Biological Control 46 (2008) 508–514

Contents lists available at ScienceDirect

Biological Control

journal homepage: www.elsevier.com/locate/ybcon

Co-vectoring of *Beauveria bassiana* and *Clonostachys rosea* by bumble bees (*Bombus impatiens*) for control of insect pests and suppression of grey mould in greenhouse tomato and sweet pepper

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ARTICLE INFO

Article history:
Received 14 February 2008
Accepted 19 May 2008
Available online 27 May 2008

Keywords:
Beauveria bassiana
Bombus impatiens
Botrytis cinerea
Clonostachys rosea
Lygus lineolaris
Trialeurodes vaporariorum
Greenhouse tomato and sweet pepper

ABSTRACT

Greenhouse cage trials were conducted to assess the effectiveness of bumble bee pollinators for the co-vectoring of two fungi (*Beauveria bassiana* [Balsamo] Vuillemin [BotaniGard 22WP® formulation] and *Clonostachys rosea* Lnk: Fr. [Endofine®]), in greenhouse tomato and sweet pepper for control of insect pests (greenhouse whitefly, *Trialeurodes vaporariorum* Westwood; and tarnished plant bug, *Lygus lineolaris* [Palisot de Beauvois]), and grey mould (*Botrytis cinerea* Pers: Fr.). Three treatments were evaluated: 6.24×10^{10} conidia of *B. bassiana* + 1.38×10^{7} conidia of *C. rosea*/g of inoculum vectored by bees (active inoculum); heat-inactivated inoculum vectored by bees; and a control with no inoculum and no bees. In each crop, the treatments were arranged in a completely randomized block design with four replications. When applied in the tomato crop, the active inoculum killed 49% of the *T. vaporariorum* and suppressed grey mould by 57% and 46%, respectively, on the flowers and leaves. In sweet pepper, mortality of *L. lineolaris* was 73% and grey mould was suppressed by 59% and 47%, respectively, on the flowers and leaves in the active inoculum treatment. The incidence of grey mould in sweet pepper and tomatoes treated with the heat-inactivated inoculum or no inoculum (control) was approximately 80% on the flowers and leaves. Thus, the combined inoculum of *B. bassiana* and *C. rosea* can potentially control *T. vaporariorum* and *L. lineolaris*, and suppress grey mould in greenhouse tomatoes and sweet peppers, when vectored simultaneously by bumble bees. Commercial greenhouse trials need to be conducted next to determine control efficacy under large scale production conditions.

1. Introduction

Crop yields produced under greenhouse conditions are generally ten times greater than those produced in the field (Cantliffe et al., 2001). However, conditions in greenhouses are often conducive to outbreaks of arthropod pests and plant diseases (Gullino et al., 1999; van Lenteren, 2000). Tomato and sweet pepper crops grown in commercial greenhouses are commonly infested by the greenhouse whitefly (*Trialeurodes vaporariorum* Westwood [Homoptera: Aleyrodidae]) and the fungal pathogen *Botrytis cinerea* Pers: Fr. (Helotiales: Sclerotiniceae), the causal agent of grey mould disease (Albajes et al., 1999; Heinz et al., 2004; Jarvis, 1997). Plant bugs, such as the tarnished plant bug (*Lygus lineolaris* [Palisot de Beauvois] [Hemiptera: Miridae]), are becoming an increasingly important pest in sweet pepper (Gillespie et al., 2003).

For both of these greenhouse crops, biological control strategies are the preferred pest management approach (Albajes et al., 1999). The cost of using biological control agents, especially in the greenhouse vegetables, is becoming more competitive and acceptable to growers. Biological control agents are environmentally friendly and are more reliable over the long term than are chemicals (Shipp et al., 2007; van Lenteren, 2007). They are also less harmful to the health and well being of growers, their workers and the plants. Moreover, the application of natural enemies does not interfere with fruit harvest or with crop management activities (i.e., no or shorter re-entry period after application) (van Lenteren, 2007).

Fungal agents, such as *Beauveria bassiana* (Balsamo) Vuillemin (Ascomycota: Hypocreales) and *Clonostachys rosea* Lnk: Fr. (Ascomycota: Hypocreales), have shown good potential for control of insect pests and suppression of plant diseases. *Beauveria bassiana* is effective against numerous insect pests including *T. vaporariorum* and *L. lineolaris* (Al-mazra'awi et al., 2006a; Butt et al., 1994; Liu et al., 2003; Shipp et al., 2003). *C. rosea* is versatile in terms of the ecological niches it occupies and is frequently found in cultivated soils where it degrades phytotoxic phenolic acids (Schroers, 2001) and in aerial plant structures (Peng et al., 1992; Sutton et al., 1997). Its antagonism to *B. cinerea* involves nutrient competition in

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doi:10.1016/j.biocontrol.2008.05.008

wounded tissue, hyperparasitism and competitive colonization of senescing and dead tissues (KÖhl and Fokkema, 1998; Morandi et al., 2000; Sutton et al., 1997; Yu and Sutton, 1997a). Both fungal agents have been reported to be compatible with each other in laboratory trials (Krauss et al., 2004).

Honey bees and bumble bees were shown in the early 1990s to effectively vector the fungal agent (*C. rosea*) to flowers for pathogen suppression (Peng et al., 1992; Yu and Sutton, 1997b) and later to vector insect pathogens for pest control (Al-mazra'awi et al., 2006a,b; Kevan et al., 2005, 2007). Bee vectored *B. bassiana* and *C. rosea* have been shown individually to have excellent potential for control of insect pests and suppression of grey mould disease. In 1997, Yu and Sutton (1997b) used bumble bees (*Bombus impatiens* [Cresson] [Hymenoptera: Apidae]) and honey bees (*Apis mellifera* L. [Hymenoptera: Apidae]) to disseminate *C. rosea* for control of grey mould caused by *B. cinerea* on flowers of raspberries (*Rubus idaeus* L.). Both pollinator species were effective in vectoring *C. rosea* (0.3–0.4 mg of inoculum per bee per day) and suppressed *B. cinerea* more in the flowers when applied using bees as compared to when *C. rosea* was sprayed on the crop. The incidence of flowers without *C. rosea* was higher in the plots that were sprayed (55–57%) than in plots treated with *C. rosea* vectored by bumble bees (6–9%) or honey bees (14–15%). Seven years later, Al-mazra'awi et al. (2006a) demonstrated that *B. bassiana* vectored by bumble bees (*B. impatiens*) in greenhouse sweet pepper caused 34–45% mortalities of *L. lineolaris* versus 9–15% in the controls and 34–40% for western flowers thrips (*Frankliniella occidentalis* [Pergande] [Thysanoptera: Thripidae]) compared to 3% in the controls. Kapongo et al. (in press) reported 54% infection level for *T. vaporariorum*, and 70% for *L. lineolaris* at a bee vectored concentration of 6.24×10^{10} conidia of *B. bassiana*/g of inoculum.

In all the bee vectoring studies to date, a carrier was used to dilute the fungal agent to the desired concentration for application to the crop. In the greenhouse cage trials by Al-mazra'awi et al. (2006a); Kapongo et al. (in press), corn flour was used as the carrier to dilute *B. bassiana* to obtain the required inoculum concentrations. In the current study, the corn flour was replaced with another beneficial fungal microorganism (*C. rosea*). Because *B. bassiana* and *C. rosea* have been shown to be compatible in laboratory trials, the possibility is suggested that the two agents can be combined into a single inoculum for application using bumble bees. Thus, the objective of this study was to evaluate the effectiveness of using bumble bees to vector a combined inoculum of *B. bassiana* and *C. rosea* for simultaneous control of insect pests and suppression of a fungal disease on greenhouse vegetable crops.

2. Materials and methods

2.1. Insect and plant cultures

Adult *T. vaporariorum* used in the trials were collected from greenhouse colonies that were maintained on potted tomato and sweet pepper plants at the Greenhouse and Processing Crops Research Centre (GPCRC), Harrow, ON. Newly emerged adults of *L. lineolaris* (24 to 48 h old) were obtained from a laboratory culture maintained at the Southern Crop Protection and Food Research Centre (SCPFRC), London, ON. The colony of *L. lineolaris* was established from field-collected adult insects on alfalfa at the SCPFRC Research Farm. The *L. lineolaris* colony was maintained at 24 °C, 60% RH and 16-h photoperiod with organic lettuce as the food source.

Bumble bee colonies (*B. impatiens*) supplied by Biobest Canada Ltd., Leamington, ON, were used during the different trials. Each colony had one queen and 25 workers. The colonies were anaesthetized with CO_2 and transferred from the commercial box to a wooden colony box ($32 \times 23 \times 24$ cm) fitted with an inoculum dispenser. The dispenser was modelled after the Yu and Sutton (1997b) dispenser and modified by Al-mazra'awi (2004). The bees were kept in the colony boxes for 2 days before use in the cage trials to allow the bees to become familiar with their new hives.

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

2.2. Inoculum preparation

A commercial formulation of *B. bassiana*, GHA strain (BotaniGard 22WP®, Laverlam International Corporation, Butte MT, USA) containing 2×10^{11} conidia of *B. bassiana*/g of product was used as the insect pest control agent. A commercial formulation of *C. rosea*, strain 88–710 (EndoFine®, AdjuvantPlus, Kingsville, ON, Canada) containing 2×10^{7} viable CFU of *C. rosea*/g of product was used for suppression of grey mould.

The viability (density of colony-forming units [CFU]) of *B. bassiana* and *C. rosea* in the commercial products was estimated before each trial to determine the correct amount of each fungal agent to use when preparing the inoculum concentration. To determine viability of the conidia, three 0.01-g samples of Botanigard 22WP® were suspended each in a flask containing 100 ml distilled water and 0.1% Tween 80. Then 200 µl of the conidial suspension was added to 1 ml of Sabouraud's dextrose broth with 1% yeast extract in a sterile test tube. The suspension was incubated at 24 ± 1 °C for 24 h. After which, four groups, each consisting of 200 conidia, were examined for percentage germination using a hemacytometer and a compound microscope. The testing protocol for *C. rosea* was as follows: Three 0.01-g amounts of EndoFine® were suspended each in a 100-ml of distilled water and 0.1% Triton-100 in a 250-ml flask. The flasks were agitated on a rotary shaker at 125 rpm for 2 h. Then 200 µL of the conidial suspension was added to 1 mL of paraquat-chloramphenicol agar (PCA) medium (0.1 mL paraquat, 200 mg chloramphenicol, and 12 g agar L^{-1} water) (Peng and Sutton, 1991). The suspension was incubated at 24 ± 1 °C for 24 h. Two hundred conidia for each of four sub-samples from each sample were examined for germination using a hemacytometer under a compound microscope. Spore germination of *B. bassiana* and *C. rosea* ranged from 96% to 98% for each of the trials. A combined inoculum of *B. bassiana* + *C. rosea* was made by mixing BotaniGard 22WP® and EndoFine® to a final concentration of 6.24×10^{10} conidia of *B. bassiana* + 1.38×10^{7} conidia of *C. rosea*/g of inoculum.

To produce the inoculum of *B. cinerea* for plant infections, 10^6 spores of B. cinerea (isolate PG-A-Fr-88-710 from strawberry [cv Redcoat]) were suspended in 0.1 ml of water + Tween 20 and spread onto potato dextrose agar medium in Petri dishes (∅9 cm). The Petri dishes were incubated for 40–45 d at 20–22 °C.

2.3. Vectoring trials

The cage trials were conducted in two greenhouse compartments (13×8 m) containing two or three fine-meshed screened cages ($520 \times 240 \times 220$ cm high) per greenhouse at the GPCRC. Each cage (treatment) contained 32 potted sweet pepper (summer and spring 2005) or tomato (summer and spring 2006) plants with a minimum of two flower sets per plant. In each cage, the plants

were arranged in two double rows, each with eight plants. The cages were maintained at 21–23 °C and 80–85% RH throughout the trials. For each crop, the three treatments were arranged in a completely randomized block design with four replications over time. The three treatments were: (T1) 6.24×10^{10} conida of *B. bassiana* + 1.38×10^{7} conidia of *C. rosea*/g of inoculum + bees (active inoculum), (T2) heat-inactiviated inoculum + bees and (T3) control treatment with no inoculum and no bees.

Each colony of bumble bees (1 queen and 25 workers) was introduced on day 1 after the plants were placed in the cages to allow acclimation of the colony to the cages. On day 2, 125 adult *L. lineolaris* and 500 adult *T. vaporariorum* were released per treatment for pepper and tomato, respectively. The wooden inoculum dispensers ($21 \times 12 \times 8.5$ cm height) filled with the appropriate inoculum (BotaniGard 22WP® and Endofine®) were placed at the exit/entry opening of the hives on day 3 after which the bees (*B. impatiens*) were allowed to forage and disseminate the inoculum. The flowers and leaves of each of 12 randomly selected plants per treatment were also artificially inoculated with spores of *B. cinerea*. Spores of a single Petri dish were spread on six leaves and flowers of a selected plant using a small camel hair brush. The selected inoculated leaves were fully expanded and located in the top canopy of the crop while the inoculated flowers were chosen on the basis that they would be fully open for pollination a few hours after the trial began. The inoculated plants were used to provide the source plants for the flower and leaf samples.

The first sample (50 adult *L. lineolaris*, 50 adult *T. vaporariorum*, and 24 leaves and flowers [two flowers/leaves per plant]) per treatment was collected on day 6 after which the dispensers were removed. The leaves were randomly selected and the flower samples consisted of open bee-visited flowers. The pest insects were collected to determine the percentage of mortality caused by *B. bassiana*, the number of *B. bassiana* propagules per *Lygus* or group of five *T. vaporariorum* and the internal infection level of insect pests by *B. bassiana*. Hives were then closed and the bees 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 sample was collected 3 days later. Also on day 10, leaves and flowers of 12 new plants were inoculated with *B. cinerea* as described for the first sampling period to provide open flowers for the second sample period. After each sampling day, the remaining inoculum in the dispenser was dried and weighed using a Mettler AT250 scale (Mettler-Toledo, Inc., USA) to determine the amount of inoculum that bumble bees delivered during the time of the experiment. Five bees were also sampled after they exited from the dispenser of each treatment at each sampling date. These samples were used to determine the amount of *B. bassiana* and *C. rosea* that individual bees carried.

Ten *L. lineolaris* and *T. vaporariorum* adults per sample were surface sterilized following Shipp et al. (2003) to estimate internal infection levels by *B. bassiana* (i.e., infection levels in the pests at the time that the samples were collected). The insects were then removed and placed in an environmental chamber (24 ± 1 °C, 80% RH) for 5 days to determine the incidence of mycosis. Another 10 *L. lineolaris* and *T. vaporariorum*, 12 flowers, 12 leaves and 5 bees per treatment sample were washed to quantify the densities of conidia (colony-forming units [CFU]) of *B. bassiana* and *C. rosea* present on the samples. Each plant/insect sample was submerged in 100 ml sterile water (only 50 ml used for *L. lineolaris* and *T. vaporariorum*) plus 0.1% Tween 20 in a 250-ml Erlenmeyer flask and agitated on a rotary shaker at 125 rpm for 2 h. Two separate aliquots of 0.1 mL of tenfold serial dilutions of the aqueous suspensions in each flask were simultaneously placed onto oatmeal agar medium (Difco, Detroit, MI) amended with 0.55% Dodine, 0.005% chlorotetracycline and 0.01% crystal violet in Petri dishes for *Beauveria* growth (Chase et al., 1986) and PCA medium for *Clonostachys* growth (Peng and Sutton, 1991). The Petri dishes were kept in the dark at 22 ± 1 °C for 5 days, after which colonies of *B. bassiana* and *C. rosea* were counted and recorded. The remaining *L. lineolaris* and *T. vaporariorum* were used to assess 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 leaves of organically grown lettuce for 7 days. Individual adults of *T. vaporariorum* were each placed in a screened sealed plastic vial containing the growing point of a tomato plant for 7 days. The tomato stems as well as the organic lettuce were replaced every second day. All bioassay cages were inspected daily for insect death. Dead insects were placed on moistened filter paper in Petri dishes and incubated at 25 °C and 80% RH. The appearance of white mycelium on the dead insects indicated *B. bassiana* infection.

The remaining 12 leaves and flowers were placed individually on moistened filter paper in Petri dishes which were sealed with Parafilm and incubated for 14 days at 25 °C and 80% RH. After incubation, ten 10-mm diameter disks were cut at random sites on each leaf and flower by means of cork borers that were surface-disinfected in sodium hypochlorite (95% concentration) and washed in sterile water before each excision. Sporulation of grey mould was assessed following the protocol of Sutton et al. (2002) using a dissecting microscope to determine the incidence and percentage area of tissue pieces with conidiophores.

Mortality of the bumble bees exposed to the *B. bassiana* + *C. rosea* treatments was determined after the second sampling period. Each bumble bee colony was then transferred back into the commercial colony box, fed pollen patties and placed in a controlled environment chamber at 23 ± 1 °C for 3 weeks after the end of the trial. The percentage mortality of *B. impatiens* was recorded weekly. Data for bee mortality from the control, in which no hive was used, were determined by using a commercial hive that was supplied at the same time as the treatment hives and fed pollen patties. The control hive (“commercial hive”) was maintained in a controlled environment chamber at 23 ± 1 °C for 5 weeks (2 week trial cage period + 3 week period after the greenhouse cage trials like the treatment hives).

2.4. Statistical analysis

Abbott’s formula (Abbott, 1925) was used to determine the percentage of adult *T. vaporariorum* and *L. lineolaris* that were killed by *B. bassiana*. Data for mortality, mycosis and internal infection of pest insects, bee mortality and incidence of grey mould on flowers and leaves were first subjected to square root transformation before statistical analysis. The transformed data for each species of insect pest and for disease incidence were initially analzsed using two way analysis of variance (ANOVA) ($P < 0.05$). Treatment means for percentage of insects that were dead or exhibiting mycosis were compared by Tukey’s multiple means comparison test (SAS Institute, 2001). With disease incidence of grey mould, incidence values for the two control treatments were averaged because there was no significant difference between the control treatments and then compared to the active inoculum treatment using protected least significant difference (LSD) test.

To compare the number of CFU on insect and plant samples, the CFU count data were arcsine transformed, subjected to PROC univariate, residual analysis and then analyzed using a repeated measurement procedure (PROC MIXED covtest, SAS Institute, 2001). The mean numbers of CFU of *B. bassiana* and *C. rosea* from each insect and plant sample were then compared using the *F*-test. The type I error rate was set at probability level of 0.05. Mortality, disease suppression and CFU data were back-transformed to their original scales for presentation in tables and figures.

3. Results

3.1. Effects of combined fungal inoculum on pest insects (T. vaporariorum and L. lineolaris)

The combined inoculum (*B. bassiana* + *C. rosea*) resulted in significantly greater percentage mortality of adult *T. vaporariorum* (49.1 ± 4.4%) as compared to the control treatments using Abbott's formula ($F_{2,21}$ = 80.80, P < 0.0001). Unadjusted mean mortality values for the three treatments were active inoculum (59.1 ± 2.5%), heat-inactivated inoculum (18.8 ± 6.6%) and control (no bee, no inoculum) (20 ± 2.5%). No significant difference was found in percentage mortality of *T. vaporariorum* between the two control treatments ($F_{2,21}$ = 80.80, P = 0.94). The mean percentage of dead *T. vaporariorum* exhibiting mycosis in the active inoculum treatment was 73.2 ± 1.6% while none of the dead *T. vaporariorum* in the control treatments showed mycosis. With respect to surface sterilized *T. vaporariorum*, internal infections were found in 36.2 ± 5.3% of the adults from the active inoculum treatment and none in the control treatments.

In sweet pepper, the combined inoculum caused significantly greater mortality of adult *L. lineolaris* (72.5 ± 1.4%) compared to the two control treatments using Abbott's formula ($F_{2,21}$ = 128.19, P < 0.0001). Unadjusted mean mortality values for the three treatments were active inoculum (75.4 ± 2.9%), heat-inactivated inoculum (10.8 ± 2.2%) and control (no bee, no inoculum) (10.8 ± 1.2%). Again, no significant difference was found between the two control values ($F_{2,21}$ = 128.19, P = 0.91). The mean percentage of dead *L. lineolaris* exhibiting mycosis in the active inoculum treatment was 90.5 ± 12.2% while no mycosis was detected in the control treatments. With respect to surface sterilized *L. lineolaris*, the mean percentage of internal infections was 51.9 ± 6.5% for the active inoculum treatment and nothing in the control treatments.

3.2. Effects of combined fungal inoculum on bumble bees (B. impatiens)

Bombus impatiens vectored simultaneously both fungi (*B. bassiana* + *C. rosea*) to tomato plants without any significant effect on bee mortality ($F_{2,18}$ = 3.19, P = 0.0894; Table 1). However, bumble bee mortality was significantly affected on the pepper crop ($F_{2,9}$ = 17.10, P = 0.0009) among the three treatments (Table 1). Bee mortality in the control hive was significantly lower than in the hive with the active inoculum treatment ($F_{2,9}$ = 17.10, P = 0.0003) and also in the hive with the heat-inactivated inoculum ($F_{2,9}$ = 17.10, P = 0.0023). No significant difference was found between the mortalities in the hives with active inoculum and heat-inactivated inoculum ($F_{2,9}$ = 17.10, P = 0.1854).

3.3. Effects of combined fungal inoculum on grey mould disease

In tomato flowers, grey mould incidence was significantly lower in plants treated with the active inoculum compared to the controls ($F_{2,21}$ = 121.62, P < 0.0001; Fig. 1). However, there were no significant differences in mould incidence between the heat-inactivated inoculum and the no inoculum control treatments ($F_{2,21}$ = 121.62, P = 0.5455). The inoculum acted in the same manner on the leaves, for which a significant difference was found between treated leaves and controls ($F_{2,21}$ = 50.30, P < 0.0001). Again, no significant difference was found between the controls ($F_{2,21}$ = 121.62, P = 0.5112; Fig. 1).

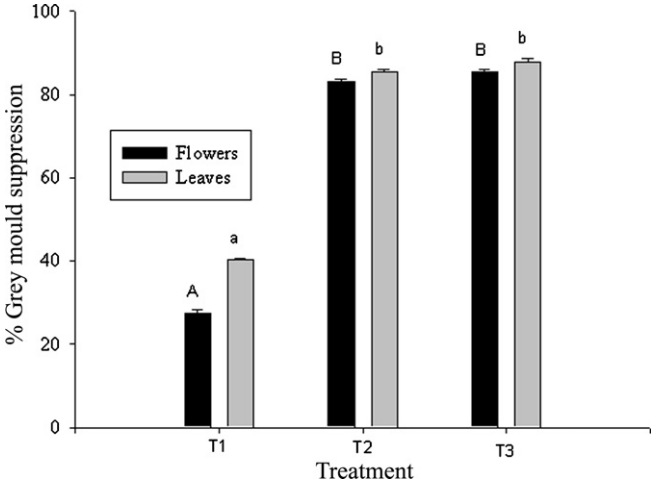

Fig. 1. Effects of the combined inoculum (*Beauveria* + *Clonostachys*) on grey mould disease. Three treatments were compared: (T1) 6.24×10^{10} conidia of *Beauveria* + 1.38×10^{7} conidia of *Clonostachys* + hive of 25 bumble bee workers (active inoculum), (T2) heat-inactivated combined inoculum (T1 autoclaved) + hive of 25 bumble bee workers, and (T3) no inoculum, no bees. Selected plants in each of the three treatments were artificially infested with *B. cinerea* spores. Data bars are mean values each with standard error (SE). Treatment means for grey mould incidence for a given treatment and plant part (flower or leaf) assigned the same letter are not significantly different (protected LSD, P < 0.05).

Results for the combined inoculum on flowers and leaves of sweet pepper were similar to those for tomatoes. Flowers from the treated and control cages showed a significant difference in the development of grey mould ($F_{2,21}$ = 253.64, P < 0.0001); no significant difference was found between the controls ($F_{2,21}$ = 121.62, P = 0.9949; Fig. 2). The assessment of grey mould on pepper leaves confirmed that the combined inoculum had a negative impact on the development of grey mould ($F_{2,21}$ = 90.86, P < 0.0001) with a higher incidence of the disease on the plants from the control cages compared to the treatment in which bumble bees were used to vector the active inoculum.

Based on the difference in incidence of grey mould between the active inoculum and control treatments, the combined inoculum of *B. bassiana* and *C. rosea* as vectored by *B. impatiens* suppressed the incidence of grey mould in flowers and leaves, respectively, by 56.8% and 46.3% in tomato (Fig. 1) and by 58.9% and 46.8% in pepper (Fig. 2).

Table 1
Mean (±SE) mortality and mycosis level for *Bombus impatiens* when vectoring the combined *Beauveria bassiana* + *Clonostachys rosea* inoculum for insect pest control and disease suppression in greenhouse cage trials of pepper and tomato

Treatments	Bee (tomato)		Bee (pepper)	
	% Dead (n = 1200)	% of dead with mycosis	% Dead (n = 1300)	% of dead with mycosis
Control hive (not exposed to cage treatments)	15.5 (1.8)a	0	7.5 (0.8)a	0
Heat-inactivated inoculum	16.7 (1.5)a	0	15.4 (0.8)b	0
Active inoculum (6.24×10^{10} *Beauveria* + 1.38×10^{7} conidia of *Clonostachys*/g of inoculum)	23.5 (3.4)a	34.1 (2.0)	18.7 (2.2)b	35.4 (2.8)

n = numbers of samples processed (the number of bees that were found alive in the hive 3 weeks after the second sampling period). Within a column, means followed by same letter are not significantly different (Tukey test, P < 0.05).

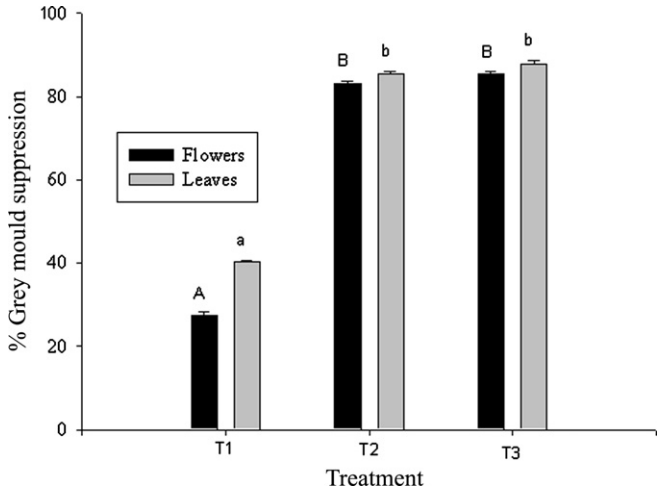

Fig. 2. Effects of the combined inoculum (*B. bassiana* + *C. rosea*) on grey mould disease. Three treatments were compared: (T1) 6.24×10^{10} conidia of *B. bassiana* + 1.38×10^{7} conidia of *C. rosea* + hive of 25 bumble bee workers (active inoculum), (T2) heat-inactivated combined inoculum (T1 autoclaved) + hive of 25 bumble bee workers, and (T3) no inoculum, no bee. Selected plants in each of the three treatments were artificially infested with *B. cinerea* spores. Data bars are mean values each with a standard error (SE) bar. Treatment means for grey mould incidence for given treatment and plant part (flower or leaf) assigned the same letter are not significantly different (protected LSD, $P < 0.05$).

3.4. Colony-forming units of B. bassiana and C. rosea deposited on flowers and leaves by B. impatiens

Spores of *B. bassiana* were detected on 84% of the tomato flowers and 76% of the pepper flowers (Table 2). Counts of *B. bassiana* were 1.6×10^4 and 1.0×10^4 CFU per flower, respectively, for tomato and pepper. Spores of *C. rosea* were recovered from 82, and 60%, respectively of tomato and pepper flowers (Table 2). Counts of *C. rosea* CFU were 4.3×10^3 and 4.8×10^3, respectively, per flower of tomato and pepper.

The average counts of active fungal spores deposited by bumble bees were 7.1×10^3 and 5.5×10^4 CFU of *B. bassiana* per cm^2 of leaves in the tomato and pepper trials (Table 2). Spores were found on 92% of the sampled tomato and pepper leaves. For *C. rosea,* 3.2×10^3 and 6.1×10^3 CFU of *C. rosea* were counted per cm^2 of sampled leaves of tomato and pepper. *C. rosea* was recovered from 90% of tomato leaves and 76% of pepper leaves.

3.5. Colony-forming units of B. bassiana and C. rosea recovered from insect pests and bees

Bumble bees emerging from the dispenser at the exit/entry of the hive carried an average 5.5×10^5 and 8.0×10^5 CFU of *B. bassiana*, and 2.6×10^4 and 5.0×10^4 CFU of *C. rosea* for the tomato and pepper trials, respectively. This represented 90–100% of the sampled bumble bees that were detected with spores in both crops (Table 2).

The mean numbers of *B. bassiana* and *C. rosea* CFU per group of five adult *T. vaporariorum* were 330 and 48, respectively, recovered from 28% and 18% of the sampled groups (Table 2). Adult *Lygus* were processed individually. They had mean numbers of 401 and 218 CFU of *B. bassiana* and *C. rosea*. Active spores of *B. bassiana* were found on 48% of the sampled individuals, while 23% of the *L. lineolaris* have been detected with live spores of *C. rosea* (Table 2).

4. Discussion

Bumble bees (*B. impatiens*) can vector simultaneously two fungal agents, *B. bassiana* and *C. rosea,* for arthropod pest control and

Table 2
Mean (±SE) number of untransformed colony-forming units (CFU)/ insect or plant sample and mean (±SE) percentage of bumble bees, flowers, leaves, *Lygus lineolaris* adults, *Trialeurodes vaporariorum* adults with detectable densities of *Beauveria bassiana* and *Clonostachys rosea* collected from cages exposed to *Bombus impatiens* vectored inoculum (6.24×10^{10} conidia of *Beauveria* + 1.38×10^7 conidia of *Clonostachys*/g of inoculum)

Insect/plant samples	*Beauveria*				*Clonostachys*			
	Tomato		Pepper		Tomato		Pepper	
	Mean CFU	% of samples with detectable CFU	Mean CFU	% of samples with detectable CFU	Mean CFU	% of samples with detectable CFU	Mean CFU	% of samples with detectable CFU
Bees, $n = 40$	5.5×10^5 (7.7×10^4)	97.5 (1.6)	8.0×10^5 (7.3×10^4)	100	2.6×10^4 (0.4×10^4)	90.0 (6.4)	5.0×10^4 (1.1×10^4)	100
Flowers, $n = 96$	1.6×10^4 (2.2×10^3)	84.3 (8.1)	1.0×10^4 (0.3×10^3)	75.58 (17.4)	4.3×10^3 (0.3×10^3)	82.3 (5.2)	4.8×10^3 (0.2×10^3)	60.4 (12.8)
Leaves (per cm^2), $n = 96$	7.1×10^3 (0.6×10^3)	91.6 (2.73)	5.5×10^4 (4.9×10^3)	91.6 (1.1)	3.2×10^3 (0.2×10^3)	89.6 (7.8)	6.1×10^3 (0.7×10^3)	76.0 (10.1)
L. lineolaris, $n = 80$	NA	NA	401.0 (55.1)	47.5 (8.3)	NA	NA	217.5 (58.9)	22.5 (5.1)
T. vaporariorum, $n = 80$	330.0 (100.2)	27.5 (8.0)	NA	NA	48.0 (18.3)	17.5 (2.4)	NA	NA

n, number of samples processed. The *T. vaporariorum* were pooled in groups of five for CFU counts. No CFU of *Beauveria* and *Clonostachys* were found on samples collected from the control treatments.

plant disease suppression, with minimal side effects on the vectoring agent. This is the first time that pollinators have been used to disseminate more than one control agent for insect pest and/or disease control at the same time on any crop. Bumble bees are the main pollinators for greenhouse tomato and sweet pepper crops (Kevan et al., 1991; Shipp et al., 1994; Morandin et al., 2001; Velthuis and Van Doorn, 2006). Thus, combining the delivery of microbial agents with pollination services provides added value to using pollinators in crops.

The density of *B. bassiana* spores recovered either on plant parts or bumble bees was about 10 times greater than that of *C. rosea* on the same plant tissues (Table 2). This can be partly explained by the fact that the concentration of *C. rosea* was approximately 6000 times less compared to *B. bassiana* in the combined inoculum. However, the number of CFU of both fungi recovered from the plant parts was enough for infection of insect pests and suppression of grey mould disease. The mixture of these fungi as vectored by the bumble bees to the plant parts killed 49% of the *T. vaporariorum* and 73% of the *L. lineolaris*.

The same mixture of bee vectored *C. rosea* and *B. bassiana* also suppressed the development of *B. cinerea* on tomato and sweet pepper plant tissues under greenhouse conditions. *C. rosea* was the fungus that suppressed the grey mould. There has been one report of a *Beauveria* isolate (isolate 11–98 from a click beetle in Tennessee, US) that suppressed the incidence of damping-off caused by *Rhizoctonia solani* Kühn in field tomato (Ownley et al., 2004). However, *B. bassiana* (GHA strain) has never been reported to have any suppressive activity against plant pathogens. The ability of *C. rosea* to suppress *B. cinerea* has been reported in numerous studies (Sutton et al., 1997; Yu and Sutton, 1997b,a; Köhl and Fokkema, 1998; Morandi et al., 2000).

These results corroborate the findings obtained by Sutton et al. (2002) in which the incidence of *B. cinerea* was high in deleafed stems of control tomato plants (83–100%), but was suppressed by 52–67% in deleafing wounds that were treated with *C. rosea*. In the present study, *C. rosea* suppressed the incidence of grey mould by 57% and 46% in the flowers and leaves of tomato, and by 58.9% and 46.8% in the flowers and leaves of pepper, respectively (Figs. 1 and 2). Similar results were reported for other crops such as raspberry, rose and strawberry in which *C. rosea* suppressed sporulation of *B. cinerea* (Yu and Sutton, 1997b; Valdebenito-Sanhueza et al., 1997; Sutton et al., 1997; Tatagiba et al., 1998; Morandi et al., 2003).

In the present study, the incidence of grey mould was higher in leaves of tomato (87%) than sweet pepper (79%) (Figs. 1 and 2). Grey mould is often reported as a more serious disease of greenhouse tomatoes than of sweet pepper in North America (Howard et al., 1994; Killebrew, 1995; Ontario Ministry of Agriculture and Food, 2005) and worldwide (Dik and Wubben, 2004). We suggest that the hairy structure of the tomato leaves may result in better deposition and colonization of *B. cinerea* spores. The opposite would occur for sweet pepper where the glabrous surface of the leaves does not offer a stable site for retaining the spores. As a consequence, most of them once deposited could easily be removed.

A previous study by Sutton et al. (2002) showed that grey mould on stems and leaves of tomato was controlled with 10^6 conidia of *C. rosea*/g of inoculum. In the present study, the density of *C. rosea* inoculum in the mixture was 1.38×10^7 conidia/g or about ten times greater than that used by Sutton et al. (2002). However, the application of the antagonistic fungus, *C. rosea,* in both studies had similar effects in suppressing grey mould disease (current and those of Sutton et al., 2002: 52–67%. vs 55–66% suppression of grey mould) despite differences in concentrations of the biological control. Therefore, it may be possible to reduce the concentration (i.e., amount) of *C. rosea* in the mixture and have the same level of control. In addition, we suggest that more biological control agents, such as *Lecanicillium lecanii, Metarhizium anisopliae* and *Trichoderma harzianum*, could be added in a mixed inoculum to be vectored by bumble bees. However, multi-agent inocula must contain and deliver effective concentrations of all constituents.

This study demonstrates that pollinators can play an important role in overall crop management practices; not only for fruit pollination, but also for arthropod and plant disease control. However, trials in commercial greenhouses are needed to determine control efficacy under commercial production conditions.

Acknowledgments

The study was funded by Improved Farming Systems and Practices Initiative, Pest Management Centre, Agriculture and Agri-Food Canada. We thank Laverlam International Corporation, Butte, MT, USA, Adjuvants Plus Inc., Kingsville, ON, Canada, Biobest Canada Limited, Leamington, Ontario and Dr. Bruce Broadbent, SCPFRC, London, Ontario, for providing us with *B. bassiana, C. rosea*, colonies of bumble bees and *L. lineolaris*, respectively. For technical assistance, we also thank E. Armstrong, G. Mosey, M. Whitfield and Y. Zhang.

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