Research Article

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Development of a new dispenser for microbiological control agents and evaluation of dissemination by bumblebees in greenhouse strawberries

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Abstract

BACKGROUND: To date, in modern agriculture, biological control strategies are increasingly becoming the preferred pest management approach. However, the success of microbiological control agents (MCAs) largely depends on efficient dissemination into the crop. The pollinator-and-vector technology employs pollinating insects like bees for a better dissemination. In this study, a new dispenser for bumblebee workers of *Bombus terrestris* L. was developed. Binab-T-vector and Prestop-Mix were used as two typical MCA products for dissemination.

RESULTS: In a first series of experiments in the laboratory for optimisation, the newly developed dispenser was a two-way type dispenser, 20 cm long, with two rectangular compartments and different entrance and exit holes. In addition, the amounts of MCA loaded on the workers were 10 times higher with the new dispenser as compared with the side-by-side passageway (SSP) dispenser. Typically, the highest amounts were recovered from the thorax and legs of the workers. In a second series of experiments under greenhouse conditions with the use of queen-right *B. terrestris* hives, successful dissemination in strawberry flowers was obtained at different distances from the hive (0–8 m, 8–18 m and 18–21 m), and the workers inoculated the first, second and third flowers that were consecutively visited. In addition, the new dispenser caused no adverse effects on worker foraging intensity, whereas a dramatic reduction was scored with an SSP dispenser. Finally, the data suggested that it is necessary to refill the newly developed dispenser at 3 day intervals.

CONCLUSIONS: The results demonstrated that, with the use of the newly developed dispenser, bumblebee workers carried high amounts of MCA, and this resulted in a successful dissemination of MCA into strawberry flowers.

Keywords: bumblebees; pollinator-and-vector; dispenser; microbiological control agents; *Botrytis cinerea*; strawberry; biological control

1 INTRODUCTION

It is now generally accepted that, in modern agriculture, biological control strategies are the preferred pest management approach, specifically in greenhouses, as they are environmentally friendly and more reliable than chemical pesticides on a long-term basis.[1,2] However, the success of microbiological control agents (MCAs) as potential candidates in the battle against pests largely depends on their efficient dissemination into the crop.[3,4]

Pronubial insects like honey bees (*Apis mellifera* L.) and bumblebees (*Bombus terrestris* L.) have the capacity to carry microparticles such as pollen and thus are of crucial importance for pollination in nature and in modern agriculture.[5] In addition, these pollinators disseminate bacterial cells and fungal spores among flowers during pollination.[6–8] In this context, Kevan *et al.*[9] outlined the different pollinator-and-vector technologies available for use by bees for the dissemination of MCAs into flowers. Hence, the pollinator-and-vector technology has already demonstrated its potential for the suppression of various economically important plant pathogens, e.g. *Moniliania vaccinii-corymbosi* JM Reade (Pezizaceae) in blueberry, *Botrytis cinerea* Pers.: Fr. (Moniliaceae) in strawberry and raspberry, *Erwinia amylovora* Burril (Enterobacteriaceae) in apple and pear and *Sclerotinia sclerotiorum* Lib. (Sclerotiniaceae) in sunflower.[10–19] Interestingly, in several cases, bees were more effective than standard spray applications. Over the years, seven types of

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hive-mounted dispenser have been developed and optimised for honey bees: two one-way dispensers where bee entry and exit occur via the same hole (Tub and Harwood types), and five two-way dispensers where the entrance and exit are separated (Peng, Gross, Tray, Triwaks and Houle types).[11,13,20–24] It is clear that the success of a given pollinator-and-dispenser system depends on the use of a suitable formulation of the MCA and on the dispenser design to guarantee efficient dissemination. Furthermore, success with honey bees is strongly influenced by bee foraging activity and disease pressure.

Alongside honey bees, bumblebees are also widely used for their pollination services in modern agriculture as they fly at low temperatures and low light intensities and incur no significant disease problems.[25,26] Firstly, with *Bombus impatiens* Cresson, the loading of the bumblebee workers with *Clonostachys rosea* Link.: Fr. (Bionectriaceae) (formerly *Gliocladium roseum* Bainier) [0.3–128 $\times$ 10^4 colony forming units (CFU) bumblebee^{-1}] was comparable with that achieved for honey bees (1.3–81 $\times$ 10^4 CFU honey bee^{-1}).[10] Secondly, bumblebee-vectored *C. rosea* was more effective in the suppression of *B. cinerea* than spray applications of the MCA. Similar results were also obtained with *B. impatiens* vectoring *Trichoderma harzianum* Rifai 1295-22 (Hypocreaceae) against *B. cinerea* in strawberry.[13] However, a few recent studies have indicated that the dispensers designed for honey bees are not optimal for bumblebees owing to several physiological and behavioural differences, a good example being the cleaning behaviour of bumblebees. Maccagnani *et al*.[27] investigated the one-way side-by-side passageway (SSP) dispenser and the two-way overlapping passageway (OP) dispenser with *B. terrestris* bumblebee workers which are widely used for pollination in the production of important vegetables such as tomato and small fruit such as strawberry. However, the performance of both dispensers was not satisfactory and resulted in the numbers of inoculated flowers being lower than for a standard spray treatment owing to the poor inoculum load generated in the *B. terrestris* workers.

Observations with the SSP dispenser suggested that bumblebee workers were poorly loaded and that foraging behaviour was affected, providing the impetus to develop a new dispenser. A study was then undertaken to evaluate its ability and efficiency to disseminate an MCA. Here, Binab-T-vector and Prestop-Mix were used as two model MCA products. The efficiency of the newly developed dispenser to load bumblebee workers with inoculum was compared with the existing SSP dispenser system. Additionally, the impact of the type of dispenser and the presence of MCA powder formulation in the dispenser on the foraging capacity of bumblebee workers was evaluated using queen-right hives. Finally, the efficacy of dissemination of MCA powder formulation onto strawberry flowers by *B. terrestris* workers using the newly developed dispenser was determined under greenhouse conditions.

2 MATERIALS AND METHODS

2.1 Insects

All experiments were performed with bumblebees obtained from a continuous mass rearing programme (Biobest NV, Westerlo, Belgium) and conducted under standardised laboratory conditions of 28–30 °C, 60–65% relative humidity and continuous darkness. The insects were provided with commercial sugar water and pollen (Soc. Coop. Apihurdes, Pinofranqueado-Cáceres, Spain) *ad libitum* as energy and protein sources respectively.

2.2 Products as example MCAs for dissemination by bumblebees

To evaluate the efficiency of the newly developed dispenser, two commercial MCAs were used as examples: Binab-T-vector is a wettable powder formulation of a 50/50 mixture of *Trichoderma atroviride* Karst. (Hypocreaceae) and *Hypocrea parapilulifera* BS Lu, Druzhin. & Samuels (Hypocreaceae), containing at least 10^6 CFU g^{-1} and developed to be vectored by bumblebees (Binab Bio-Innovation AB, Helsingborg, Sweden), and Prestop-Mix is a wettable powder formulation of *Gliocladium catenulatum* Gilman & Abbott J1446 (Bionectriaceae), containing 10^7–10^9 CFU g^{-1} and developed to be delivered by pollinators (Verdera Oy, Espoo, Finland). The products were stored in accordance with the manufacturers' guidelines.

Before the different experiments in this project were started, the actual numbers of CFU g^{-1} in the two powdered MCA products were determined using plating-out bioassays on potato dextrose agar (PDA). Means of $7.0 \pm 1.9 \times 10^7$ CFU g^{-1} and $1.1 \pm 0.4 \times 10^8$ CFU g^{-1} were determined for Binab-T-vector and Prestop-Mix respectively.

2.3 Optimisation of the dispenser length for an optimal loading of bumblebee workers

In this series of experiments, a two-way dispenser of 25 cm length and two similar dispensers of 30 and 40 cm length were used. The transparent plexiglass cover lid of the 25 cm dispenser was marked at 5 cm intervals.

Before the start of the experiment, the dispenser was filled with MCA powder formulation (Binab-T-vector) at 0.11 g cm^{-2}, representing a homogeneous powder layer thickness of 3 mm. Bumblebee workers were kept individually in falcon tubes covered with aluminium foil to ensure darkness. Subsequently, the falcon tube was connected to the dispenser, and the bumblebee worker was allowed to walk through the dispenser over a selected distance, i.e. 5, 10, 15, 20, 25, 30 or 40 cm, and was then collected. These experiments were conducted in darkness under red light to avoid disturbing the typical walking behaviour of the bumblebees. The time needed by the worker to pass through the dispenser was recorded for test distances of 20, 25, 30 and 40 cm. For each distance, a total of 30 individual bumblebees was tested.

After collection, the workers were killed and the numbers of CFU loaded onto their bodies were determined. Each individual worker was gently shaken in 25 mL physiological solution (8.5 g L^{-1} NaCl) for 60 min. The numbers of CFU per worker were determined by plating out dilutions of the physiological solution on PDA medium. Four replicates were undertaken for each dilution and were treated as one value. Petri dishes were incubated at room temperature, and after 36 h the numbers of CFU were counted.

Data were expressed as mean CFU bee^{-1} $\pm$ SEM and were tested for normal distribution with Kolmogorov–Smirnov tests ($P = 0.05$). After confirmation of normality, one-way analysis of variance (ANOVA) was followed by Tukey–Kramer *post hoc* tests ($\alpha = 0.05$). All statistical analyses were done in SPSS 16.0.

2.4 Loading of powder MCA formulation on bumblebee workers

2.4.1 *Effect of the type of dispenser*

For this series of experiments, two types of dispenser were used, i.e. a one-way SSP dispenser and the newly developed two-way dispenser.

A one-way SSP design was constructed as a hive-mounted dispenser where the exit and entrance were the same (position and size), as described by Maccagnani *et al*.[27] Briefly, this dispenser was made of PVC plastic with a length of 20 cm, a width of 11.3 cm and a height of 5 cm, and consisted of two equal compartments A and B (each with a width of 5.4 cm) which were parallel to each other. Compartment A could be directly connected to the nest/hive via a plastic tube through which bumblebee workers were able to enter the dispenser. In compartment A, which contained transparent plastic cross-plates, bumblebees were loaded with inoculum by zigzag-fashion walking through the layer of MCA powder formulation. At the end of the zigzag passageway, bumblebee workers entered compartment B via an opening. Compartment B was darkened and had one exit hole through which bumblebees left the dispenser. On the opposite side to the exit hole, compartment B was connected to the nest/hive. A bumblebee-in-closer was placed in all holes directly connected to the bumblebee hive in order to guarantee the unidirectional pathway of bumblebees. The bumblebee-in-closers were transparent plastic conical tubes of 3 cm length.

In addition, a new two-way dispenser was developed that consisted of two rectangular compartments: an exit compartment A (length 20 cm, width 5 cm, height 4 cm) and an entrance compartment B (length 6 cm, width 2.5 cm, height 4 cm) (Fig. 1). The dispenser exit compartment length was set at 20 cm on the basis of the results of the experiments detailed in Section 2.3. On one side, the exit compartment (A) contained one rectangular opening (3 × 3.5 cm) which was in direct contact with the hive; on the opposite side there were two exit holes (diameter 2 cm) in close proximity (at 0.3 and 2.0 cm) to the entrance hole of the dispenser, allowing the bumblebee workers to start their foraging. For the entrance compartment (B) there were two 2 cm diameter holes opposite to each other: one hole served as the entrance hole of the dispenser, and the other was in direct connection with the nest/hive. The latter hole was coloured blue on the outside to attract incoming bumblebees. A unidirectional pathway in both compartments was obtained by placing bumblebee-in-closers in the holes.

To evaluate the effectiveness of the SSP dispenser and the newly developed dispenser in the loading of bumblebee workers with inoculum, compartment A was filled with a 3 mm thick layer of an MCA powder formulation (Binab-T-vector), leaving a margin of 2 mm from the opening in the dispenser. This dose corresponded to 0.11 g cm^{-2}. A grid was placed at the bottom of compartment A to maintain a homogeneous distribution of the MCA powder formulation during the experiment. Each dispenser was connected to a queen-right bumblebee hive that consisted of one queen, her brood and 50 workers. In these hives, pollen had been removed 1 day before the start of the experiment to stimulate the workers to leave the hive and search/forage for food. An empty 50 mL falcon tube was placed at the dispenser exit at an angle of 45° upwards to collect individual workers as they left the dispenser. In total, 30 individual workers were collected per hive. After killing (by freezing), the different workers were individually separated into different body parts: head, thorax, abdomen, forelegs, midlegs and hindlegs, and then individually shaken in physiological solution as described in Section 2.3. The numbers of CFU per worker body part were determined as described in Section 2.3. In addition, the values of CFU per body part were expressed in relation to surface area. The surface areas of the head, thorax and abdomen of the workers with the SSP dispenser were calculated as the surface areas of a sphere with a diameter of 4.03 ± 0.28 mm, a sphere with a diameter of 4.97 ± 0.52 mm and a cylinder of 6.13 ± 0.58 mm length and 5.75 ± 0.63 mm diameter respectively, and for the forelegs a decapitated cone with diameters of 0.64 ± 0.29 mm and 0.44 ± 0.21 mm and a length of 8.62 ± 1.16 mm, for midlegs a decapitated cone with diameters of 0.86 ± 0.33 mm and 0.45 ± 0.30 mm and a length of 11.22 ± 1.45 mm and for hindlegs a decapitated cone with diameters of 0.81 ± 0.46 mm and 0.72 ± 0.50 mm and a length of 13.94 ± 1.01 mm respectively. For the workers in the new dispenser, the respective sizes were 4.18 ± 0.31 mm, 4.85 ± 0.45 mm, 6.60 ± 0.75 mm, 6.08 ± 0.53 mm, 0.46 ± 0.22 mm, 0.45 ± 0.18 mm, 9.22 ± 1.55 mm, 0.61 ± 0.34 mm, 0.53 ± 0.30 mm, 12.28 ± 1.11 mm, 0.70 ± 0.37 mm, 0.75 ± 0.49 mm

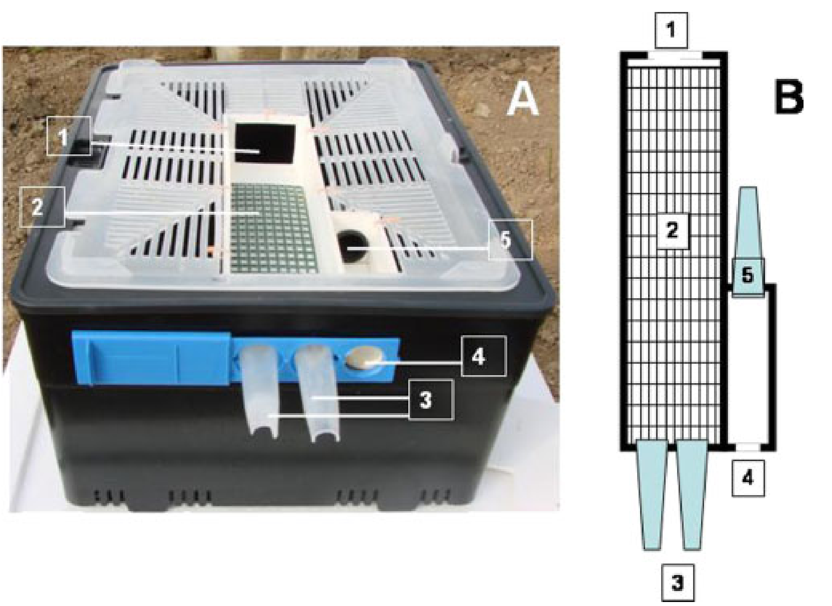

Figure 1. The newly developed two-way dispenser. (A) Photograph from the front without the cover lid, and (B) schematic drawing, top view. (1) Connection of the exit compartment to the bumblebee hive; (2) exit compartment with a grid at the bottom that contains the powder MCA formulation; (3) exit holes with bumblebee-in-closer; (4) entrance hole; (5) bumblebee-in-closer, connecting the entrance compartment to the bumblebee hive. Dispenser length 20 cm.

and 14.65 ± 1.51 mm. The sizes were determined with a slide rule with an accuracy of 0.1 mm.

After confirmation of normal distribution with Kolmogorov–Smirnov tests ($P = 0.05$), data were analysed with two-way ANOVA ($\alpha = 0.05$) with 'body part' and 'dispenser type' as fixed factors. Then, means were separated with an independent-sample *t*-test ($P = 0.05$), as these factors showed a significant interaction.

2.4.2 Effect of flying activity

In these experiments, a bumblebee hive containing one queen, her brood and 20 workers was used, connected to the newly developed two-way dispenser filled with MCA powder formulation (Binab-T-vector) at 0.11 g cm^{-2}. This set-up was placed in a transparent plexiglass flight cage (60 × 60 × 60 cm) in the laboratory. Ten bumblebee workers were randomly collected immediately after leaving the dispenser, and also ten workers at 60 s after flying activity in the flight cage. The workers were killed by decapitation and kept individually in a falcon tube until analysis. The number of CFU per worker was determined by shaking the worker bodies in physiological solution and then by plating out on PDA media as in Section 2.3.

In an extra experiment, the effect of flying activity on the loading of MCA powder formulation on the worker body parts was also evaluated. A dispenser filled with Prestop-Mix at 0.11 g cm^{-2} was used. Individual body parts of ten workers immediately after leaving the dispenser and of ten workers after a flight of 60 s in the flight cage were collected in a falcon tube, and the numbers of CFU were determined as described in Section 2.3.

2.5 Foraging activity of bumblebee workers in a greenhouse when a dispenser is connected to the hive

2.5.1 Effect of the presence of an SSP dispenser and the newly developed dispenser

In a controlled greenhouse of 1.5 ha with sweet pepper plants, the foraging activity of bumblebee workers when an SSP dispenser or the newly developed dispenser was connected to the hive was investigated, and this was compared with the activity for hives without a dispenser (controls). New queen-right bumblebee hives each containing one queen, her brood and 50 workers were used. For each dispenser, two *B. terrestris* hives were used, and the experiment was repeated 5 times. At day 0, the dispensers were connected to the hives and the numbers of workers entering and leaving the nest per 30 min were counted at 7 a.m.–9 a.m. and 4 p.m.–6 p.m. on day 3. In the control groups (hives without a dispenser), the foraging activity was followed at the same intervals.

Normal distribution was confirmed with Kolmogorov–Smirnov tests ($P = 0.05$). Data were then analysed with two-way ANOVA with 'dispenser' (without dispenser, SSP dispenser, new dispenser) as a fixed factor and 'repeats' as a random factor. Factors and interactions were removed from the model when not significant ($P > 0.05$). Means were then separated with paired *t*-tests and corrected with a Bonferroni correction ($P = 0.017$).

2.5.2 Effect of the presence of an MCA powder formulation in the newly developed dispenser

In this experiment, the impact on the foraging activity of bumblebee workers of the presence of a dispenser that was either empty or loaded with MCA powder formulation (Binab-T-vector) and connected to a bumblebee hive was investigated.

For each treatment, two queen-right *B. terrestris* hives consisting of a queen, her brood and 50 workers were used. The hives were supplied with sugar water placed under the hive, but pollen was supplied outside the hive so that the bumblebee workers needed to forage for food. An empty newly developed dispenser was connected at 10 p.m. to the hive, and the next morning the foraging activity frequency (i.e. the numbers of bumblebee workers that flew in and out) was counted for 30 min intervals during two periods of the day: 7 a.m.–9 a.m. and 4 p.m.–6 p.m. Any reduction in activity was attributed to the presence of an empty dispenser. The dispensers were filled with Binab-T-vector at 0.11 g cm^{-2} in the evening (10 p.m.) to avoid any disturbance of the hive. In the control, the dispenser was kept empty. The next day the foraging activity was determined for 30 min intervals over the course of three periods: 7 a.m.–9 a.m., 11 a.m.–1 p.m. and 4 p.m.–6 p.m. The experiment was repeated 5 times.

Normal distribution was confirmed with Kolmogorov–Smirnov tests ($P = 0.05$). The effect of the presence of an MCA powder formulation (empty versus filled dispenser) was evaluated with a one-way ANOVA ($P = 0.05$).

2.6 Dissemination of MCA powder formulation into strawberry flowers in the greenhouse

2.6.1 Dissemination over distances of 8, 18 and 21 m

In three closed strawberry greenhouses of 480 m^2 each, the ground floor was divided into three zones (A, B and C) with a hive + dispenser at the centre and two circles of 8 and 18 m diameter. This allowed the distance of vectoring over a distance of 0–8 m (zone A), 8–18 m (zone B) and 18–21 m (zone C) to be determined. Queen-right bumblebee hives consisting of one queen, her brood and 50 workers were used. An SSP dispenser or a newly developed dispenser was connected to the hives. The dispensers were filled with the MCA powder formulation (Binab-T-vector) at 0.11 g cm^{-2}, as described above. During these 3 week experiments, the temperature and relative humidity were recorded with data loggers in the hives. In addition, the powder quantities in the dispensers were monitored weekly, and they were refilled if necessary. The experiment was repeated twice.

At weekly intervals, flowers were randomly collected that had been visited by bumblebees in zone A (0–8 m), zone B (8–18 m) and zone C (18–21 m). Typically, flowers that have been visited by bumblebees show brown anthers. The collected flowers (20 per zone) were put individually into falcon tubes. For each tube, physiological solution was added and gently shaken for 60 min, and the numbers of CFU were determined by plating-out assays (36 h at 25 °C) using *Trichoderma* selective medium (TSM).[28] In total, four plates were scored per dilution.

Normal distribution was confirmed with Kolmogorov–Smirnov tests ($P = 0.05$). Data were then analysed by two-way ANOVA with 'zone' (A, B, C) and 'dispenser' (SSP dispenser, new dispenser) as fixed factors. Factors and interactions were removed from the model when not significant ($P > 0.05$). Means were separated with paired *t*-tests ($P = 0.05$).

2.6.2 Dissemination to first, second and third strawberry flowers visited

In closed greenhouse compartments of 14 m^2, 20 strawberry plants were placed at a distance of 2.7 m from a queen-right hive consisting of one queen, her brood and 50 workers. Before the start of the experiment, the old flowers and fruits were removed from the strawberry plants. The hive was then connected to the new dispenser, which was filled with a 3 mm thick layer of MCA powder formulation (Prestop-Mix), leaving a margin of 2 mm from the opening in the dispenser; this dose corresponded to 0.11 g cm^{-2}.

The first, second and third flowers consecutively visited by a given individual bumblebee worker after leaving the dispenser were immediately collected on departure of the bee and put individually into falcon tubes. The numbers of CFU per flower were determined as described above, except that PDA was used as medium to determine *G. catenulatum* J1446 after 48 h. The experiment was performed with ten workers and repeated twice.

The mean CFU $flower^{-1}$ ± SEM was calculated for the first, second and third flowers visited. Normality was confirmed with Kolmogorov–Smirnov tests ($P = 0.05$), and the significance between flowers visited was determined with a paired *t*-test ($P = 0.05$).

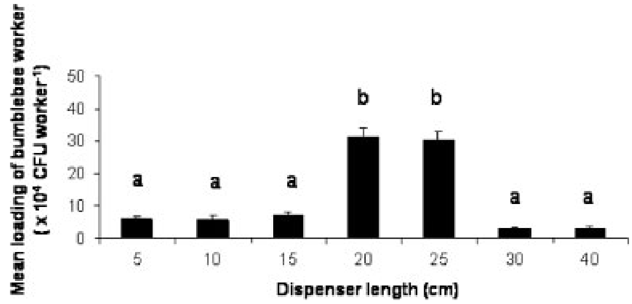

Figure 2. Effect of the length of the newly developed dispenser on the loading of MCA (Binab-T-vector) on workers of *Bombus terrestris*. The numbers of CFU per bee are expressed as means (± SEM). Values followed by a different lower-case letter are significantly different after ANOVA followed by a Tukey–Kramer *post hoc* test with $\alpha = 0.05$.

2.6.3 Dissemination during 1 week without dispenser refilling

In the manner described above, pollen was placed at a distance of 2.7 m from a queen-right hive containing one queen, her brood and a minimum of 100 workers in a closed greenhouse compartment of 14 m^2. Old flowers and fruit were removed from the strawberry plants before the start of the experiment.[29] The hive was then connected to a newly developed dispenser filled with a 3 mm thick layer of the MCA powder formulation (Prestop-Mix), leaving a margin of 2 mm from the opening in the dispenser, giving a dose corresponding to 0.11 g cm^{-2}. During the 7 days of this experiment, the dispenser was not refilled. This experiment was repeated 3 times.

Foraging bumblebee workers were collected immediately (day 0) and at 1, 2, 3, 4, 5, 6 and 7 days after leaving the hive (ten workers per time point). These workers were put individually in falcon tubes with physiological solution and gently shaken for 60 min. The numbers of CFU per worker were determined as described above. PDA medium was used to determine *G. catenulatum* J1446 after 48 h and 72 h; four plates were used per dilution. In addition, at the end of the experiment, at day 7, the amounts of Prestop-Mix remaining in the dispenser was determined.

In this experiment, the level of 10^4 CFU $worker^{-1}$ was used as a binomial variable to evaluate loading. This value was also used by Bilu *et al.*[23] when they investigated the frequency to refill their dispenser. Therefore, when the honey bees were carrying $\geq 10^4$ CFU, the workers were considered to be carrying enough inoculum to disseminate; in the case of $< 10^4$ CFU, the workers were considered not to be carrying enough inoculum to disseminate.

3 RESULTS

3.1 Optimisation of the dispenser length for an optimal loading of bumblebee workers

Figure 2 shows that maximal loading was reached with a dispenser length of 20 and 25 cm, resulting in $31.4 \pm 2.5 \times 10^4$ CFU $worker^{-1}$ and $30.3 \pm 2.7 \times 10^4$ CFU $worker^{-1}$ respectively. At shorter distances of 5, 10 and 15 cm and longer distances of 30 and 40 cm, significantly lower numbers of CFU $worker^{-1}$ were recovered ($F = 65.483$; df = 207; $P < 0.001$). The time that the foraging workers needed to pass through the dispenser was also determined. For dispenser lengths of 20 and 25 cm this ranged from 3 s to 5.5 min and from 3 s to 5.8 min respectively. In contrast, workers needed time periods ranging from 3 s to 30.1 min and from 60 s to 5 h to pass through dispensers of 30 cm and 40 cm length. Note that the workers in the longer dispensers (30 and 40 cm) started to clean their heads and legs before leaving the dispenser, while for dispenser lengths of 20 and 25 cm such typical cleaning behaviour was not observed.

3.2 Loading of powder MCA formulation on bumblebee workers

3.2.1 Effect of type of dispenser

In a first experiment it was shown that, in the newly developed dispenser of 20 cm length, significantly ($P < 0.05$) higher numbers of CFU bee^{-1} were counted when compared with the SSP dispenser: $43.3 \pm 1.6 \times 10^4$ CFU $worker^{-1}$ and $49.4 \pm 10.8 \times 10^3$ CFU $worker^{-1}$ respectively. The mean total body surface area of the workers did not differ significantly between experiments ($P > 0.05$) using the two dispenser types. The bumblebee worker body surface averaged 352.8 ± 54.0 mm^2 in the experiments with the newly developed dispenser, and 340.7 ± 30.7 mm^2 in the experiments with the SSP dispenser.

The second experiment demonstrated how the loading of the bumblebee worker with MCA powder formulation (Binab-T-vector) was distributed over the different body parts of the workers (Table 1). With the SSP dispenser, the highest amounts of MCA recovered were on the thorax (27 530 ± 2028), followed by the abdomen (17 993 ± 2772), representing 56 and 36% of the initial whole-body load ($49.4 \pm 2.0 \times 10^3$ CFU $worker^{-1}$). The head and legs contained only 535–1633 CFU per body part, which equates to 1–3% of the total body load. When calculated per unit of surface area (mm^2), the thorax gave the maximum value of 362 ± 26 CFU mm^{-2}, followed by the abdomen with 171 ± 26 CFU mm^{-2}; the other parts scored a much lower load of 17–24 CFU mm^{-2}. In contrast, use of the newly developed dispenser resulted in a higher loading of the worker body parts compared with the SSP dispenser (Table 1). The distribution of the MCA over the different body parts was significantly different (two-way ANOVA; $F = 22.210$, $P = 0.001$) between the SSP dispenser and the newly developed dispenser, except for the abdomen. The thorax and abdomen were loaded with $117.6 \pm 19.4 \times 10^3$ CFU and $25.1 \pm 2.8 \times 10^3$ CFU respectively, equating to 57 and 12% of the total body load. It was also conspicuous that the workers that walked through the newly developed dispenser contained a significantly higher load on their legs, specifically 12 500, 20 067 and 24 367 CFU on the forelegs, midlegs and hindlegs respectively (independent sample *t*-test; $P = 0.001$). Interestingly, the amounts on the head, thorax, forelegs, midlegs and hindlegs were 5-, 5-, 26-, 27- and 16-fold higher than with the SSP dispenser (Table 1). This was also reflected in the numbers of CFU per body part surface

Table 1. Overview of the loading with MCA (Binab-T-vector) on the bumblebee, in CFU, for the different body parts analysed (head, thorax, abdomen, prolegs, midlegs and hindlegs) using the one-way SSP dispenser and the newly developed two-way dispenser

	SSP dispenser			New dispenser		
Body part	Mean CFU (± SEM) [% of total body load]	Mean surface (± SEM) (mm^2)	Mean CFU mm^{-2} (± SEM)	Mean CFU (± SEM) [% of total body load]	Mean surface (± SEM) (mm^2)	Mean CFU mm^{-2} (± SEM)
Head	923 (±241) [2]	51.2 (±2.9)	17 (±4)	4892 (±1526) [2]	55.3 (±1.5)	94 (±31)
Thorax	27 530 (±2028) [56]	78.5 (±1.5)	362 (±26)	117 601 (±19391) [57]	74.6 (±2.4)	1666 (±208)
Abdomen	17 993 (±2772) [36]	111.4 (±6.9)	171 (±26)	25 136 (±2806) [12]	126.9 (±2.8)	207 (±15)
Prolegs	535 (±3) [1]	30.2 (±2.8)	18 (±2)	12 500 (±400) [6]	27.2 (±1.5)	468 (±52)
Midlegs	808 (±14) [2]	47.7 (±1.2)	17 (±1)	20 067 (±2917) [10]	45.7 (±3.7)	461 (±103)
Hindlegs	1633 (±125) [3]	69.5 (±0.9)	24 (±2)	24 367 (±3867) [12]	68.9 (±4.6)	366 (±64)

Comparison of the CFU mm^{-2} for each body part between the SSP dispenser and the newly developed dispenser with an independent sample *t*-test resulted in the following: for head ($t = 57.013$, df = 58, $P = 0.001$), for thorax ($t = 25.978$, df = 58, $P = 0.001$), for abdomen ($t = 4.086$, df = 58, $P = 0.23$), for forelegs ($t = 56.870$, df = 58, $P = 0.001$), for midlegs ($t = 58.810$, df = 58, $P = 0.001$) and for hindlegs ($t = 28.712$, df = 58, $P = 0.001$).

unit (mm^2), and here the high loading of the legs is to be noted. However, the load per surface achieved for the abdomen was not different between the SSP dispenser and the newly developed dispenser (independent sample *t*-test; $P = 0.23$).

Finally, note that, with the SSP dispenser, all workers started to clean their head and their legs before leaving the dispenser. In contrast, such typical cleaning behaviour was not observed in the newly developed dispenser.

3.2.2 *Effect of flying activity*

Results of the flight cage experiment demonstrated that for Binab-T-vector the workers carried a low amount of $1.8 \pm 1.0 \times 10^4$ CFU $worker^{-1}$ after 60 s of flying activity, representing a loss of 95% of the initial load. When this experiment was performed with Prestop-Mix, the percentage of MCA that was lost during a flight of 60 s was 81%. The thorax and legs of the workers contained $2.9 \pm 1.0 \times 10^3$ CFU $thorax^{-1}$ and $1.3 \pm 0.4 \times 10^3$ CFU $legs^{-1}$ after a flight of 60 s, and these values corresponded respectively to 26 ± 9% and 43 ± 12% of the initial amount on these body parts the moment the worker left the dispenser.

3.3 Foraging activity of bumblebee workers in a greenhouse when a dispenser is connected to the hive

3.3.1 *Effect of the presence of an SSP dispenser and the newly developed dispenser*

The foraging activity (i.e. the numbers of workers that are leaving and entering the dispenser per 30 min) for the different hives without a dispenser between 7 a.m. and 9 a.m. and between 4 p.m. and 6 p.m. did not differ ($P > 0.05$) at 16.3 ± 0.7 and 12.5 ± 1.6 workers respectively.

Interestingly, when the newly developed two-way dispenser was connected to the hive there was no significant effect on the foraging intensity, and 13.3 ± 1.8 workers were counted per 30 min between 7 a.m. and 9 a.m., and 12.0 ± 1.1 workers per 30 min between 4 p.m. and 6 p.m., which agreed with the respective numbers of 14.8 ± 0.9 and 13.0 ± 1.1 in the control groups (hives without a dispenser). In contrast, when an SSP dispenser was connected to the hive there were only 4.7 ± 0.6 workers counted per 30 min between 7 a.m. and 9 a.m., and 5.7 ± 0.8 mean workers per 30 min between 4 p.m. and 6 p.m. The statistical analysis confirmed that there was no significant interaction between the factors 'dispenser' and 'repeats' (two-way ANOVA; $P = 0.256$), but the main effect of the factor 'dispenser' was significant (two-way ANOVA; $P = 0.001$). As a consequence, and based on the paired *t*-test ($P < 0.017$), it was decided not to continue to work with an SSP dispenser in the following experiment (Section 3.3.2).

3.3.2 *Effect of the presence of an MCA powder formulation in the newly developed dispenser*

These experiments demonstrated that filling the newly developed dispenser with MCA powder formulation (Binab-T-vector) did not affect the foraging activity of the bumblebee workers. A total of 13.9 ± 1.1 workers per 30 min were counted with the empty dispenser, and 16.1 ± 2.0 workers per 30 min when it was filled (one-way ANOVA; $F = 2.336$, $P = 0.104$).

3.4 Dissemination of MCA powder formulation into strawberry flowers in the greenhouse

3.4.1 *Dissemination over distances of 8, 18 and 21 m*

In the different greenhouse compartments, the temperature showed a minimum of 10 °C at night and a maximum of 25 °C during the day, and the relative humidity was 84.0 ± 1.2% during the experimental period.

The bumblebee workers deposited 97.5 ± 41.3 CFU $flower^{-1}$ in zone A (0–8 m), 77.3 ± 15.4 CFU $flower^{-1}$ in zone B (8–18 m) and 105 ± 38 CFU $flower^{-1}$ in zone C (18–21 m) when the newly developed dispenser was used. In contrast, the mean numbers of CFU per flower for the different zones were much lower with an SSP dispenser: 32.0 ± 27.2 in zone A, 7.7 ± 7.7 in zone B and 5.0 ± 5.0 in zone C. Two-way ANOVA showed no significant interaction between the factors 'zone' and 'dispenser' ($F = 0.004$, $P = 0.996$). Moreover, the main effect for 'zone' was not significant ($P = 0.110$), while the type of dispenser had a significant impact ($P = 0.001$).

3.4.2 Dissemination to first, second and third flowers

With the newly developed dispenser filled with MCA powder formulation (Prestop-Mix), the mean numbers of CFU per flower found in the first, second and third strawberry flowers visited were 23.4 ± 0.4, 14.7 ± 6.3 and 16.5 ± 2.0 respectively. These greenhouse results demonstrated that workers of *B. terrestris* delivered the MCA powder formulation during their first, second and third visits. The numbers were highest in the first flower visited and then decreased for the second flower visited, although this reduction was marginal ($P = 0.069$). However, the numbers of CFU in the second and third flowers were not significantly different ($P = 0.738$).

3.4.3 Dissemination during 1 week without dispenser refilling

At the start of the experiment, i.e. immediately after filling the dispenser, all bumblebees that left the new dispenser were loaded with an average of $86.0 \pm 32.9 \times 10^4$ CFU bee^{-1}. The percentage of workers with $\geq 10^4$ CFU on their body decreased from 100% at day 0 to 75% and 50% on days 1 and 2 after dispenser filling respectively. Later, this value dropped to 20–30% at days 3, 4 and 5, and at day 6 and day 7 it was only 0–5%. After 7 days, the bumblebees had dispersed 2.3 ± 0.4 g of the Prestop-Mix product.

4 DISCUSSION

The present paper reports on the development of a new dispenser to exploit *B. terrestris* bumblebee workers as vectors for the dissemination of MCA powder formulations. Here, two commercial MCA products were used as examples: Binab-T-vector and Prestop-Mix. Interestingly, both products have recently been reported as safe for use in combination with microcolonies of *B. terrestris*.[30] In the pollinator-and-vector system, the loading of bee workers with MCA occurs as the insects walk through a dispenser filled with a powder formulation that contains the MCA or antagonist.[9,10–14,31–33] Over the years, different dispensers have been developed, and their efficacy varies depending on the type of dispenser (one-way versus two-way) and the insect species used (honey bees versus bumblebees). The results of this project also demonstrated that the length of the dispenser plays a critical role in obtaining a high load, with 20–25 cm being optimal. This finding led to the development of a new dispenser with a two-way structure and a length of 20 cm. The data demonstrated that a much better whole-body loading was achieved with the newly developed dispenser as compared with a one-way SSP dispenser. For Binab-T-vector containing $7.0 \pm 1.9 \times 10^7$ CFU g^{-1} powder formulation, a 10 times higher loading of bumblebee workers was realised, specifically $31.3 \pm 2.5 \times 10^4$ CFU worker^{-1}. Similarly, the loading of *B. terrestris* workers reached $86.0 \pm 32.9 \times 10^4$ CFU worker^{-1} using the newly developed dispenser for Prestop-Mix containing $1.1 \pm 0.4 \times 10^8$ CFU g^{-1} powder formulation. Previously, Maccagnani *et al.*[12] achieved a mean loading of workers of *A. mellifera* with 23.8×10^5 CFU bee^{-1} when they used *T. harzianum* formulated at a mean of 2.5×10^{10} CFU g^{-1} powder. Therefore, taking into account the numbers of CFU per gram formulation and the loading of CFU per bee, their loading efficacy was up to 40 times lower than those achieved here with the newly developed dispenser. Similarly, the experiments of other investigators using *Bombus* bumblebees with different dispenser types have generally shown a relatively low efficacy of loading. Kapongo *et al.*,[33] using a one-way dispenser, achieved about 70×10^4 CFU bee^{-1} for Botanigard 22WP containing *Beauveria bassiana* Bals.-Criv. Vuill. (Cordycipitaceae) at 6.3×10^{10} CFU g^{-1}, and about 3×10^4 CFU bee^{-1} for Endofine containing *C. rosea* at 1.4×10^7 CFU g^{-1}. To a similar extent, Albano *et al.*,[24] using a two-way Houle dispenser, achieved $7.2 \pm 2.3 \times 10^4$ CFU bee^{-1} for Rootshield containing *T. harzianum* T-22 at 9.8×10^6 CFU g^{-1}. The two-way OP dispenser also resulted in similar low levels for *T. harzianum* and *Gliocladium virens* Miller, Giddens, Foster: $4.3 \pm 4.2 \times 10^4$ CFU bee^{-1}.[27] Therefore, it can be concluded that the new dispenser developed in the present project facilitates the dissemination of MCAs in a more efficient manner than existing dispenser systems.

To explain the higher loading achieved on bumblebee workers with the newly developed dispenser, the present results demonstrated that the thorax and legs play a crucial role. These two body parts contribute 57% and 28%, respectively, to the total load of MCA achieved on worker bodies. The thorax carried 5 times more CFU mm^{-2} (1666) when the new dispenser was used, whereas this value was only 362 CFU mm^{-2} with an SSP dispenser. Also, the legs carried about 20–30 times more CFU mm^{-2} with the new dispenser (366–468) compared with an SSP dispenser (17–24). Importantly, it is these two body parts (thorax and legs) that make contact with the flower organs during pollination, and so they will play an important role in disseminating/vectoring MCA into the flower (Mommaerts V, unpublished data). The present observations also agree with those of Peng *et al.*,[11] who observed that high MCA amounts adhered to the femoral setae of *A. mellifera*. Similarly, Kovach *et al.*[13] reported that these body parts are also important in the loading of *A. mellifera* workers, with 58% of the total found on the legs and 23% on the thorax; however, the total numbers of CFU on the *Apis* workers were lower in these tests than in the present experiments with *Bombus* workers. Finally, it is important to note that the workers in the present experiments with the SSP dispenser showed typical cleaning behaviour, whereas this was not observed with the newly developed dispenser. However, cleaning behaviour was seen with dispensers with lengths of 30 cm and longer, where much longer passage times were recorded compared with the optimal dispenser length of 20–25 cm. The authors postulate here that the cleaning behaviour of the workers may explain the longer time needed to pass through the longer dispensers, as well as the lower degree of loading obtained for these workers. Therefore, it is recommended that any dispenser be designed so as not to trigger cleaning behaviour in the workers and to maximise their loading with MCA powder formulation. In contrast, and to explain the lower body loading obtained with a dispenser length of 15 cm or lower, it is suggested that shorter dispenser lengths allowed the workers to pass through the dispenser too rapidly, which, in turn, resulted in a lower MCA loading. It has been seen that, in the two-way concept, the dispenser length, the design with opposite holes, their diameter and their position, the blue colour attraction and the bumblebee-in-closers all play an important role in maximising dispenser efficiency.

The use of an efficient pollinator-and-vector system must, alongside maximising load, also guarantee no impairment of the foraging behaviour of the workers when the dispenser is connected to the hive, i.e. the workers need to visit flowers at the same frequency/activity as with a hive without a dispenser. Bilu *et al.*[23] reported a strong reduction in *A. mellifera* activity when a one-way Hardwood dispenser was connected to a honey bee hive. Similarly, in the present experiments a dramatic reduction of about 60% in foraging activity was observed with the one-way SSP dispenser connected to a hive of *B. terrestris*. Furthermore, note that the activity of honey bee workers was reduced when they needed to pass through the one-way Tub and Hardwood dispensers and the two-way Triwaks dispenser filled with Trichodex.[23] Therefore, these results indicate that there is a need for a training period to find the exit and entrance of the dispenser for the workers before the workers can forage for the flowers.[23] Here, it is believed that, the more complex the dispenser, the more impact there will be on worker foraging activity. Therefore, it is suggested that a relatively simple design of two-way dispenser is most appropriate in a pollinator-and-vector system. With the new dispenser investigated here, no loss of foraging activity of the bumblebee workers was observed. This was also the case when the dispenser was filled with an MCA powder formulation.

In the context of MCA transport by workers, the authors also think that the MCA powder formulation could reduce the loading of the bumblebee workers during their foraging activity. For instance, for Binab-T-vector it was observed that 95% of the initial loading present at the moment the workers left the dispenser was lost during 60 s of flying. The period of 60 s of flying is representative of the time needed by the workers to reach the flowers in a crop in the greenhouse (Mommaerts V, unpublished data). Under the same conditions, a loss of 81% was recorded for Prestop-Mix. In addition, the decrease in loading for Prestop-Mix on the two most important body parts was followed, and it was found that the decrease was highest on the thorax, with a loss of 74%, while this value was 57% for the legs. The authors believe that this may be due to the activity of the wings on the dorsal side of the thorax, and that the legs on the ventral side are less exposed to wing turbulence. So, although this experiment was conducted in a flight cage under laboratory conditions, it is believed that the results are indicative, and that such flight cage experiments can be useful in the development of an efficient MCA powder formulation for a pollinator-and-vector system. As indicated in this project, the type of powder formulation may play a role in the rate of loss and the interaction with the amounts of initial load; however, future research is required to make firm conclusions.

This study demonstrated the efficiency of the newly developed dispenser and that bumblebee workers are able to disseminate an MCA powder formulation (Binab-T-vector) homogeneously into flowers of strawberry plants under greenhouse conditions. Interestingly, the distribution of the MCA product was affected by the type of dispenser used, but not by the zone. Consequently, the numbers of around 100 CFU flower^{-1} with the new dispenser were equal over the different distances 0–8 m, 8–18 m and 18–21 m, demonstrating that the flowers can be protected over an area of 21 m around a hive. In contrast to the present findings, Maccagnani *et al*.[27], using an OP dispenser, found a reduction in the amounts of *Trichoderma* vectored by *B. terrestris* workers to tomato flowers with increased distance from the hive (0–4–8–12–18–24 m). In this case there was a small gradual decrease between 4 and 12 m, but then a strong decrease between 12 and 18 m, which was followed by a nearly total loss between 18 and 24 m. Although not determined, the present authors believe that this difference might be explained by a lower efficacy of the dispenser and/or differences in the characteristics of the MCA powder formulation, which may have served to produce a lower worker loading. Furthermore, flower properties may be important, as tomato flowers only produce pollen and no nectar, while bumblebees are highly attracted to strawberry flowers by the presence of both pollen and nectar.[34,35] Additionally, tomato flowers are visited by bumblebees with a typical buzzing behaviour, causing turbulence in the flower, which may also cause a (variable) reduction in CFU flower^{-1}.

On the dissemination efficacy, it should be said that the numbers of CFU in the flowers did not exceed 1% when expressed as a percentage of the loading on the bumblebee worker. When expressed as a percentage of the worker body load (about 3.9×10^4 CFU worker^{-1}) immediately after leaving the dispenser, the transport to the flower was about 0.025%. However, given a 95% loss of the MCA formulation during a flight of 60 s (as seen in the present flight cage experiments), which is considered to be the mean time to forage from the hive + dispenser to the flower, then the percentage of dissemination of MCA from the bumblebee to the flower was about 0.5%. Nonetheless, the authors consider these percentages to be relatively low, although they are consistent with other studies performed with *A. mellifera*, *B. terrestris* and *B. impatiens* vectors where transport ranged from 0.2 to 1.7%.[11,19,24,27] In addition, although transmission percentages depended largely on the crop and the attractiveness of the flowers, Albano *et al*.[24] also reported a higher rate of transmission with bumblebees than with honey bees.

When the behaviour of workers visiting flowers was examined in more detail, it was clear that the workers deposited MCA in the different flowers that were consecutively visited. As may be expected, the highest amounts were deposited in the first flower, but the second and third flowers visited still received a relatively high amount. Recently, in a study by Albano *et al*.[24], *B. impatiens* and *A. mellifera* workers transported Rootshield containing *T. harzianum* T-22 at 9.8×10^6 CFU g^{-1} powder, and achieved respective numbers of 123 ± 196 CFU and 119 ± 222 CFU into strawberry flowers after a single visit. However, it should be noted that, for this formulation, containing *T. harzianum* and a carrier free of clay, the authors reported large variability in transmission rates. Taken together, although the recovered numbers of CFU per flower after a single visit are relatively low, it can be postulated that they will accumulate by repeated worker visits over several days as one bumblebee will visit one flower over and over during the day.[36]

An additional important aspect for an efficient biocontrol strategy is to guarantee a continuous dissemination of the MCA into the flowers. As seen in the present project, the percentage of workers with $\geq 10^4$ CFU on their body decreased from 100% at day 0 to 75% at day 1 and 50% at day 2 after dispenser filling, suggesting that refilling of the dispenser is recommended at 3 day intervals. This agrees with recent experiments of Al-Mazra'awi *et al*.[37] using *B. impatiens* colonies, who reported a stable worker load of $\geq 10^4$ CFU at days 0, 1 and 2 after filling of their dispenser. In addition, and as discussed, it is evident that the dispenser design and initial filling, the foraging activity of the workers and the size of the hive all play an important role in the dispenser refill frequency required to guarantee efficient dissemination of the MCA powder formulation in the flowers of the crop.[13,24,3,27,33]

In conclusion, this study has examined the development of a new two-way dispenser that allows the use of *B. terrestris* as a vector to disseminate MCA powder formulation in an efficient manner. Further experiments, especially under greenhouse conditions, are necessary to determine the effectiveness of this vectoring system in the biocontrol of important plant pathogens such as *B. cinerea* in strawberry.

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