collected per treatment as they exited from the dispenser to determine the number of *B. bassiana* propagules per bee.

Ten *L. lineolaris*, 10 *T. vaporariorum* adults and 24 nymphal *M. persicae*/*T. vaporariorum* per sample were surface sterilized to estimate internal infection levels (i.e. infection levels in the pests at the time that the samples were collected). The samples were surface-sterilized by immersing in 70% ethanol for 15–20 s, followed by 5% bleach containing

0.3% NaOCl amended with 0.05% Tween-20 for 3 min and two rinses of sterile distilled water amended with Tween-20 for 1–2 min (Shipp et al. 2003) The surface sterilized insects were then placed on water agar plates and incubated at 24 ± 1°C and 80% RH for 5 days to determine the incidence of mycosis.

Another 10 *L. lineolaris*, 10 *T. vaporariorum* adults 24 nymphal *M. persicae*/*T. vaporariorum* and 5 *B. impatiens* per treatment sample were washed to determine the amount of conidia [colony forming units (CFU)] that individual pests and bees carried. The samples were agitated individually in flasks containing 100 ml sterilized distilled water and 0.1% Tween 80 for the bees (in 50 ml of sterilized distilled water for groups of five individuals for adult *T. vaporariorum* and groups of six individuals for immature *T. vaporariorum* and *M. persicae*) on a rotary shaker at 125 rpm for 2 h. Three 0.1 ml aliquots of tenfold serial dilutions of each suspension were spread on oatmeal agar Petri plates amended with 550 μg ml^{-1} Dodine, 400 μg ml^{-1} penicillin G., 1,000 μg ml^{-1} streptomycin sulphate and 5 μg ml^{-1} crystal violet (Beilharz et al. 1982), incubated at 25 ± 1°C for 4–5 days, after which colonies of *B. bassiana* were counted and recorded.

The remaining 30 *L. lineolaris* and *T. vaporariorum* were used to determine the percentage mortality caused by *B. bassiana* over time. Each adult *L. lineolaris* was placed in an aerated Petri dish (9-cm ∅) and fed fresh organically grown lettuce leaves for 7 days. Individuals of *T. vaporariorum* were placed in an aerated sealed plastic vial on the growing point of a tomato plant for 7 days. The tomato growing points and organic lettuce were replaced every second day. All bioassay cages were inspected daily for dead insects. Dead insects were placed on moistened filter paper-lined Petri dishes and incubated at 25°C and 80% RH for 7 days. Presence of white mycelium on the dead insects was used to indicate the incidence of *B. bassiana* infection. *Myzus persicae* and *T. vaporariorum* nymphal mortalities could not be determined this way because of high handling mortality.

Mortality of the bumble bees (*B. impatiens*) exposed to the *B. bassiana* treatments was determined after the second sampling period. At this time, each bumble bee colony was transferred back into the commercial hive box and fed pollen patties for 3 weeks to simulate a typical time period that the hives would be kept in commercial greenhouses. The hives were maintained in controlled environment chambers at 23 ± 1°C. Incidence of bumble bee mortality was recorded weekly.

Twelve plants (three in each row) per treatment were selected randomly at each sampling period. One flower and three leaves were collected individually from the selected plant. The leaves were sampled at different positions of the plant canopy which was divided into three sections (top, middle and bottom). One leaf was collected from each of the three positions. The flower and leaf samples then were processed individually following the protocol described earlier for insect pests and bees to determine the number of CFU of *B. bassiana*. The leaves were washed in 100 ml sterilized distilled water and 0.1% Tween 80, and the flowers in 50 ml of sterilized distilled water.

Statistical analysis

Abbott's formula (Abbott 1925) was used to determine percent mortalities of adult *T. vaporariorum* and *L. lineolaris* for the three *Beauveria* treatment concentrations. All mortality, mycosis and internal infection data for *T. vaporariorum*, *L. lineolaris* and *M. persicae*, and bee mortalities were subjected to arcsine square root transformation before statistical analysis. The transformed data for each insect species were first analysed using two-way analysis of variance (ANOVA). Mean percentages of killed insects and

insects exhibiting mycosis were then compared using Tukey multiple comparison (SAS Institute 2001) to determine if significant differences occurred among them.

To compare the number of CFU on the insect and plant samples, and to determine the vertical distribution of *B. bassiana* in the crop canopy, the CFU counts of *B. bassiana* at each leaf position of the canopy and on the different insect and plant samples were arcsine transformed and subjected to PROC univariate, residual analysis and then analysed using a repeated measurement procedure (PROC MIXED covtest, SAS Institute 2001). The mean numbers of CFU of *B. bassiana* from each insect and plant sample and at each of the three leaf positions in the canopy were then compared using the F-test. Mortality and CFU data were back-transformed to their original scales for presentation in the tables.

Results

Mortality of pest insects by Beauveria bassiana

The highest percentages of dead insects were recorded at the concentrations of 6.24×10^{10} and 2×10^{11} conidia of *B. bassiana* per g of the inoculum (Table 1). A significant difference in percent mortality of insect pests was found among the three concentrations of *B. bassiana* ($F_{2,\ 21} = 39.41$, $P < 0.0001$). Comparison between the middle and high concentrations indicated that there was no significant difference in mortality rates between these concentrations. The type of crop (pepper and tomato) did not have any impact on the performance of the different concentrations of *B. bassiana* ($F_{1,\ 21} = 0.16$, $P = 0.8524$).

The rate of internal infection of *B. bassiana* increased with the concentration of *B. bassiana* (Table 1). On tomato, it varied from 11.0 to 35.0% in adult whiteflies and 17.0–32.0% in whitefly nymphs from low to high concentration. A similar trend was found on sweet pepper in which the infection rate varied between 29 and 58% in adult *L. lineolaris* and 22–30% in *M. persicae*. None of the insect pests in either the heat inactivated inoculum or the control treatments were infected (Table 1).

Effects of Beauveria bassiana on bumble bees (*Bombus impatiens*)

The highest percentage of bumble bee death occurred in the colonies in which the highest concentration of *B. bassiana* was used (Table 2). Comparison of treatments regarding the percentages of bee mortality showed a significant difference among treatments ($F_{4,\ 40} = 321.12$, $P < 0.0001$) with the greatest percentage of dead bumble bees in the high concentration of *B. bassiana* compared to the other two concentrations. The percentages of mycosis among the dead bees were higher (73%) at the highest inoculum level than at the middle and lower levels (44–56%) (Table 2).

Vertical distribution of spores of Beauveria bassiana in the crop canopy

The numbers of spores of *B. bassiana* deposited by the bumble bees were not evenly distributed at each canopy height (Table 3) and each bee carried about 0.21 and 0.20 mg of inoculum per day in the tomato and pepper trials, respectively. The distribution of spores on the top, middle and bottom sections of tomato plants differed significantly ($F_{2,\ 81} = 11.13$, $P < 0.0001$) with the highest number on the top leaves. Treatment effects

Table 1 Mean (SE) mortality, mycosis and internal infection level of *Trialeurodes vaporariorum*, *Lygus lineolaris* and *Mysus persicae* exposed to three concentrations of bumble bee-vectored *Beauveria bassiana* and two control treatments

Treatments (conidia of *Beauveria* per g of inoculum)	*T. vaporariorum* adults (five trials)			*L. lineolaris* adults (four trials)			*T. vaporariorum* nymphs	*M. persicae* nymphs
	% Dead ($n = 300$)	% of dead with mycosis	% Internal infection ($n = 100$)	% Dead ($n = 240$)	% of dead with mycosis	% Internal infection ($n = 80$)	% Internal infection ($n = 240$)	% Internal infection ($n = 192$)
Control treatment (no bees, no inoculum)	0a	0a	0a	0a	0a	0a	0a	0a
Heat inactivated inoculum	0a	0a	0a	0a	0a	0a	0a	0a
Low concentration (9×10^9)	17.9 (2.1)b	69.2 (1.5)b	11.0 (2.7)b	33.0 (5.0)b	71.0 (6.6)b	28.7 (2.9)b	17.0 (1.3)b	21.5 (3.5)b
Middle concentration (6.24×10^{10})	53.9 (3.4)c	81.5 (1.4)c	34.0 (4.0)c	69.7 (3.6)c	85.0 (2.7)c	53.7 (4.6)c	29.0 (1.7)c	33.5 (3.3)c
High concentration (2×10^{11})	55.9 (4.2)c	81.4 (1.4)c	35.0 (4.0)c	67.1 (5.2)c	90.0 (3.2)c	57.5 (4.5)c	32.0 (1.6)c	29.5 (5.3)c

n, numbers of samples processed (a total of 40 individuals per sample for each of the 2 sampling periods per trial were collected, 30 for mortality and 10 for internal infection). Within a column, means followed by different letters are significantly different at $P < 0.05$ using an F-test. All treatment mortalities were adjusted using Abbott's formula

Table 2 Mean (SE) mortalities of *Bombus impatiens* exposed to three concentrations of *Beauveria bassiana* and two control treatments

Treatments (conidia of *Beauveria* per g of inoculum)	Bees (Tomato)		Bees (Pepper)	
	% Dead ($n = 1600$)	% of dead with Mycosis	% Dead ($n = 1240$)	% of dead with Mycosis
Control hive (not exposed to cage trials)	6.5 (1.4)a	0a	5.5 (0.8)a	0a
Heat inactivated inoculum	7.0 (0.4)a	0a	6.1 (0.4)ab	0a
Low concentration of *Beauveria* (9×10^9)	9.0 (1.3)ab	53.8 (5.1)b	10.9 (0.6)b	43.5 (7.3)b
Middle concentration of *Beauveria* (6.24×10^{10})	14.8 (2.3)b	55.5 (3.3)b	10.9 (0.9)b	54.7 (4.2)b
High concentration of *Beauveria* (2×10^{11})	42.0 (1.8)c	73.4 (4.5)c	45.4 (2.4)c	73.1 (5.7)c

n, numbers of samples processed (the number of bees that were found in the hive 3 weeks after the second sampling period). Within a column, means followed by different letters are significantly different at $P < 0.05$ using an F-test

on deposited spores also differed among canopy sections with the greatest number of CFU found on the top leaves at the highest concentration of *Beauveria* ($F_{2,\ 81} = 57.86$, $P < 0.0001$). No interaction was found between inoculum density and density of spores of *B. bassiana* on the leaves ($F_{4,\ 81} = 1.25$, $P = 0.2959$). Similar results were obtained in sweet pepper. As in tomato, the distribution of spores of *B. bassiana* differed significantly among the three levels in the leaf canopy in peppers ($F_{2,\ 63} = 28.42$, $P < 0.0001$) with the highest number of spores deposited on the top leaves. There was a significant difference among the three concentrations of spores of *B. bassiana* applied ($F_{2,\ 63} = 29.24$, $P < 0.0001$). The results also indicated that there was no interaction between treatments and distribution of spores in the canopy ($F_{4,\ 63} = 0.12$, $P = 0.9761$).

Number of CFU of Beauveria bassiana deposited by bumble bees on leaves and flowers

Colony forming units of *B. bassiana* were detected on a majority of the tomato leaf samples from the treatments in which the bees vectored high, middle and low concentrations of the pathogen. On pepper, essentially all leaves had CFU of *B. bassiana* from the high, middle and low concentrations. Counted CFU/cm^{-2} ranged from 714 to 76 on tomato leaf samples and from 596 to 172 on sweet pepper, for the high, middle and low concentrations of *B. bassiana* (Tables 4, 5). No CFU of *B. bassiana* was detected on the leaves in the heat inactivated inoculum and control treatments.

Colony forming unit counts of *B. bassiana* on the tomato flower samples varied from 30.7×10^3 to 2.8×10^3 for the high, middle and low concentration treatments. The percentages of flowers having CFU of *B. bassiana* were again high (>75%) for the high, middle and low concentration treatments. In the pepper trials, 22.0×10^3 to 2.1×10^3 CFU of *B. bassiana* were found on flowers from the high, middle and low concentration treatments. Those CFU of *B. bassiana* were detected on 85–58% of the flower samples corresponding to high, middle and low concentration treatments (Tables 4, 5).

Number of CFU of Beauveria bassiana on insects (pests and pollinator)

The numbers of CFU of *B. bassiana* found on the different insects and the percentage of those insects that had detectable CFU are presented in Tables 4 and 5, respectively, for the

Table 3 Mean (SE) number of colony forming units (CFU cm^{-2}) of *Beauveria bassiana* on leaves at different plant canopy positions that were exposed to three concentrations of bumble bee-vectored *Beauveria bassiana* and one control treatment

Treatments (conidia of *Beauveria* per g of inoculum)	Tomato (five trials), mean CFU per cm^{-2} ($n = 120$)			Pepper (four trials) mean CFU per cm^{-2} ($n = 96$)		
	Position of leaves in plant canopy			Position of leaves in plant canopy		
	Top	Middle	Bottom	Top	Middle	Bottom
Heat inactivated inoculum	0	0	0	0	0	0
Low concentration of *Beauveria* (9×10^9)	93.0 (17.9)c	85.4 (28.7)b	49.2 (11.3)a	301.8 (45.0)b	102.6 (30.1)a	112.8 (46.3)a
Middle concentration of *Beauveria* (6.24×10^{10})	1,028.1 (238.1)b	272.1 (50.6)a	281.9 (71.0)a	1,038.5 (151.5)b	322.8 (76.3)a	400.2 (149.9)a
High concentration of *Beauveria* (2×10^{11})	1,174.9 (331.3)b	551.0 (87.2)a	417.2 (101.6)a	1,186.5 (148.9)b	384.7 (73.0)a	285.7 (63.8)a

n, numbers of processed samples (a total of 36 leaves for each of the 2 sampling periods per trial were collected, 12 per canopy position). Within each row, means CFU for tomato or pepper followed by different letters are significantly different at $P < 0.05$ using an F-test

Table 4 Mean (SE) number of colony forming units, CFU/insect or plant sample, and mean (SE) percentage of *Bombus impatiens*, tomato flower and leaf samples with detectable densities of *Beauveria bassiana* as collected from cages exposed to three concentrations of bumble bee-vectored *B. bassiana*

Treatments	Low concentration of *Beauveria*		Middle concentration of *Beauveria*		High concentration of *Beauveria*	
	Mean number of CFU	% of samples with CFU	Mean number of CFU	% of samples with CFU	Mean number of CFU	% of samples with CFU
B. impatiens ($n = 50$)	1.0×10^4 (1.7×10^3)a	75.0 (22.6)	1.9×10^5 (3.8×10^4)b	100	1.6×10^6 (3.1×10^5)c	100
Flowers ($n = 120$)	2.87×10^3 (0.4×10^3)a	75.0(14.12)	2.3×10^4 (2.5×10^3)b	95.0 (3.4)	3.07×10^4 (3.2×10^3)b	88.3 (9.6)
Leaves (per cm^{-2}) ($n = 120$)	75.8 (12.0)a	65.2 (16.23)	527.4 (104.6)b	87.5 (12.2)	714.3 (130.1)c	88.8 (11.0)
Trialeurodes vaporariorum adults ($n = 100$)	68.5 (2.8)a	N/A	355.8 (9.1)b	N/A	543.8 (50.9)c	N/A
T. vaporariorum nymphs ($n = 240$)	30.2 (2.5)a	N/A	163.6 (6.2)b	N/A	266.8 (21.6)c	N/A

Beauveria concentrations were 9×10^9 (low), 6.24×10^{10} (middle) and 2×10^{11} (high concentration) of conidia of *Beauveria* per g of inoculum. *n* numbers of samples processed (a total of 5 *B. impatiens*, 10 *T. vaporariorum* adults, 12 flowers, 12 leaves and 24 *T. vaporariorum* nymphs were collected per sample for 2 sampling periods for each of the five trials). N/A: The percentage of individual *T. vaporariorum* adults and nymphs with detectable spores of *Beauveria* was not determined because they were processed in groups of five adults and six nymphs per sample for CFU counts. Within a row, means followed by different letters are significantly different at $P < 0.05$ using an F-test

Table 5 Mean (SE) number of colony forming units, CFU/insect or plant sample and mean (SE) percentage of *Bombus impatiens*, pepper flowers, pepper leaves, *Lygus lineolaris* adults and *Myzus persicae* nymphs with detectable densities of *Beauveria bassiana* as collected from cages exposed to three concentrations of bumble bee-vectored *B. bassiana*

Treatments	Low concentration of *Beauveria*		Middle concentration of *Beauveria*		High concentration of *Beauveria*	
	Mean number of CFU	% of samples with CFU	Mean number of CFU	% of samples with CFU	Mean number of CFU	% of samples with CFU
B. impatiens ($n = 40$)	3.3×10^5 (1.1×10^5)a	77.5 (11.3)	1.7×10^6 (9.0×10^5)b	87.50 (11.8)	1.6×10^6 (7.4×10^5)b	92.5 (5.5)
Flowers ($n = 96$)	2.1×10^3 (0.3×10^3)a	57.6 (30.2)	1.17×10^4 (2.8×10^3)b	75.0 (8.6)	2.2×10^4 (2.4×10^3)c	84.6 (11.4)
Leaves (per cm^2) ($n = 96$)	172.4 (26.6)a	98.6 (1.5)	587.2 (98.3)b	100	596.37 (95.9)b	100
L. lineolaris adults ($n = 80$)	77.3 (29.3)a	18.17 (3.6)	290.3 (25.3)b	33.7 (7.4)b	252.7 (29.7)b	26.2 (6.3)
M. persicae nymphs ($n = 192$)	39.0 (9.1)a	N/A	277.5 (30.5)b	N/A	229.7 (41.6)b	N/A

Beauveria concentrations were 9×10^9 (low), 6.24×10^{10} (middle) and 2×10^{11} (high concentration) of conidia of *Beauveria* per g of inoculum. *n* numbers of samples processed (a total of 5 *B. impatiens*, 10 *L. lineolaris* adults, 12 flowers, 12 leaves and 24 *L. lineolaris* nymphs were collected per sample for 2 sampling periods for each of the four trials). *Myzus persicae* nymphs were pooled in groups of six nymphs for CFU counts. N/A: The percentage of individual *M. persicae* nymphs with detectable spores of *Beauveria* was not determined because they were processed in groups of six. Within a row, means followed by different letters are significantly different at $P < 0.05$ using an F-test

tomato and sweet pepper trials. Approximately 96 to 76% of the bumble bees from the high, middle and low concentration treatments from both crops carried spores of *B. bassiana* after leaving the dispenser. On tomato, bumble bees had 1.6×10^6 to 1.0×10^4 CFU of *B. bassiana* for the high, middle and low concentration treatments. On pepper, they carried 1.6×10^6 to 3.3×10^5 CFU of *B. bassiana* from the high, middle and low concentration treatments. On the pest insects, the numbers of CFU ranged from 544 to 69 for groups of five adult *T. vaporariorum* and from 253 to 77 per adult *L. lineolaris*, for the high, middle and low concentration treatments. Colony forming units of *B. bassiana* varied from 267 to 30 for groups of six *T. vaporariorum* nymphs, and from 230 to 39 for groups of six *M. persicae*, for the high, middle and low concentration treatments. No *B. bassiana* was found on any of the insects from the heat inactivated inoculum and control treatments.

Discussion

In the current study, individual bumble bees carried ca. 0.20 mg of inoculum per day when exiting the dispenser which was approximately half of what Al-mazra'awi et al. (2006a) and Yu and Sutton (1997) recorded. Al-mazra'awi et al. (2007) reported that the quantity of conidia of *B. bassiana* that was picked up by honey bees was less when the moisture content of the inoculum was lower. In the trials by Al-mazra'awi et al. (2006a), the humidity was 70% compared to 80–85% RH in our trials.

Distribution of spores in the plant canopy in our study showed a difference among the three plant heights (top, middle and bottom). Foraging behaviour of the bees and the growth phenology of the plant were probably the main determinants for the vertical distribution pattern of spore deposition. Bumble bees are attracted to the flowers for pollen and nectar (Kevan 2005), and flowers in general occur in the top third of the plant canopy. Thus, the leaves in the top section of the canopy would receive also larger amounts of inoculum from the bees. In addition, inoculum powder probably was shed also from the bees by buzz pollination of the flowers whereby high frequency muscle vibrations of the bees are used to shake the pollen off the flowers and grooming by the bees on the leaves. This inoculum ended up being deposited throughout the plant canopy. The horizontal distribution of inoculum among plants could not be assessed because of the small size of the cage (5.20×2.40 m) and bumble bees are able to fly more than 200 m (Heinrich 1979; Loose et al. 2005).

The spores of *B. bassiana* vectored by the bumble bees and deposited on the flowers and leaves infected the *T. vaporariorum* and *L. lineolaris* present on the plants. Adult *L. lineolaris* mortalities were greater than for adult *T. vaporariorum* (68% vs. 55%) for the pooled middle and high concentration treatments, and 33% vs. 18% for the low concentration treatment. There are several possible reasons for these differences. First, *L. lineolaris* are more mobile insects than whiteflies (Howard et al. 1994). The latter only move a short distance before settling down on another leaf (Arnó et al. 2006). By frequently moving, *L. lineolaris* is more likely to contact spores of *B. bassiana* either in the air or on the plant surface. Second, *L. lineolaris* feed on the upper parts of the plants such as leaves, flowers and growing points where most of the infective spores of *B. bassiana* were deposited. In contrast, whiteflies feed on the underside of the plant leaves (Arnó et al. 2006) which are less exposed to deposition of *B. bassiana*'s spores carried by the bumble bees.

Individual aphid and whitefly nymphal mortalities were not determined because mortality from handling these insects was too great. However, the internal infection rates

recorded for the aphids and whitefly nymphs were high enough to show that the *B. bassiana* induced mortality among them. The internal infection level for adults was always lower than the bioassay mortalities (Table 1). This can be explained by the fact that *B. bassiana* spores must first attach, then penetrate the insect cuticle and multiply in the haemocoel. This infection process may take several hours to 2 days (Jaronski 1997). In the present study, insect pests were immediately processed after their collection to determine their internal infection levels. Thus, the spores attached to insects used in the bioassay trials had a longer time to germinate and penetrate the host compared to those samples that were processed for surface sterilization shortly. Similar internal infection levels for immature *T. vaporariorum* in complete greenhouse cucumber spray trials at GPCRC resulted in >60% reduction in adult *T. vaporariorum* population levels after four weekly spray applications of *B. bassiana* (Shipp et al. 2003). In addition, Wraight et al. (2000) reported >90% control of immature *Bemisia argentifolii* Bellow and Perring in field trials on cucumber, melon and cantaloupe where *B. bassiana* was applied using a portable air-assisted sprayer.

Findings from our study indicate that *B. bassiana* can have an impact on bumble bee pollinators. Vandenberg (1990) reported that *B. bassiana* reduced honey bee longevity at concentrations of 9×10^7 and 9×10^8 spores per bee when sprayed on the bees or mixed in 50% sucrose syrup and fed to the bees. However, when whole-hives of honey bees were exposed to the same concentration of *B. bassiana* (strain GHA), as used in the middle concentration of our study, for three times at 5-day intervals, infection level was <1% for workers and no infection occurred in the brood (Goettel and Jaronski 1997). Brood temperature for honey bees is ca. 32–36°C which is too high for germination and development of *B. bassiana* (Ekesi et al. 1999). The hive temperatures for *B. impatiens* were not measured in the current study, but may also impact the susceptibility of *B. impatiens* to infection by *B. bassiana*, especially when colony size exceeds the initial stocking rate of 50 bees per hive. In commercial greenhouses, hives populations will grow to >100 bees per hive before the hive is replaced.

In our study, exposure of *B. impatiens* to the high concentration of *B. bassiana* for 3 days and then repeated again for another 3-day exposure period after 4 days resulted in 42–45% mortality compared to the same exposure periods for the other treatments after a 5 weeks period. However, the percentage mortality of bumble bees exposed to the middle and low concentrations were not statistically different (9–15%) and, under practical commercial production conditions, not much higher than the control mortalities (5–7%). Evaluation of these bee-vectored *B. bassiana* concentrations under commercial production conditions for greenhouse tomato over a 7 weeks period in 2006 found that pollination level and fruit yield and quality were all within acceptable commercial standards (J.P.K., unpublished data). The high and middle concentrations of *B. bassiana* also killed the highest percentages of *T. vaporariorum* and *L. lineolaris* (ca. 62%). Therefore, the middle concentration of *B. bassiana* as vectored by bumble bee pollinators was considered the optimal concentration level.

Our study has shown that bumble bees can be used to vector effectively *B. bassiana* to greenhouse tomato and sweet pepper to infect greenhouse pests such as *T. vaporariorum, M. persicae* and *L. lineolaris*. This is a new technique for the application of microbial control agents which combines pest control with flower pollination (Kevan et al. 2005). Future research will test if other control agents compatible with *B. bassiana* could replace some or all of the corn flour that was added in the preparation of the inoculum, so that bumble bees could vector multiple agents for potential control of arthropod pests and suppression of plant diseases.

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