Research Article

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Bombus terrestris as pollinator-and-vector to suppress *Botrytis cinerea* in greenhouse strawberry

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Abstract

BACKGROUND: *Bombus terrestris* L. bumblebees are widely used as commercial pollinators, but they might also be of help in the battle against economically important crop diseases. This alternative control strategy is referred to as pollinator-and-vector technology. The present study was designed to investigate the capacity of *B. terrestris* to fulfil this role in greenhouse strawberry flowers, which were manually inoculated with a major plant pathogen, the grey mould *Botrytis cinerea* Pers.: Fr. A model microbiological control agent (MCA) product Prestop-Mix was loaded in a newly developed two-way bumblebee dispenser, and, in addition, the use of the diluent Maizena-Plus (corn starch) was tested.

RESULTS: Importantly, loading of the MCA caused no adverse effects on bumblebee workers, with no loss of survival or impairment of flight activity of the workers during the 4 week flowering period. Secondly, vectoring of Prestop-Mix by bumblebees resulted in a higher crop production, as 71% of the flowers developed into healthy red strawberries at picking (preharvest yield) as compared with 54% in the controls. In addition, these strawberries were better protected, as 79% of the picked berries remained free of *B. cinerea* after a 2 day incubation (post-harvest yield), while this percentage was only 43% in the control. Overall, the total yield (preharvest × post-harvest) was 2–2.5 times higher than the total yield in the controls (24%) in plants exposed to bumblebees vectoring Prestop-Mix. Thirdly, the addition of the diluent Maizena-Plus to Prestop-Mix at 1 : 1 (w/w) resulted in a similar yield to that of Prestop-Mix used alone, and in no negative effects on the bumblebees, flowers and berries.

CONCLUSIONS: This greenhouse study provides strong evidence that *B. terrestris* bumblebees can vector a MCA to reduce *B. cinerea* incidence in greenhouse strawberries, resulting in higher yields. Similar yields obtained in the treatments with Prestop-Mix and Prestop-Mix + Maizena-Plus suggest an equally efficient dissemination of the biocontrol agent into the flowers with only half the initial concentration of Prestop-Mix, which illustrates the importance of the diluent.

Keywords: *Bombus terrestris*; pollinator-and-vector; *Botrytis cinerea*; strawberry; greenhouse; dispenser; diluent

1 INTRODUCTION

Strawberry [*Fragaria x ananassa* (Weston) Duchesne ex Rozier (Rosales: Rosaceae)] is a fruit crop grown worldwide, but diseases such as the grey mould *Botrytis cinerea* Pers.: Fr. (Helotiales: Sclerotiniaceae) frequently limit yield.[1–5] Damage by this pathogen occurs at flowering,[6] when conidia originating from infested crop debris or dispersed by wind start to infect the petals, stamens and pistils and then colonise the fruit.[1,7] In the field, disease symptoms become visible when berries are ripening, and thus disease management strategies are needed at flowering. To date, *B. cinerea* management is still reliant on chemical control to a large extent, although the number of reports of resistance development in fungal pathogens against fungicides is increasing.[8–11] The use of fungicides has also been shown to have an adverse effect on pollen germination, which, in turn, results in reduced fruit formation.[12] Biocontrol agents as an alternative to chemical control were first applied against post-harvest diseases (for reviews, see Droby *et al*.[13] and Sharma *et al*.[14]). However, owing to regulatory actions such as the reduction of registered active ingredients, studies to control preharvest diseases with environmentally friendly strategies are now increasing.[15] Indeed, multiple research groups have achieved *B. cinerea* suppression when using *Apis mellifera* L. (Hymenoptera: Apidae) and/or *Bombus impatiens* Cresson (Hymenoptera: Apidae) to vector *Clonostachys rosea* (Link./Fr) Schoers, Samuels, Seifert & Gams (formerly: *Gliocladium roseum* Bainier) (Hypocreales: Bionectriaceae) and strains of *Trichoderma harzianum* Rifai (Hypocreales: Hypocreaceae).[12,16–19] Next to the

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successful suppression obtained with these vectors, a recent study demonstrated the potential of the worldwide used greenhouse pollinator *Bombus terrestris* L. (Hymenoptera: Apidae) in laboratory or semi-field experiments.[20]

Within agriculture/horticulture, bumblebees represent an important group of pollinators next to honey bees and solitary bees. Indeed, bumblebees have been used since 1987 to pollinate a variety of greenhouse crops.[21] At present, several species are commercially available, but the most common and widely used bumblebee, in global terms, is *B. terrestris*.[21] This pollinator has also been widely used for the pollination of strawberries.[21] The absence of pollination in strawberry results in a high proportion of deformed fruit that are of low (zero) economic value.[22]

The aim of this project was to investigate the capacity of *B. terrestris* to vector into strawberry flowers under greenhouse conditions the commercial biocontrol agent Prestop-Mix, which is used here as a model product, using the newly developed two-way bumblebee dispenser of Mommaerts *et al*.[20] Prestop-Mix contains the fungus *Gliocladium catenulatum* Gilman & Abbott J1446 [now *Clonostachys rosea* f. *catenulata* (Gilman & Abbott) Schroers J1446] (Hypocreales: Bionectriaceae), a control agent for various diseases of the rhizosphere and phyllosphere.[23–26] Here, the efficiencies of transport/dissemination into the flowers and the subsequent biocontrol of *B. cinerea* were evaluated by scoring the numbers of healthy red strawberry fruits before/at harvest (representing preharvest yield) and also after incubation for 2 days at optimal conditions for *B. cinerea* growth (representing post-harvest yield). The efficacy of the pollinator-and-vector system was evaluated through examination of the total yield to produce a high number of healthy, non-infected red fruits (preharvest × post-harvest). In addition, based on previous results demonstrating 80% loss of the product owing to bumblebee flight,[20] the effect of the use of a diluent, corn starch Maizena-Plus, on the biocontrol capacity of the vectoring of Prestop-Mix with bumblebees was determined.

2 MATERIAL AND METHODS

2.1 Bumblebee hives and strawberry plants

Bombus terrestris bumblebee hives obtained from a mass rearing programme at Biobest NV (Westerlo, Belgium) were used for the greenhouse experiment. Each hive contained a queen, her brood and a minimum of 75 workers.

Strawberry plants of *Fragaria vesca* L. var. ostara (Rosales: Rosaceae) were used as a model greenhouse crop. The young plants were purchased from Schoubs IVO NVBA (Tongeren, Belgium) and grown in soil containing black : white peat (7 : 3; Peltracom, Belgium) in 20 cm diameter pots. For this experiment, a total of 500 strawberry plants were used. Before the start of the experiment, the plants were kept in a greenhouse compartment that was separated from the treatments until the appearance of flower buds. Thereafter, the plants were transferred to their meshed tent compartment where the different treatments were performed. In the greenhouse, the climatic conditions were controlled by a Priva CD750 climate control unit set at 18–25 °C and 82–83% relative humidity. The plants were manually watered on a weekly basis and fertilised with Osmocote-Exact Standard High K containing an extra-high potassium level (Scotts International BV, The Netherlands), at 2 mL of granules per plant once before the start of the experiment.

2.2 Prestop-Mix, Maizena-Plus and *Botrytis* spore suspension

The biofungicide Prestop-Mix, based on *G. catenulatum* J1446 (now *C. rosea* f. *catenulata* J1446) [powder formulation containing 10^7–10^9 colony-forming units (CFU) g^{-1} and developed to be delivered by pollinators; Verdera Oy, Espoo, Finland], was used in this study as a model product for vectoring by bumblebees to suppress the plant pathogen *B. cinerea*. The product was stored in accordance with the manufacturer's guidelines. Before use, the numbers of CFU of *C. rosea* f. *catenulata* J1446 were determined through plating out on potato dextrose agar (PDA) medium, and scored as $4.5 \pm 0.1 \times 10^7$ CFU g^{-1} product.

In addition to Prestop-Mix alone as the product to be vectored, a mixture of Prestop-Mix combined with Maizena-Plus (corn starch) at 1 + 1 (w/w) was also prepared. Maizena-Plus consisted of corn starch with 2% protein and was purchased as a food-grade powder product in Delhaize (Belgium). The powder products were put at 0.11 g cm^{-2} in the newly developed two-way dispenser of Mommaerts *et al*.[20]

To produce the inoculum of *B. cinerea* for flower infection, petri dishes of three-week-old fully grown *B. cinerea* strain B05.10 from tomato (as obtained via Dr Rudy Aerts, Katholieke Hogeschool Kempen, Belgium) were used. The spores from the petri dishes were suspended in 10 mL of sterile water and collected in a 50 mL sterile falcon tube in a laminar flow unit. The resulting suspension was centrifuged for 15 min. Subsequently, the wash water was discarded, and a further 10 mL of sterile water was added under laminar flow. This process was repeated 3 times. Following this, the concentration (spores mL^{-1}) was determined using a Bürker count chamber, and the concentration was adjusted to obtain a final concentration of 10^5 spores mL^{-1}. The suspension of *B. cinerea* spores was stored at 10 °C until use, which was within 3 days after preparation.

2.3 Dispenser

This study used the newly developed two-way dispenser for bumblebees as described by Mommaerts *et al*.,[20] except for one small modification where the front, non-transparent, grey plastic plate was replaced with a transparent plexiglass plate because the greenhouse experiments were done during the low-light-intensity winter period of November–December 2009.

2.4 Experimental design for the different treatments in the greenhouse

The experiment was conducted in a greenhouse compartment of 24 × 63 × 3.75 m containing four fine-meshed tents (width 6 m, length 15 m, height 2 m) in Westerlo (Belgium). In each tent, a total of 100 strawberry plants were placed when flower buds appeared. Temperature and relative humidity were measured continuously during the 8 week experimental period (W1–8) using data loggers. Data loggers were distributed among the plants (two data loggers were placed at the level of the plants in each plot).

In each plot, 100 strawberry plants were arranged in two rows with a distance of 5 cm between the rows and 5 cm in the row. Then, each week (W1–4) buds that opened in the same week were labelled with strips of the same colour, and for each week another colour was used. Each of the four tents was subjected to a different treatment: T1: 'control' = manual infection with *B. cinerea* and no pollination by bumblebees; T2: 'Maizena-Plus' = manual infection with *B. cinerea* and dissemination of Maizena-Plus by bumblebees via a dispenser; T3: 'Prestop-Mix' = manual infection with *B. cinerea* and dissemination of Prestop-Mix by

bumblebees via a dispenser; T4: 'Prestop-Mix + Maizena-Plus' = manual infection with *B. cinerea* and dissemination of mixture of Prestop-Mix and Maizena-Plus (1 + 1, w/w) by bumblebees via a dispenser.

2.5 Release of bumblebee workers as vectors and inoculation of *Botrytis cinerea*

Following labelling, the plants were exposed to visitation by bumblebees on a daily basis to facilitate pollination and the vectoring of the microbiological control agent (MCA). The inoculation of *Botrytis* spores into the flowers was similarly conducted on a daily basis.

On day 1, at 9 a.m., the new dispensers were filled with 0.11 g cm^{-2} of powder in T2, T3 and T4. The hives were then opened and flower visitation was allowed for 90 min. Thereafter, all strawberry plants were covered with a mesh as described by Shafir *et al*.[18] to prevent further pollination. Then, *B. terrestris* hives were provided with artificial pollen grains (Soc. Coop. Apihurdes, Pinofranqueado-Cáceres, Spain) and sugar water placed at a distance of 2 m from the hives. Before darkness, i.e. at 5 p.m., the exit holes of each dispenser were closed, whereas the entrance hole was left open to guarantee that all foraging bumblebees returned to their hive.

On the second day, at 9 a.m., the plants were uncovered and the dispensers were opened to allow the bumblebee workers to visit the flowers for a period of 90 min. Subsequently, all plants were covered. In the evening, i.e. at 5 p.m., all dispenser exit holes were closed, as was described for day 1. Then, the labelled open flowers in all plots were manually inoculated with 10 µL of the prepared *Botrytis* spore suspension, representing 10^3 spores $flower^{-1}$, with the use of a micropipette. After the inoculation, all plants were again covered for the night with plastic foil to maintain a high relative humidity and thus increase spore germination success for the pathogen (Aerts R, private communication).

On day 3, at 9 a.m., the foil was removed from the inoculated plants and the dispensers were opened so that the bumblebee workers could forage on the flowers over a 90 min period. All plants were then covered again.

This process was performed during week 1, and repeated in weeks 2, 3 and 4 of the flowering period. To guarantee successful vectoring of the MCA, the dispensers in T2, T3 and T4 were refilled every 3 days with 0.11 g cm^{-2} of freshly prepared product.

At the end of the 4 week flowering period, the number of labelled flowers that had been visited by bumblebees was set at 200 for each treatment, and their development into strawberry fruits was followed. This number of 200 flowers per treatment is considered sufficient to obtain a reliable evaluation of the efficacy of biocontrol of *Botrytis*, based on Yu and Sutton.[17] In the case of T4, however, it was only possible to score a total of 187 labelled flowers for fruit development in W5–8. In normal conditions, the flowers that were visited by bumblebees in weeks 1, 2, 3 and 4 should result in red strawberry fruits in weeks 5, 6, 7 and 8 respectively.

2.6 Foraging activity of *Bombus terrestris* before and during the greenhouse experiment

Three days before the start of the greenhouse experiment, a new dispenser was fitted in a *B. terrestris* hive containing a queen, her brood and a minimum of 75 workers. The experiment was done with five independent hives. Then, the flight activity was determined for each hive by counting the numbers of bumblebee workers (foragers) flying in and out over a 30 min period each day at 9 a.m. Three out of the five hives were then selected at random, and all three showed no significant ($P > 0.05$) differences in flight activity. One hive was introduced into each tent at the start of the opening of the first flower buds (start of week 1): for T2 this was hive 3 initially, but this was replaced in week 2 with hive 4; for T3 this was hive 1; for T4 this was hive 2. During the greenhouse experiment, the foraging intensity of the bumblebee workers for the different hives was evaluated on a weekly basis over the course of the flowering period (W1–4). The numbers of bumblebees that were flying in and out were recorded every 10 min over the course of 90 min. In addition, the numbers of dead bumblebee workers present in each tent were recorded on a weekly basis. At the end of the flowering period, i.e. after week 4, all hives were removed.

All statistical analyses were performed using SPSS v.16.0. For all data, normality was confirmed with Kolmogorov–Smirnov tests ($P = 0.05$). The foraging activity before the start of the greenhouse experiments for vectoring was analysed by one-way ANOVA followed by a *post hoc* Tukey–Kramer test ($\alpha = 0.05$). To compare the foraging activity of the hives in the different treatments during the greenhouse experiment, two-way ANOVA was performed with 'treatment' and 'time' as fixed factors. Factors and interactions were removed from the model when not significant ($P > 0.05$). Then, means were separated with a paired *t*-test and corrected with a Bonferroni correction ($P = 0.008$).

2.7 Effect of inoculation manipulation on *Botrytis cinerea* growth in the flowers

In parallel with treatments T1–4, a series of plants was kept in a separate area of the greenhouse. Then, at the moment of manual flower inoculation with the *Botrytis* spore suspension in the treatments in week 1, the flowers of these separated plants were simultaneously inoculated with 10 µL of sterile water. Ten flowers randomly selected and collected on day 3 were treated. In T1, the flowers were inoculated with 10 µL of the spore suspension at day 2, and there was no use of bumblebees (as described above). From this plot, again ten flowers were selected at random on day 3 for further evaluation.

The ten flowers collected on day 3, either in the tent of T1 or from the separate plants, were incubated at 10 °C for 24 h. Five flowers were then examined under a stereoscopic microscope for presence of *B. cinerea* mycelium, and the other five flowers were individually rinsed to determine the number of CFU of *B. cinerea* present per flower. Each sample was gently shaken on a rotary shaker in 15 mL of physiological solution for 60 min. Then, a tenfold serial dilution was made of the aqueous solution and 100 µL was placed on PDA medium. This was repeated twice. For *B. cinerea*, the numbers of CFU were scored after 3 days of incubation at 22 °C.

Comparison of mycelium growth and the numbers of CFU of *Botrytis* in the flowers of the plants of T1 and those that were separately treated with water allowed evaluation of the impact of the manipulation of inoculation on the flower performance/development, and the impact of this, in turn, on the growth of *B. cinerea* and the natural presence of *Botrytis* in the greenhouse.

2.8 Efficacy of vectoring to suppress *Botrytis cinerea*: pre- and post-harvest and total yields

To determine the efficacy of the treatment, the numbers of labelled flowers (W1–4) were compared with the numbers of

fruits obtained (W5–8), as, under normal conditions, the flowers visited by bumblebees in weeks 1, 2, 3 and 4 will result in red strawberry fruits in weeks 5, 6, 7 and 8 respectively.

The preharvest yield (%) was calculated as a percentage of the numbers of healthy red strawberry fruits directly after collection (W5–8) based on the total number of healthy flowers labelled (W1–4). During the flowering period (W1–4), the numbers of flowers that stayed healthy (score = 0) or had died (score = 1) were recorded twice per week and compared with the total numbers of labelled flowers. Similarly, during weeks 5 to 8, the number of healthy red fruits was scored and distinguished from infected ones which showed the presence of the typical grey mycelium of *B. cinerea*. This was done using a binary scoring system: 0 = not infected, 1 = infected.

In addition to the preharvest yield percentage, the fresh weight of the healthy red strawberries directly after collection was also determined. All healthy strawberry fruits collected at weeks 5, 6, 7 and 8 (see above) for the four different treatments were individually weighed. Statistical analysis by Kolmogorov–Smirnov ($P = 0.05$) confirmed that all data were normally distributed. Then, the effect on fresh strawberry fruit weight per sampling week and per plot was determined by one-way ANOVA followed by a *post hoc* Tukey test ($\alpha = 0.05$).

The post-harvest yield (%) was calculated as a percentage of the numbers of healthy fruits after incubation based on the total numbers of fruits incubated. Immediately after harvesting, all healthy red strawberry fruits were placed individually in closed plastic pots with moist filter paper on the bottom.[27] Then, following 2 days of incubation at 22 °C in a 16 : 8 h light : dark photoperiod, fruits were again individually scored by visual inspection for the presence of *B. cinerea* mycelium with the use of a binary system: 0 = not infected, 1 = infected.

At the end of the greenhouse experiment, the total yield was calculated to indicate the numbers of healthy red strawberry fruits that were obtained by healthy flowers in the different treatments (preharvest evaluation) and that were free from *Botrytis* inside the fruit (post-harvest evaluation). This percentage of total yield, including the pre- and post-harvest yields, was given as follows:

$$\% \text{ total yield} = \% \text{ preharvest yield} \times \% \text{ post-harvest yield}$$

For the total yield, the effect of treatment was analysed by one-way ANOVA followed by a *post hoc* Duncan test ($\alpha = 0.05$).

3 RESULTS

3.1 Temperature and RH during the greenhouse experiment

During the period of the greenhouse experiment, the mean temperature in weeks 1, 2, 3 and 4 of the flowering period ranged between 18.5 and 20.6 °C (Fig. 1). For the strawberry fruit sampling period, the temperature was lower at 16.7–18.2 °C. The lower temperatures during the fruit sampling period can be explained by this period falling between mid-November and the end of December 2009, and this winter period was characterised by freezing outdoor temperatures. In parallel, the relative humidity at the level of the plants during the flowering period ranged between 87 and 97%, while it ranged between 65 and 83% during the fruit sampling period.

3.2 Foraging activity of *Bombus terrestris* before and during the greenhouse experiment

Before the start of the greenhouse experiment, the mean foraging activities (i.e. the numbers of workers flying in and out in 30 min) of

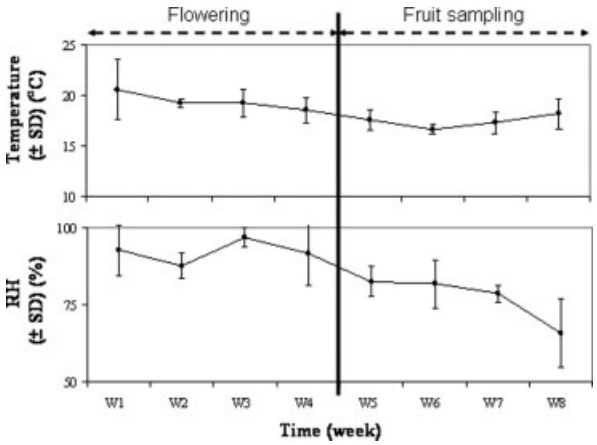

Figure 1. Overview of the temperature and relative humidity (RH) during the greenhouse trial with 4 weeks of flowering (W1–4) and 4 weeks of fruit sampling (W5–8).

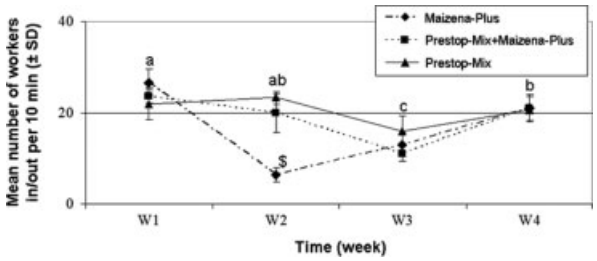

Figure 2. Foraging activity of bumblebee workers in the different treatments (T2–4) during the flowering period (W1–W4). The $ symbol indicates the replacement of hive 3 with hive 4 in week 4. Two-way ANOVA showed a significant effect of the factor 'time'. Values followed by a different letter (a to c) were significantly different (paired *t*-test; $P = 0.008$).

the five *B. terrestris* hives were 13.0±2.1 for hive 1, 19.0±2.7 for hive 2, 19.3 ± 2.7 for hive 3, 25.3 ± 5.4 for hive 4 and 11.0 ± 2.3 for hive 5. Statistical analysis confirmed a homogeneous foraging activity ($\alpha = 0.131$) in the five hives. For the greenhouse experiment, hives 1, 2 and 3 were selected at random for further use. However, in T2, hive 3 was replaced with hive 4 at the second week of the greenhouse experiment because all workers died at the end of the first week of vectoring.

The foraging activities of hive 1 (T3), hive 2 (T4) and hive 3 (T2) during the 4 weeks of the greenhouse experiment are given in Fig. 2, indicating that the temperature registered (Fig. 1) did not negatively affect the foraging behaviour of the bumblebees. Two-way ANOVA showed no significant ($P = 0.167$) interaction between the factors 'treatment' and 'time', and no significant ($P = 0.234$) effect of the factor 'treatment', whereas the factor 'time' resulted in a significant ($P = 0.001$) effect on the foraging activity. One week after the start of the greenhouse experiment, the foraging activities were 22.0 ± 3.5 for hive 1, 23.7 ± 1.5 for hive 2 and 26.7 ± 2.9 for hive 3. For week 2 the foraging activity in all treatments was also equal, showing no influence of the treatment. Moreover, the foraging activities for all nests in week 2 were not significantly different when compared with weeks 1 and 4 (paired *t*-test; $P = 0.644$ and $P = 0.698$ respectively). However, it should be remarked that for T2 no data were collected at week 2 because of the replacement of hive 3 with hive 4. In week 3, for all treatments the foraging activities were equal among the different treatments but were lower when compared with the

Table 1. Overview of the pre- and post-harvest yield percentages and total efficacy of vectoring to suppress *Botrytis cinerea*. For each treatment (T1–4) and per week, the number of healthy flowers, the number of healthy red fruits at pre- and post-incubation, the mean fresh weight of the produced healthy red fruits and the percentage of yields are given. Per treatment, the mean (± SD) of the 4 weeks is given in the bottom row

Treatment	Number of healthy flowers	Number of healthy red fruits	% yield (preharvest)	Mean fresh weight of fruits (g)[a]	Number of healthy fruits after incubation	% yield (post-harvest)	% total yield (pre- and post-harvest)[b]
1	W1: 77	W5: 20	26	W5: 4.3 a	7	35	9
	W2: 22	W6: 16	73	W6: 4.1 a	7	44	32
	W3: 44	W7: 22	50	W7: 3.0 a	7	32	16
	W4: 44	W8: 30	68	W8: 0.6 a	18	60	41
Mean (± SD)			54(±21)			43(±13)	24 (±14) a
2	W1: 87	W5: 36	41	W5: 5.7 b	14	39	16
	W2: 54	W6: 25	45	W6: 4.3 a	15	60	27
	W3: 26	W7: 14	54	W7: 3.4 a	6	43	23
	W4: 26	W8: 16	62	W8: 1.8 b	9	56	35
Mean (± SD)			51(±9)			50(±10)	25(±8) a
3	W1: 65	W5: 45	69	W5: 4.7 ab	36	80	55
	W2: 44	W6: 22	50	W6: 4.1 a	15	68	34
	W3: 22	W7: 20	91	W7: 3.0 a	10	50	46
	W4: 13	W8: 10	77	W8: 0.8 a	7	70	54
Mean (± SD)			72(±17)			67(±13)	47(±10) b
4	W1: 52	W5: 34	65	W5: 4.8 ab	23	68	44
	W2: 30	W6: 19	63	W6: 3.0 a	19	100	63
	W3: 10	W7: 8	80	W7: 2.0 a	5	63	50
	W4: 17	W8: 13	77	W8: 0.8 a	11	85	65
Mean (± SD)			71(±9)			79(±17)	56(±10) b

[a] Analyses with one-way ANOVA resulted for W5 in two groups ($F = 2.273$; df = 131; $P = 0.083$), for W6 in one group ($F = 1.881$; df = 69; $P = 0.141$), for W7 in one group ($F = 2.213$; df = 81; $P = 0.093$) and for W8 in two groups ($F = 5.141$; df = 55; $P = 0.003$). The values per treatment of the same week followed by a different letter (a or b) are significantly different after a *post hoc* Tukey test with $\alpha = 0.05$.
[b] Analyses with one-way ANOVA resulted for the percentage total yield in two groups ($F = 8.291$; df = 15; $P = 0.003$). The values per treatment followed by a different letter (a or b) are significantly different after a *post hoc* Duncan test with $\alpha = 0.05$.

activities obtained in weeks 1, 2 and 4 (paired *t*-test; $P < 0.008$). For week 4, again all treatments had equal foraging activities, but the activities were significantly lower than in week 1 (paired *t*-test; $P = 0.001$).

The worker mortalities (i.e. the numbers of dead workers found outside the hive) were similar in T2, T3 and T4. However, for week 1 no data were collected for T2 (Maizena-Plus) owing to a total loss of workers (100% mortality) observed at the end of that week (which cannot be explained). The numbers of dead workers scored during weeks 2 to 4 were 20, 19 and 23 in treatments T2, T3 and T4 respectively. Thus, T3 (Prestop-Mix) and T4 (Prestop-Mix + Maizena-Plus) resulted in 0–15% worker mortality compared with T2 (Maizena-Plus alone).

3.3 Effect of inoculation manipulation on *Botrytis cinerea* growth in the flowers

Examination of the flowers 24 h after pathogen inoculation showed that *B. cinerea* was able to germinate, and the presence of mycelium confirmed growth of the pathogen. In contrast, no mycelium was present in the control flowers inoculated with water. The mean numbers of CFU per flower (± SD) for *B. cinerea* were 546 ± 121 in week 1, 743 ± 131 in week 2, 721 ± 50 in week 3 and 720 ± 131 CFU in week 4. In contrast, for the water control, no CFU were present in the flowers, and this was the case in all samples at the end of the 4 weeks (W1–W4).

3.4 Effect of the release of bumblebee workers as vectors and inoculation of *Botrytis cinerea*

In T1 and T2 the loss of flowers was low at 5.5 and 3.5% respectively. This showed that manual inoculation of the pathogen *B. cinerea*, bumblebee visiting and Maizena-Plus did not result in high losses of flowers. In contrast, in T3 and T4 a higher percentage of loss of flowers, at 28 and 40% respectively, was scored over the 4 weeks of flowering (W1–4). Here, the loss was due to overpollination caused by the bee-to-flower ratio being too high. This resulted in *B. terrestris* workers biting into the flowers during the 90 min foraging period, as was seen in T3 and T4.

3.5 Efficacy of vectoring to suppress *Botrytis cinerea*: pre- and post-harvest and total yields

For the preharvest suppression of *B. cinerea*, Table 1 demonstrates that the number of flowers resulting in healthy fruits was highest for T3 and T4. The preharvest yield ranged between 50 and 91% and between 63 and 80%, corresponding to a mean preharvest yield of 72 ± 17% and 71 ± 9% respectively. In contrast, for T1 and T2, the protection of the flowers was lower, resulting in a lower yield of 26–73% and 41–62% or a mean respective preharvest yield of 54 ± 21% and 51 ± 9%.

When all healthy (non-infected) red strawberry fruits, as collected at picking, were weighed, it was clear that, over the different weeks, the fresh weights were equal for the different treatments (Table 1). There was only one exception for T2 in week

8; however, it should be remarked here that at week 8 the fruits in all treatments had a tendency to be smaller.

For the post-harvest suppression of *B. cinerea*, a similar trend was observed. As shown in Table 1, for T3 and T4 a high number of healthy red fruits, as collected at picking, developed no disease symptoms after incubation, while the opposite was observed for T1 and T2. Consequently, the mean post-harvest yield was higher for T3 and T4 at 67 ± 13% and 79 ± 17%, respectively, as against 43 ± 13% and 50 ± 10% for T1 and T2 respectively.

Finally, when considering the total yield (preharvest yield × post-harvest yield), vectoring Prestop-Mix + Maizena-Plus (T4) resulted in the highest yield of 56 ± 10%; this was 47 ± 10% for Prestop-Mix alone (T3). Meantime, the total yield percentages were significantly lower ($P < 0.05$) at only 24 ± 14% and 25 ± 8% for T1 and T2 respectively.

4 DISCUSSION

A prerequisite for the use of pollinators as MCA vectors is that the product(s) in the dispenser pose no negative lethal or sublethal effects towards the pollinator, in the present case bumblebee workers. In the present greenhouse experiment it was confirmed that there were no adverse lethal effects on worker survival and no sublethal effects on the foraging activity with the use of Prestop-Mix and Maizena-Plus (T3 and T4). These results are in agreement with a previous study by Kovach *et al*.,[12] who did not report any negative effects on bee health after vectoring *T. harzianum*.

This greenhouse study investigated the capacity of *B. terrestris* to suppress the pathogen *B. cinerea* in greenhouse strawberry flowers at a high pathogen pressure. The conditions of the present greenhouse experiment were expected to be very favourable for *B. cinerea* infection, as 18.5–20.6 °C and 88–97% relative humidity were measured during the flowering period and 16.7–18.2 °C and 66–83% relative humidity during the fruit sampling period. Indeed, Wilcox and Seem[28] and Cota *et al*.[5] reported that *B. cinerea* infection is favoured at moderate temperatures of 15–25 °C and high relative humidities. Secondly, the pathogen *B. cinerea* was inoculated directly into the open strawberry flowers, and hyphal growth of *B. cinerea* on the anthers and the petals was observed within 24 h of inoculation. Indeed, it has been reported that flowers, and particularly open flowers, are the most susceptible plant tissues,[6,29] *Botrytis cinerea* can easily colonise flower anthers[30] and in strawberry the petals and stamens also play a dominant role in the infection by *B. cinerea*.[27] Under these conditions, this greenhouse study confirmed the usefulness of *B. terrestris* bumblebees as vectors of a commercially available model MCA (Prestop-Mix) to protect strawberry flowers against the major plant pathogen *B. cinerea* to give significantly higher preharvest and post-harvest yield percentages of healthy strawberry fruits. Specifically, for the treatments T3 and T4, a better mean preharvest yield of 71–72% was scored, as opposed to 54% for T1 and 51% for T2. Similarly, the strawberry fruits were better protected, as the post-harvest yields were also higher in T3 and T4. The present results therefore provide strong evidence that *B. terrestris* bumblebees distributed satisfactory levels of MCA into the flowers for the suppression of *B. cinerea*. Similarly, bumblebees have been demonstrated to vector *C. rosea* in tomato and sweet pepper flowers, resulting in the successful suppression of *B. cinerea* (57–59%).[19] Also, Kovach *et al*.[12] reported a reduction in the number of infected strawberries with bumblebees and honey bees vectoring *T. harzianum* that was equally efficient as the spraying of chemical (standard) fungicides such as vinclozolin (Ronilan 4F; BASF Corp.), captan (Captan 50WP; Micro FLo), benomyl (Benlate 50WP; Dupont) and iprodione (Rovral 4F; Rhone Poulenc AG Co.). For honey bees in an open field situation, it is to be noted that the vectoring of *Trichoderma* T39 against *B. cinerea* can reduce the number of symptomatic strawberry fruits at harvest, although this was only the case when the disease pressure was considered not to be high, with the number of infected strawberry fruits in the control plots being <65.[18]

Next to the crop production yield when expressed as a percentage of fruits produced per number of flower, it is to be noted that there were no adverse effects on the mean strawberry weights in the present greenhouse experiments. Similarly, Kovach *et al*.[12] used bumblebees (*B. impatiens*) and honey bees (*A. mellifera*) for the vectoring of *T. harzianum* 1295-25 into strawberry flowers without adverse effects on the weight of the strawberry fruits. However, it should be remarked that, in some cases, such as the T3 and T4 treatments, overpollination was observed. As a consequence, this resulted in the presence of bite marks on flowers, and, in turn, this was responsible for the high flower loss observed in both treatments. Similarly, Velthuis and van Doorn[21] reported damaged flower tissues after flower overvisitation by *B. terrestris*, which eventually led to flower loss and malformed fruits. Therefore, it is recommended that future vectoring experiments be performed in larger greenhouses, preferentially of 1 ha, as the experimental set-up in greenhouse compartments in this study was responsible for the generation of inappropriate ratios of bees to flowers.

In this study it was possible efficiently to suppress *B. cinerea* when flowers were first exposed to the MCA and 24 h later to the pathogen. Similarly, different authors[17,31–33] have also reported high efficiencies when other MCAs, such as *C. rosea, Bacillus licheniformis* (Weigman) Chester N1 (Bacillales: Bacillaceae), *Ulocladium atrum* Preuss (Pleosporales: Pleosporaceae), *T. harzianum* T22 and *Trichoderma atroviride* P. Karsten LC52 (Hypocreales: Hypocreaceae), were present prior to the pathogen *B. cinerea*. It is noteworthy that, in many cases, the mechanism of biocontrol remains unknown. However, Chatterton and Punja[26] demonstrated that, in the case of root diseases, control by *C. rosea* f. *catenulata* was mediated by the production of glucanases, and that this process was regulated by the environmental pH. Interestingly, different antagonism mechanisms have been reported for diverse MCAs, such as the production of antibiotics and toxic metabolites, competition for nutrients and space and mycoparasitism.[34] Therefore, the present authors envisage that, next to the preventive capacity, which until now has been the only focus of vectoring studies, it might be of interest also to evaluate the curative potentials. In the latter case, it can be envisaged that future work could investigate combinations of MCAs or a combination of a MCA with a fungicide. Hence, it is realistic to suggest that other pathogens, such as powdery mildews, may be targeted, because previous assays have demonstrated the presence of *Beauveria bassiana* (Balsamo) Vuillemin (Hypocreales: Crodycipitaceae) strain GHA on the surfaces of tomato and sweet pepper leaves after vectoring with bees.[19,35]

Although many plant protection products (PPPs) are useful in pollinator-and-vector strategies, often their formulation is not specifically developed for dissemination by pollinators, and thus optimisation by adding a diluent to the formulation is likely in order to enhance adhesiveness on the vector body and reduce powder loss during flight. In the present study it was interesting that the use of the food-grade diluent powder Maizena-Plus, containing corn starch, resulted in the same yields but with only half the amount of

MCA. As a consequence, the authors envisage that future studies will be useful to optimise available formulations of PPPs in the context of pollinator-and-vector technology. For example, in order further to improve both transport and deposition of the product by the vector (bee), other diluents as well as combinations of potential diluents may be investigated. Similarly, the relation between diluent and vector used might be of interest, as three different pollinators (i.e. honey bees, bumblebees and mason bees), each with different characteristics, can be used as vectors. Finally, before the commercial implementation of this type of control strategy can be recommended to growers, it is advisable to perform demonstration studies in larger greenhouses, as satisfactory yield levels must be guaranteed.

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REFERENCES

1 Boff P, Kastelein P, de Kraker J, Gerlagh M and Kohl J, Epidemiology of grey mould in annual waiting-bed production of strawberry. *Eur J Plant Pathol* **107**:615–624 (2001).
2 Williamson B, Tudzynski B, Tudzynski P and Van Kan JAL, *Botrytis cinerea*: the cause of grey mold disease. *Mol Plant Pathol* **8**:561–580 (2007).
3 Zhang HY, Wang L, Dong Y, Jiang S, Cao H and Meng RJ, Postharvest biological control of gray mold decay of strawberry with *Rhodotorula glutinis*. *Biol Control* **40**:287–292 (2007).
4 Cota VL, Maffia LA, Mizubuti ESG, Macedo PEF and Antunes RF, Biological control of strawberry gray mold by *Clonostachys rosea* under field conditions. *Biol Control* **46**:515–522 (2008).
5 Cota LV, Maffia LA, Mizubuti ESC and Macedo PEF, Biological control by *Clonostachys rosea* as a key component in the integrated management of strawberry gray mold. *Biol Control* **50**:222–230 (2009).
6 Mertley JC, Mackenzie SJ and Legard DE, Timing of fungicide applications for *Botrytis cinerea* based on development stage of strawberry flowers and fruit. *Plant Dis* **86**:1019–1024 (2002).
7 Sutton JC, Epidemology and management of *Botrytis* leaf blight on onion and gray mold of strawberry: a comparative analysis. *Can J Plant Pathol* **12**:100–110 (1990).
8 Dianez F, Santos M, Blanco R and Tello JC, Fungicide resistance in *Botrytis cinerea* isolates from strawberry crops in Huelva (southwestern Spain). *Phytoparasitica* **30**:529–534 (2002).
9 Leroux P, Fritz R, Debieu D, Albertini C, Lanen C, Bach J, *et al*, Mechanisms of resistance to fungicides in field strains of *Botrytis cinerea*. *Pest Manag Sci* **58**:876–888 (2002).
10 Bardas GA, Myresiotis CK and Karaoglanidis GS, Stability and fitness of anilinopyrimidine-resistant strains of *Botrytis cinerea*. *Phytopathology* **98**:443–450 (2008).
11 Kretschmer M, Leroch M, Mosbach A, Walker AS, Fillinger S, Mernke D, *et al*, Fungicide-driven evolution and molecular basis of multidrug resistance in field populations of the grey mould fungus *Botrytis cinerea*. *PLoS Pathog* **5**:e1000696 (2009).
12 Kovach J, Petzoldt R and Harman GE, Use of honeybees and bumble bees to disseminate *Trichoderma harzianum* 1295-22 to strawberries for *Botrytis* control. *Biol Control* **18**:235–242 (2000).
13 Droby S, Wisniewski M, Macarisin D and Wilson C, Twenty years of postharvest biocontrol research: is it time for a new paradigm? *Postharv Biol Technol* **52**:137–145 (2009).
14 Sharma RR, Singh D and Singh R, Biological control of postharvest fruits and vegetables by microbiological antagonists: a review. *Biol Control* **50**:205–221 (2009).
15 Montesinos E, Development, registration and commercialization of microbial pesticides for plant protection. *Internat Microbiol* **6**:245–252 (2003).
16 Peng G, Sutton JC and Kevan PG, Effectiveness of honeybees for applying the biocontrol agent *Gliocladium rosea* to strawberry flowers to suppress *Botrytis cinerea*. *Can J Plant Pathol* **14**:117–129 (1992).
17 Yu H and Sutton JC, Effectiveness of bumblebees and honeybees for delivering inoculum of *Gliocladium roseum* to raspberry flowers to control *Botrytis cinerea*. *Biol Cont* **10**:113–122 (1997).
18 Shafir S, Dag A, Bilu A, Abu-Toamy M and Elad Y, Honeybee dispersal of the biocontrol agent and *Trichoderma harzianum* T39: effectiveness in suppressing *Botrytis cinerea* on strawberry under field conditions. *Eur J Plant Pathol* **116**:119–128 (2006).
19 Kapongo JP, Shipp L, Kevan P and Sutton JC, Co-vectoring of *Beauveria bassiana* and *Clonostachys rosea* by bumblebees (*Bombus impatiens*) for control of insect pests and suppression of grey mould in greenhouse tomato and sweet pepper. *Biol Cont* **46**:508–514 (2008).
20 Mommaerts V, Kurt P, Vandeven J, Jans K, Sterk G, Hoffmann L, *et al*, Development of a new dispenser for microbiological control agents and evaluation of dissemination by bumblebees in greenhouse strawberries. *Pest Manag Sci* **66**:1199–1207 (2010).
21 Velthuis HHW and van Doorn A, A century of advances in bumblebee domestication and the economic and environmental aspects of its commercialization for pollination. *Apidologie* **37**:421–451 (2006).
22 Kakutani T, Inoue T, Tezuka T and Maeta Y, Pollination of strawberry by the stingless bee *Trigona minangkabau* and the honey bee *Apis mellifera*: an experimental study of fertilization efficiency. *Res Popul Ecol* **35**:95–111 (1993).
23 Punja ZK and Yip R, Biological control of damping-off and root rot caused by *Pythium aphanidermatum* on greenhouse cucumbers. *Can J Plant Pathol* **25**:411–417 (2003).
24 Rose S, Parker M and Punja ZK, Efficacy of biological and chemical treatments for control of *Fusarium* root and stem rot on greenhouse cucumbers. *Plant Dis* **87**:1462–1470 (2003).
25 Verma N, MacDonald L and Punja ZK, Inoculum prevalence, host infection, and biological control of *Colletotrichum acutatum*: causal agent of blueberry anthracnose in British Columbia. *Plant Pathol* **55**:442–450 (2006).
26 Chatterton S and Punja ZK, Chitinase and β-1,3-glucanase enzyme production by the mycoparasite *Clonostachys rosea* f. *catenulata* against fungal plant pathogens. *Can J Microbiol* **55**:356–367 (2009).
27 Boff P, Köhl J, Gerlagh M and Kraker J, Biocontrol of grey mold by *Ulocladium atrum* applied at different flower and fruit stages of strawberry. *Biocontrol* **47**:193–206 (2002).
28 Wilcox WF and Seem RC, Relationship between strawberry gray mold incidence, environmental variables and fungicide applications during different periods of the fruiting season. *Phytopathology* **84**:264–270 (1994).
29 Sutton JC, Li DW, Peng G, Yu H, Zhang PG and Valdebenito-Sanhueza RM, *Gliocladium roseum*: a versatile adversary of *Botrytis cinerea* in crops. *Plant Dis* **81**:316–328 (1997).
30 Bristow PR, McNicol RJ and Williamson B, Infection of strawberry flowers by *Botrytis cinerea* in strawberry leaves. *Ann Appl Biol* **109**:545–554 (1986).
31 Kim HJ, Lee SH, Kim CS, Lim EK, Choi KH, Kong HG, *et al*, Biological control of strawberry gray mold caused by *Botrytis cinerea* using *Bacillus licheniformis* N1 formulation. *J Microbiol Biotechnol* **17**:438–444 (2007).
32 Yohalem DS, Paaske K, Kristensen K and Larsen J, Single application prophylaxis against gray mold in pot rose and pelargonium with *Ulocladium atrum*. *Biol Cont* **41**:94–98 (2007).
33 Robinson-Boyer L, Jeger MJ, Xu X-M and Jeffries P, Management of strawberry grey mould using mixtures of biocontrol agents with different mechanisms of action. *Biocontrol Sci Technol* **19**:1051–1065 (2009).
34 Punja ZK and Utkhede RS, Using fungi and yeasts to manage vegetable crop diseases. *Trends Biotechnol* **21**:400–407 (2003).
35 Al-mazra'awi MS, Shipp JL, Broadbent AB and Kevan PG, Biological control of *Lygus lineolaris* (Hemiptera: Miridae) and *Frankliniella occidentalis* (Thysanoptera: Thripidae) by *Bombus impatiens* (Hymenoptera: Apidae) vectored *Beauveria bassiana* in greenhouse sweet pepper. *Biol Cont* **37**:89–97 (2006).

