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Investigation of hive-mounted devices for the dissemination of microbiological preparations by *Bombus terrestris*

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Abstract

Two types of hive-mounted devices for the dissemination of fungal antagonists (*Trichoderma harzianum* Rifai and *Gliocladium virens* Miller, Giddens, Foster) of *Botrytis cinerea* Persoon: Fries were applied to *Bombus terrestris* (L.) (Hymenoptera Apidae) hives. Their efficacy in contaminating worker bees was assessed. It was also verified whether the amount of inoculum delivered onto tomato flowers by bumblebees was comparable to that delivered by spray treatments. The first device (SSP-dispenser) consisted of two distinct passageways arranged side by side, one for exiting and one for entering bumblebees; the exit passageway had oblique barriers forcing exiting workers to follow a zig-zag route. The second device (OP-dispenser) had an exit corridor constituted of two tube-like, partially overlapping passageways with a hole in the overlapping sections; to enter the hive, the bumblebees passed through a hole adjacent to the device. The OP-dispenser proved to be the most effective in bee contamination: 100.0% of the captured bumblebees were contaminated, and carried a mean inoculum load of 4.3×10^4 CFU/bee, while only 12.5% of the bees captured at the exit of the SSP-dispenser were contaminated, and carried a mean inoculum load of 1.4×10^2 CFU/bee. Irrespective of the type of dispenser used, in the greenhouses, where the antagonist was transferred onto the flowers by the bees, inoculum density on flowers was always significantly lower than in those treated with the spray. When the OP-dispenser was used, in the greenhouse treated with bumblebees, a significant negative correlation was observed between the inoculum density on flowers of the sample plants and their distance from the hive.

Key words: dissemination device, carrier, *Bombus terrestris,* biological control, *Botrytis cinerea*, fungal antagonist, *Trichoderma harzianum*, *Gliocladium virens*, Hymenoptera Apidae, tomato.

Introduction

Flowers are often important pathways of plant disease infection. The pathogen infects them symptomlessly under favourable conditions, and progressively colonises other tissues or fruits. Disease symptoms become visible when infected tissues ripen, senesce or die, as in the case of *Botrytis cinerea* Persoon: Fries on strawberries, raspberries, and tomatoes (Jarvis, 1962; Bristow *et al*., 1986; Jarvis, 1992; Eden *et al*., 1996). Effective biological plant disease control depends both on the use of suitable antagonistic strains, and on the methods and strategies for introducing, promoting and maintaining the antagonist in the crop (Sutton and Peng, 1993). The use of microbiological preparations could be particularly effective if dissemination of the antagonist occurs directly on the flower parts during bloom (Tronsmo and Ystaas, 1980; McNichol *et al*., 1985; Peng and Sutton, 1991). Pronubial insects are well known for delivering pollen, fungi and bacteria (Free, 1970; Harrison *et al*., 1980, Dag *et al*. 2000). Hence, while performing their pollination service, pronubial insects might also serve as carriers of antagonistic agents, providing potential disease control as well as economic advantages (Sutton and Peng, 1993; Sutton, 1995).

Studies on *Apis mellifera* L. (Hymenoptera Apidae) as vectors of biocontrol agents against various diseases have already been conducted in Canada, the USA, New Zealand, and Italy (Corliss and Adams, 1992; Peng *et al*., 1992; Thomson *et al*., 1992; Johnson *et al*., 1993a, b; Gross *et al*., 1994; Vanneste, 1996; Maccagnani *et al*., 1999; Bilu *et al*., 2004). Also other bee species have been studied for this purpose, such as the solitary bee *Osmia cornuta* (Latreille) for disseminating antagonistic bacteria against *Erwinia amylovora* Winslow-Burril *et al*., for the biological control of fire blight on pear (Maccagnani *et al*., 2005), and various species of the genus *Bombus* against various fungal diseases.

Bombus impatiens Cresson (Hymenoptera Apidae) has been investigated for grey mould control on raspberries (Yu and Sutton, 1997) and strawberries (Kovach *et al*., 2000). Hive-mounted dispensers, specifically designed for bee contamination, were developed. Bumblebees engaged in 'buzz pollination' (Buchmann, 1992) induce precocious petal drop. The petals do not remain adherent to small fruits, and thus an important pathway of grey mould infection is excluded (Jarvis, 1992; Benuzzi and Vacante, 2004). In Italy, 30,000-50,000 colonies of *B. terrestris* (L.) are purchased annually for pollination service on various crops, most of all on tomatoes. In addition to pollination and indirect disease control, these pronubial insects may also perform an active role of prevention by conveying microbiological preparations directly onto flower parts, provided that the bees themselves are previously contaminated with propagules of the antagonist (Sutton and Peng, 1993).

The aim of the studies carried out in 1999 and in 2000 was to investigate the effectiveness of two dispensers for microbiological preparations, mounted on the entrance of bumblebee hives, in contaminating bumblebees, and to verify whether tomato flowers visited by contaminated bees carried similar amounts of inoculum as those treated with spraying suspensions, the primary method used to apply biocontrol agents to the phylloplane (Sutton and Peng, 1993).

Materials and methods

The dispenser with side-by-side passageways (SSP-dispenser)

The SSP-dispenser was modified from the one devised by Yu and Sutton (1997). It was made of 8-mm-thick plywood, and it was 200 mm long, 80 mm wide and 35 mm high; a 2-mm-thick, removable transparent plastic sheet was used as cover. The dispenser comprised two side-by-side passageways, each 35 mm wide: A) a zigzag passageway with diagonal walls, which was normally illuminated to attract outgoing bees; the powder of the antagonistic fungi was placed in this passageway; B) a straight passageway darkened with a sheet of red adiactinic paper, transparent to humans but not to Hymenopterans in order to discourage outgoing bees to use this passageway; C) to induce incoming bees to enter (figure 1). Outgoing bees hence entered the zigzag passageway, crawled on the microbiological preparation, and exited trough a hole at the end of the zigzag passageway, which was connected with the straight passageway. At this point, they reached the opening at the front end of the dispenser. Incoming bees entered the straight passageway through the front hole, and proceeded towards a hole connecting the dispenser with the bee hive.

The dispenser with overlapping passageways (OP-dispenser)

The dispenser was made of plywood and plastic and consisted of two overlapping passageways, each 140 mm long and 25 mm wide; the upper passageway was 25 mm high, and the lower one 10 mm (figure 2). Each passageway was covered with a 2-mm-thick, removable, transparent plastic sheet. The microbiological powder preparation was placed in the lower passageway. Outgoing bees exited the hive through a hole connected with the upper passageway of the dispenser, crawled to another hole connecting the upper passageway with the lower one, crawled along this narrow passageway on the microbiological preparation, and exited the dispenser through a hole at the front end.

Incoming bees entered the colony box through a conical tube, which was set deep within the main chamber, its major opening in line with the entrance hole of the box. This tube also hindered outgoing bees from exiting the hive through the entrance hole.

The microbiological preparations

In 1999, the SSP-dispenser was tested. The microbiological preparation used was a water-soluble, powdery formulation of *Trichoderma harzianum* Rifai and *Gliocladium virens* Miller, Giddens, Foster [$1x10^8$ colony-forming units (CFU)/g]. The spraying suspension was prepared by diluting the product in water. The concentration of the ready-to-use spray was $1x10^5$ CFU/ml.

In 2000, we tested the OP-dispenser. The biocontrol agent, a water-soluble powder preparation was provided by the DI.VA.PRA (Turin, Italy). It consisted of three different strains of *T. harzianum* (13/3 RDB PH1, 15/2 RDB5 and 4/18 RDB1) ($3.5x10^9$ CFU/g). A highly concentrated suspension of propagules was supplied for the spray treatment. Once diluted, the spray had a concentration of $1x10^7$ CFU/ml. During the study period, the products were stored in a refrigerator at 4±1° C.

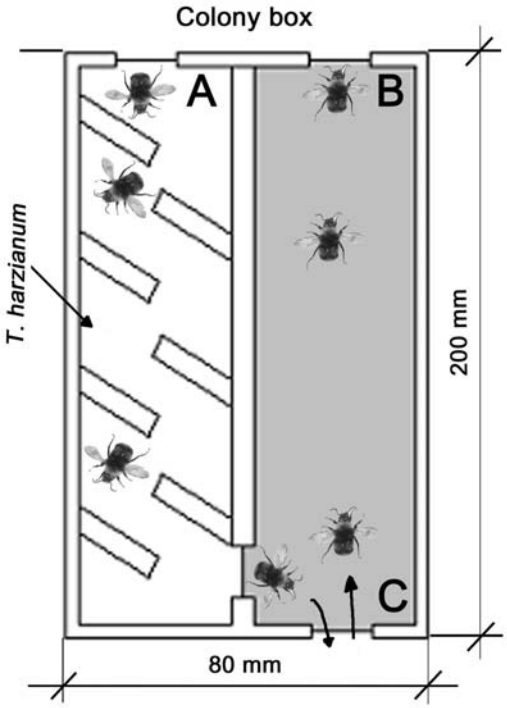

Figure 1. 1999: SSP-dispenser (side-by-side passageways). A: colony box exit; B: colony box entrance; C: opening used by both outgoing and incoming bees.

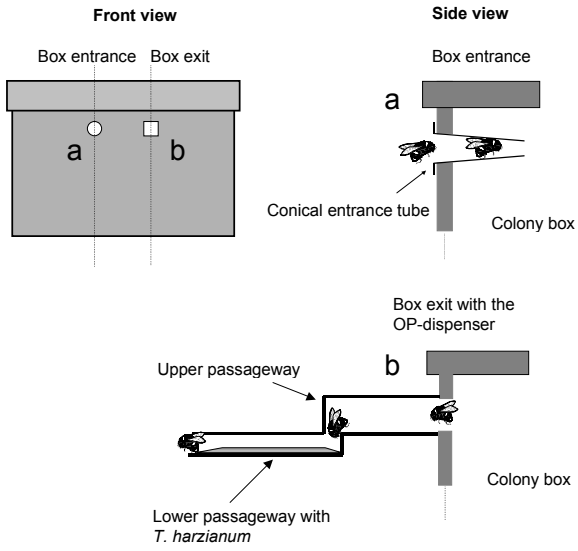

Figure 2. 2000: colony box with OP-dispenser (overlapping passageways). a: colony box entrance; b: colony box exit with OP-dispenser.

The experimental scheme

Both in 1999 and 2000, the studies were carried out in two tomato (cultivar Arletta) greenhouses, located near Bologna (Northern Italy). The greenhouses were 800 m² in size, and tomato plants were arranged in 4 rows. Both greenhouses had plastic covering on top, and net covering (mesh size 5x5 mm) on the sides. In each greenhouse, 20 sample plants (4 plants per replicate, 5 replicates per treatment) were selected in a randomised block design. In one greenhouse, the sample plants were treated with the spraying suspension, while in the other greenhouse the microbiological preparation was delivered onto flowers by bumblebees. In both 1999 and 2000, the *B. terrestris* test colony, purchased at BIO-PLANET (Cesena, Italy), was placed in the greenhouse at 5-10% bloom of the first truss (on May 5, 1999, and April 26, 2000). In both years, during bloom, every day, 1.5 g of the microbiological powder preparation were placed inside the dispensers in the late afternoon hours; the spraying suspension was applied twice a week in the evening hours with an sprayer knap sack.

Measured parameters

Dispenser efficacy

In order to assess for the efficacy of the dispensers in contaminating the bees, at mid-bloom 16 bumblebees (8 while exiting the dispenser and 8 while flying) were captured, killed with ethyl acetate, and then analysed for the presence of inoculum on their bodies. The antagonistic fungi were re-isolated on a selective medium. Mean inoculum density on bumblebees (CFU/bee), and the percentage of contaminated bees were determined.

Efficacy of bumblebees in inoculum dissemination

To compare the efficacy of the bumblebees in delivering the biological agent on the flowers with that of the spray treatments, flowers were collected randomly from the sample plants (8 flowers per plant in 1999 and 2 flowers in 2000) during full bloom, and then analysed for the presence of inoculum. In both years, for both treatments (bee-delivered and spray treatment), the mean inoculum density on flowers (CFU/flower), and the percentage of contaminated flowers were established. In each year, the inoculum density and the percentage of contaminated flowers were compared between treatments using non-parametric analyses of variance (Kruskal-Wallis test).

Furthermore, in 2000, in the greenhouse treated with bumblebees, we determined whether a correlation existed between the inoculum density on the flowers of the sample plants, and the distance of the plants from the bumblebee hive (Spearman Test). All analyses were performed with STATISTICA® 6.0.

Results

Dispenser efficacy

The SSP-dispenser was not effective in contaminating the bumblebees with the microbiological preparation: only 12.5% of the captured bees were contaminated. The OP-dispenser, instead, was highly effective: 100.0% of the analysed bees were contaminated with the antagonist. The mean inoculum density recorded on the bees, which had crawled through the two dispensers, confirm the contamination percentages: bees exiting the OP-dispenser carried a mean inoculum load of 43,194.3±42,660.9 CFU/bee, which is remarkably higher than that on bees exiting the SSP-dispenser, i. e. 135.4±400.0 CFU/bee (table 1).

Efficay of bumblebees in inoculum dissemination

The mean inoculum densities on flowers and the mean percentages of contaminated flowers recorded for the different treatment in 1999 and 2000 are reported in table 2.

With both the SSP-dispenser and the OP-dispenser, the flowers treated by bumblebees carried a significantly lower amount of CFU/flower than those treated with the suspension of propagules (Kruskal-Wallis test: $H_{(1,\ 10)} = 6.8597$, $p = 0.0088$ for the SSP-dispenser; $H_{(1,\ 10)} = 6.8182$, $p = 0.009$ for the OP-dispenser) (table 2).

Table 1. Percentage of contaminated bees and mean inoculum densities recorded for the two dispensers.

Dispenser	Contaminated bumblebees (%)	CFU/bee (mean±sd)
1999: with side-by-side passageways (SSP)	12.5	135.4±400.0
2000: with overlapping passageways (OP)	100.0	43,194.3±42,660.9

Table 2. Mean inoculum densities on flowers and mean percentages of colonised flowers (mean±sd)[a].

Year	Treatment	Percentage of colonised flowers (%)	CFU/flower
1999	Bumblebees	19.4±0.1 (a)	69.7±30.9 (a)
	Spray	77.5±0.2 (b)	1206.7±370.0 (b)
2000	Bumblebees	67.5±33.8 (*a*)	135.3±106.0 (*a*)
	Spray	100.0±0.0 (*b*)	5800.0±1394.8 (*b*)

The percentages of colonised flowers reflect the results obtained for the inoculum densities: in the greenhouse with bumblebees exiting through the SSP-dispenser, *G. virens* and *T. harzianum* were re-isolated on a selective medium in 19.4±0.1% of the flowers analysed, whereas a significantly higher percentage was recorded for the spray treatment (77.5±0.2%) (Kruskal-Wallis test: $H_{(1, 10)} = 6.9018$; $p = 0.0086$) (table 2). Also in the greenhouse treated by bumblebees exiting the OP-dispenser, the percentage of colonised flowers differed significantly from that recorded for the spray treatment: *T. harzianum* was detected on 67.5±33.8% of the flowers treated by bumblebees, and on 100.0±0.0% of the flowers treated with the spraying suspension (Kruskal-Wallis test: $H_{(1, 10)} = 5.5385$; $p = 0.0186$) (table 2).

In 2000, in the greenhouse with the OP-dispenser, a significant negative correlation emerged between the mean CFU/flower/plant and the distance of the sample plant from the bumblebee hive (Spearman test: $R = -0.5115$, $p = 0.0007$).

Discussion

The SSP-dispenser showed several functional limits, as evidenced by the absence of antagonistic propagules on most of the captured and analysed bees (table 1). Inoculum density on bumblebees was also rather low in comparison to other data reported in literature (Maccagnani *et al*., 1999; Yu and Sutton, 1997; Kovach *et al*., 2000). According to these authors the insects (bumblebees and honeybees) carried approximately $10^4 – 10^5$ CFU/bee. The SSP-dispenser was also not efficient in separating outgoing and incoming bees: many bees exited the colony box through the darkened straight passageway thus eluding the powder preparation, whereas others crawled through the zigzag passageway, but walked along the side walls. Furthermore the bees promptly smeared the zigzag passageway with their liquid excrements which, once kneaded with the antagonistic powder preparation, caused the latter to lose its consistency making it no longer suitable for bee contamination.

The OP-dispenser, instead, intercepted all and only outgoing bees, and was never used by incoming bees to enter the hive, thus minimising bee colony contamination with the antagonist and reducing inoculum waste. This was also due to the efficacy of the conical tube inserted into the entrance hole in preventing bumblebees from exiting the hive through this hole. Moreover, since the bees defecated only in the upper passageway, the biocontrol agent maintained its consistency, adequate for bee contamination. The reduced height of the lower passageway (10 mm), which hindered bees from crawling along the side walls, provided for an effective and consistent contamination of the bumblebees with *T. harzianum* propagules. In fact, the mean inoculum density carried by the bumblebees (table 1) was comparable to that reported by the Authors cited above.

For both the SSP-dispenser and the OP-dispenser, the inoculum density on flowers and the percentage of colonised flowers were significantly lower than in the spary treatments (table 2). Nevertheless, according to Kovach *et al*. (2000), increased inoculum densities do not necessarily provide a higher level of disease control. Pronubial insects are actually more efficient in delivering the propagules of the antagonist directly onto those flower parts that require protection from *B. cinerea*, whereas the spray treatment also covers other flower parts, which are not relevant for obstructing the access of grey mould, but increase the incidence of detection in the bioassay. Even though in trials carried out on strawberries from 1994 to 1997 there was a higher incidence and density of *T. harzianum* on sprayed than on bee-treated flowers, the bee-delivered treatment was more effective in disease control (Kovach *et al*., 2000).

The main disadvantage of treatments with spraying suspensions is the waste of time and material, since flowers are also treated when they are still closed (Jarvis, 1962; Sutton, 1990; Sutton and Peng, 1993; Vanneste, 1996; Yu and Sutton, 1997). Bumblebees visit flowers every day, again and again, and only when they are open.

Concerning the distribution of *T. harzianum* on the crop, the decline in inoculum density on flowers of sample plants located at increasing hive distances (figure 3) demonstrates the importance of placing the bumblebee hive in the centre of the greenhouse. As bumblebees might loose part of their load during flight, the visits to flowers that are farther away from the colony, determine a decrease in the quantity of inoculum that can be deposited on the flowers. Further studies are warranted to establish the number of flowers a bumblebee may visit subsequently while still transferring sufficient propagules of the antagonist for grey mould control. The methods and practices usually applied in order to obtain good pollination service from any pronubial species are undoubtedly appropriate for optimising its use as a biocontrol agent carrier (Vanneste, 1996; Maccagnani *et al*. 2005).

This innovative strategy of biological grey mould control is not compatible with the use of chemical pesticides, which are toxic to pollinating insects, during bloom. Thus, it does not only meet the needs and demands of the public, consumers and the environment,

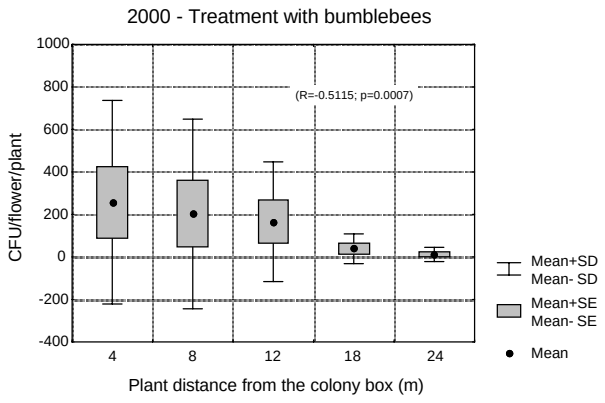

Figure 3. Mean inoculum density (CFU/flower) recorded on the sample plants at different hive distances.

but it could also be a viable option especially for growers applying integrated and biological pest management farming methods (Maccagnani *et al*., 2003a, b). Furthermore, chemical pesticides do often not provide effective grey-mould control because of the increasing occurance of benzimidazole- and dicarboximide-resistant strains of *B. cinerea* (Pepin and MacPherson, 1982; Northover and Matteoni, 1986; Staub, 1991; Faretra and Gullino, 2000).

Although this biological grey mould control technique needs to be improved, the OP-dispenser, easy to build and not expensive, was effective in contaminating the bumblebees. The technique could also be used to transport other powdery materials to flowers of different crops: biocontrol agents, pollen and even chemical products, provided that they do not compromise bee activity or harm the colony (Yu and Sutton, 1997).

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