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Honey bee dispersal of the biocontrol agent *Trichoderma harzianum* T39: effectiveness in suppressing *Botrytis cinerea* on strawberry under field conditions

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Abstract

Botrytis cinerea, which causes grey mould, is a major pathogen of many crops. On strawberry, isolates of *Trichoderma* spp. can effectively control *B. cinerea*, but frequent application is necessary. Bees can be used to disseminate biological control agents to the target crop. We tested the ability of honey bees to disseminate *Trichoderma harzianum* T39 to control *B. cinerea* in strawberry in the field during the winter in Israel over two consecutive seasons. We used the recently developed 'Triwaks' dispenser for loading the bees with the *T. harzianum* inoculum. During both years, grey mould developed in late January in untreated control plots; at low to medium disease levels it was partially controlled by fungicide treatment, and was best controlled in bee-visited plots. At high disease levels neither chemical nor biological control was effective. To assess the spatial distribution of inoculum by bees, we sampled flowers up to 200 m from the hives and found effective levels of *T. harzianum* even at 200 m. The approach used in this study provides an effective control of grey mould in strawberry in conditions of low to medium grey mould incidence.

Introduction

The ubiquitous pathogen *Botrytis cinerea* infects leaves, stems, flowers and fruits, causing grey mould, and is responsible for severe losses in many fruit, vegetable and ornamental crops (Elad et al., 2004). Chemical control remains the most commonly employed method to control the disease. However, chemical control has undesirable environmental side effects, and may negatively affect pollination, seed set, and fruit formation (Yi et al., 2003). In strawberry, for example, fungicides can decrease pollen germination and achene or seed set, thus reducing yield (Eaton and Chen, 1969a, b; Kovach et al., 2000). Furthermore, fungal pathogen populations may develop resistance, rendering chemical control ineffective (Hunter et al., 1987; Elad et al., 1992; Dianez et al., 2002). There is also a growing consumer demand for produce that is free of chemical residues. This issue is especially problematic in strawberry, since fruits are collected every few days, leaving a short period for chemical compounds to decompose (Stensvand and Christiansen, 2000). There is, therefore, great interest in developing effective alternative means for control of grey mould, such as biological control methods.

Isolates of *Trichoderma* spp. are known for their ability to control plant pathogens (Elad and

Freeman, 2002). The first biocontrol agent to be commercialized, registered and used in greenhouse crops and vineyards was isolate T39 of *T. harzianum* (TRICHODEX), which effectively controlled diseases caused by *B. cinerea*, *Sclerotinia sclerotiorum* and *Cladosporium fulvum* in greenhouse-grown tomato and cucumber and in vineyards (Elad, 2000). On strawberry, *Trichoderma* isolates, and *T. harzianum* T39 in particular, have effectively controlled *B. cinerea* under laboratory and greenhouse conditions (Trosmo and Dennis, 1977; Freeman et al., 2004). However, frequent application is necessary. More frequent applications of the T39 isolate, every 2 days, resulted in better control than less frequent applications of every 7 or 10 days (Freeman et al., 2004). However, frequent application of a biocontrol agent, especially in the field, is costly and labour-intensive, and frequent entry with sprayers may cause mechanical damage to the foliage and fruit. An efficient and inexpensive solution for continuous dissemination of biocontrol agents to strawberry in greenhouses and in the field is needed.

Honey bees and bumblebees have been used to transfer inoculum of fungi, bacteria and viruses from the hive to flowers (Kevan et al., 2003). Recently, the potential use of a solitary bee, *Osmia cornuta*, has also been investigated (Maccagnani et al., 2006). The technique is especially useful in the large variety of crops that are pollinated by bees (Delaplane and Mayer, 2000). It has been applied to control fire blight (*Erwinia amylovora*) in apple and pear (Thomson et al., 1992; Johnson et al., 1993; Vanneste, 1996; Cornish et al., 1998) and grey mould in strawberry and raspberry (Peng et al., 1992; Sutton, 1995; Yu and Sutton, 1997; Maccagnani et al., 1999; Kovach et al., 2000). These two pathosystems are similar in that the flower serves as an infection site (Thompson, 1986; Bristow et al., 1986). The technique was also evaluated for the dissemination of an insect-pathogenic fungus to control pollen beetles (*Meligethes aeneus*) in oilseed rape (Butt et al., 1998), *Trichoderma* to control head rot (caused by *S. sclerotiorum*) on sunflowers (Escande et al., 2002), the bacterium *Bacillus thuringiensis* to control the moth *Cochylis hospes* on sunflower (Jyoti and Brewer, 1999), and viruses to control *Heliothis* in clover (Gross et al., 1994).

The effectiveness of using bees as a biocontrol agent depends on several factors. Honey bees can disseminate *Trichoderma* inoculum to strawberry and effectively control grey mould (Maccagnani et al., 1999; Kovach et al., 2000). The success of the technique, however, depends on the type of inoculum dispenser (Bilu et al., 2004; Maccagnani et al., 2005), the *Trichoderma* strains, the carrier, and the attractiveness of the strawberry cultivar to the bees (Kovach et al., 2000). In the present study, we tested the commercial use of honey bees as vectors of isolate T-39 of *T. harzianum* to control grey mould in strawberry in the field in Israel, during the winter. We used the 'Triwaks' dispenser type, which we have recently developed and found effective in dispensing biological agents onto honey bee foragers (Bilu et al., 2004). The effectiveness of bee-carried *T. harzianum* was compared with chemical fungicide treatment. In addition, the *T. harzianum* population density on flowers visited by bees was tested in relation to the distance from the hive.

Materials and methods

Inoculum and dispensers

A commercial preparation of *T. harzianum* (T39) (Trichodex, 22P, Makhteshim Ltd., Beer Sheva, Israel) was used. The carrier substance is silica, and the *T. harzianum* concentration in the formulated product is 10^9 colony-forming units (CFU) g^{-1}. The 'Triwaks' dispenser consists of a $25 \times 25 \times 5$-cm wooden box with a 15-cm extended base that fits into the opening of a standard Langstroth hive. It is a two-way dispenser designed to separate outgoing bees from incoming bees for optimal dispensing of the biological agent. When loaded in the morning with 8 g of Trichodex, the 'Triwaks' dispenser maintained a stable level of dispensing throughout the day, dusting outgoing bees with an average of 6×10^4 CFU per bee, and releasing about 6×10^5 CFU per minute from the colony (Bilu et al., 2004).

Strawberry plots

Experiments were carried out in a commercial 6.5 ha strawberry (*Fragaria annanasa* cv. Tamar) field in the central coastal plain of Israel (moshav Zofit). Strawberries were planted in the beginning of October in 1.2 m wide beds, 13 plants per meter.

Beds were covered from 16:00 h to 08:00 h with 0.5 m high polyethylene tunnels according to commercial practice. The polyethylene cover was folded alongside the beds during the day, except on rainy days (35 of 97 days in the 2002–2003 season, and 32 of 81 days in the 2003–2004 season), to reduce the occurrence of humidity-promoted fruit-rot diseases and to allow insect and wind pollination.

We used a randomized complete block design, with four rows (blocks), each containing four treatments. Each treatment plot was 6 m long, but we only recorded data in the middle 5 m, leaving 0.5 m on either side as separation between treatment plots. The four treatments were: (1) control, (2) fungicide, (3) bee-transmitted *T. harzianum*, and (4) fungicide and bee-transmitted *T. harzianum*. The control and fungicide plots were kept covered continuously with a white monofilament 10% shade net, with mesh size of 3×11 mm (Polysack, Nir Yitzkhak, Israel), which allowed adequate ventilation but did not allow bees to visit the plants and to dispense *T. harzianum* inoculum. Data loggers (Hobo, Onset Computer Corporation, Bourne, MA, USA) placed in the net-covered and non-covered plots were used to monitor relative humidity and temperature at canopy height. No significant difference in microclimate conditions was found between the net-covered and non-covered plots (Figure 1).

The fungicide and the combined fungicide and biocontrol treatments were sprayed according to commercial practice, approximately every 2 weeks, alternating 0.25% Mythos (30SC pyrimethanil, AgrEvo, Germany), 0.1% Rovral (50WP iprodione, Bayer Crop Science, Germany) and 0.1% Switch (WG, 2.5% fludioxonil and 37.5% cyprodinil, Syngenta AG, Switzerland). Spraying was done during the mornings of days with no rain with a hand-held sprayer until run-off (500–800 l ha^{-1}). The control and biocontrol only treatments were covered with polyethylene during spraying in order to prevent fungicide drift from reaching them.

Experimental set-up

The same protocol was followed over two seasons. In early December, ten standard Langstroth honey bee hives with ten frames each were placed at the edge of a commercial strawberry field, 25–50 m away from the experimental plots. The hives were of similar strength, with the adult bee population housing 9–10 frames with 2–3 combs of brood. After a few days, the hives were fitted with the dispensers, and the bees were allowed to adjust to them for a few more days. In the first season we began administering the inoculum on 22 December, 2002, and in the following season on 14 December, 2003. On mornings when the weather was favourable for bee activity, we cleaned the dispensers from any residual inoculum, and loaded each dispenser with 8 g of inoculum. We did not load the dispensers on rainy days, when bees were not active and the strawberry tunnels remained covered. We collected grey mould symptomatic fruitlets and fruits throughout the field plots weekly and counted and discarded them.

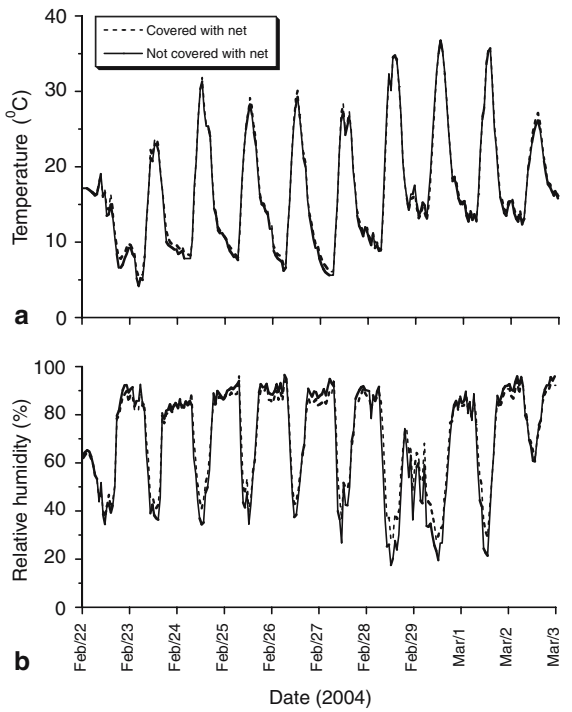


Figure 1. (a) Mean air temperature and (b) mean relative humidity during ten consecutive days in 2004 in plots covered with a white monofilament 10% shade net ($n = 3$; dashed line) and in plots not covered with a net ($n = 2$; solid line). Temperatures increased during the day and were lower at night, and relative humidity decreased during the day and was higher at night. Temperature and relative humidity were similar in the covered and non-covered plots.

Dispersal distance

During the second season, we assessed the number of viable *T. harzianum* propagules that reached strawberry flowers in the field. We collected flowers at four distances from the bee hives, 25, 50, 100, and 200 m. At each distance we had four replicate sampling plots. The plots were along four transects that emanated from the hives. At each plot we collected three samples of 10 flowers each from a subplot that was not covered and that had been exposed to bee visits, and three samples of 10 flowers each from a subplot that had been covered with a net (except at 200 m, where we did not have nets). We collected 3 day-old flowers on a day when the weather was favourable for bee activity, following two consecutive days of good weather. The number of viable *T. harzianum* propagules in each sample of 10 flowers was evaluated by the dilution method. Flowers of each sample were immersed in 20 ml sterile H_2O containing 0.01% Tween 80 and shaken for 30 min on an orbital shaker at 150 min^{-1}. Ten-fold serial dilutions were made and 0.1 ml aliquots were spread on each of four agar media plates containing half strength potato dextrose agar supplemented with 50 ppm rose bengale. Colonies that developed were counted within 4 days (Bilu et al., 2004). We averaged the counts from the four plates of each sample.

Statistical analysis

We analyzed each year separately by MANOVA with repeated-measures (sampling date) for each plot using JMP 6 (SAS Institute). The reported probability values are based on univariate adjusted Geisser-Greenhouse degrees of freedom. Further analyses were suggested by visual observation of the data in the control plots during both seasons, which showed three levels of disease incidence: low (below a mean of 15 symptomatic fruits per plot per sampling date), medium (between 15 and 65 symptomatic fruits), and high (>65 symptomatic fruits). Since we removed the symptomatic fruit during each sampling date, the total number of symptomatic fruit sampled during periods of low, medium, and high disease incidence represented a cumulative assessment of the severity of disease during each period. We divided these totals by the number of days in each period for a measure of the mean number of symptomatic fruits per plot per day. We used ANOVA and Tukey's test to compare between treatments during each period separately, with row number included as a random variable. Conducting separate analyses provides a better understanding of the disease dynamics at a price of reducing the power of each analysis; due to autocorrelation throughout the season, the separate analyses should not be interpreted as independently confirming one another.

We used two-way ANOVA to test the effect of distance and transect on the amount of inoculum that reached flowers, conducting separate analyses for netted and un-netted plots. We used a sign-test to test whether netted and un-netted plots differed in amount of inoculum that reached flowers.

Results

During both seasons, the number of symptomatic fruits was low initially and only started to escalate towards the end of January (Figures 2 and 3). Disease incidence in the control treatment first reached a mean of >15 symptomatic fruits per plot only after 24 January in both years. In 2002–2003, the number of symptomatic fruits increased over the season (Time: $F = 191$, $df = 1.7, 14.9$, $P < 0.0001$), and differed between the treatments (Treatment: $F = 5.15$, $df = 3, 9$, $P = 0.024$), with the treatment effect being consistent over the season (Treatment × Time: $F = 2.28$, $df = 5, 14.9$, NS). In 2003–2004, the number of symptomatic fruits increased over the season (Time: $F = 26.7$, $df = 1.9, 17.4$, $P < 0.0001$), and differed between the treatments (Treatment: $F = 77.2$, $df = 3, 9$, $P < 0.0001$), with the treatment effect changing over the season (Treatment × Time: $F = 9.4$, $df = 5.8, 17.4$, $P < 0.0001$).

In 2002–2003, during the low disease incidence period, there were about two symptomatic fruits per plot per day, and there was no difference between treatments (Figure 2, ANOVA, $F_{3,9} = 0.32$, NS). In 2003–2004, during most of the low disease incidence period disease incidence was almost nil, except for the end of the period, in which levels began to rise in the control treatment. There were small but statistically significant differences between treatments (ANOVA, $F_{3,9} = 6.69$, $P = 0.011$), with the two treatments

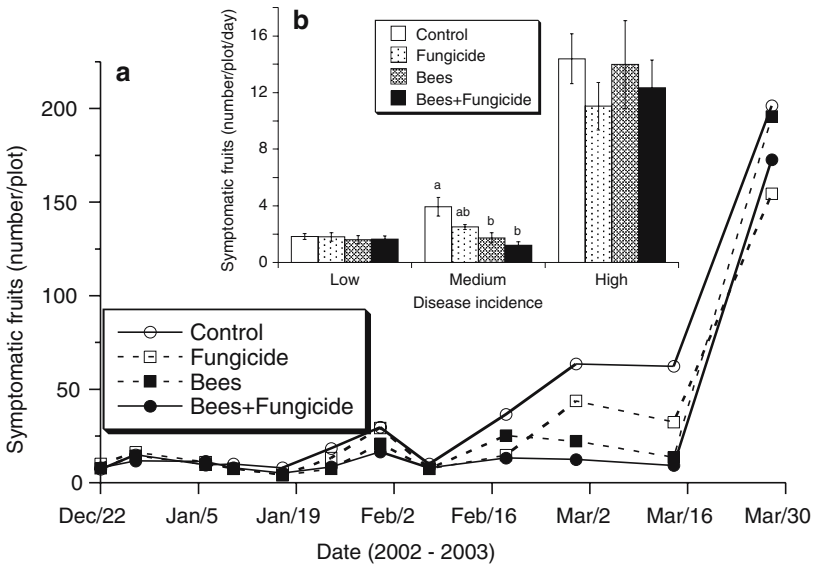
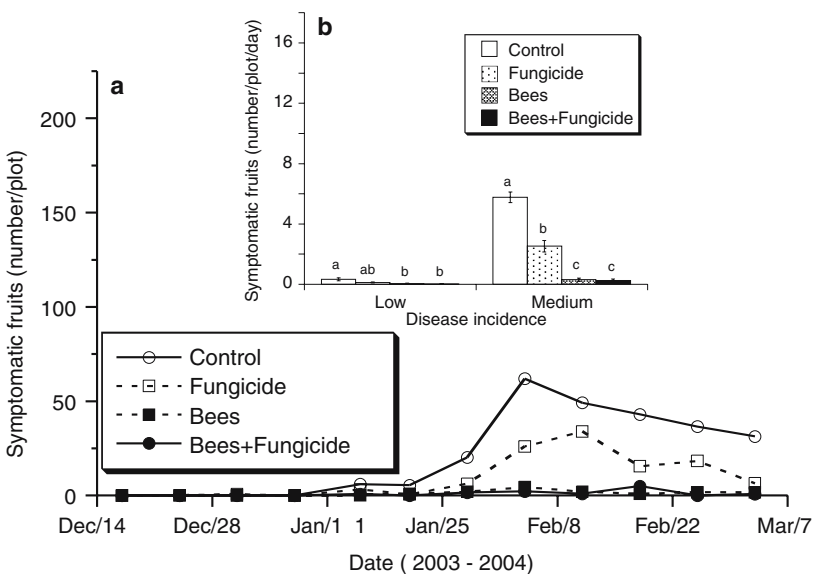


Figure 2. (a) Mean number of strawberry fruits infected by *Botrytis cinerea* per plot during the 2002–2003 season. The four treatments were untreated control, fungicide standard, *Trichoderma harzianum* T39 vectored by honey bees, and a combination of fungicides and *T. harzianum*. (b) Mean (±SE) number of infected fruits per plot per day during periods of low (<15 symptomatic fruits per plot per sampling date), medium (15–65 symptomatic fruits), and high (>65 symptomatic fruits) disease levels ($n = 4$). Treatments marked by the same letters are not significantly different from each other ($P < 0.05$).

with bee-carried *T. harzianum* having lower disease incidence than the control (Figure 3).

The disease levels during the medium disease incidence period were similar in both seasons, with a mean of 4–6 symptomatic fruits per day in the control treatment. In 2002–2003, there were significant differences between treatments (ANOVA, $F_{3,9} = 11.3$, $P = 0.0021$), with the two treatments



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with bees having lower disease incidence than the control, and the fungicide treatment showing an intermediate level (Figure 2). Similarly, in 2003–2004 there were significant differences between treatments (ANOVA, $F_{3,9} = 81.4$, $P < 0.0001$), with the two treatments with bee-carried *T. harzianum* having the lowest levels, and the fungicide treatment showing an intermediate level of disease (Figure 3).

Average monthly rainfall totals in our study area over the last 5 years from December to March were 103, 153, 110, and 47 mm, respectively. In 2002–2003, rains continued unusually late in the winter, with monthly rainfall totals from December to March of 132, 91, 201, and 127 mm, respectively. Disease levels increased greatly at the end of the season, and during this high disease period the treatments were no longer effective in controlling *B. cinerea* (ANOVA, $F_{3,9} = 1.0$, NS). In 2003–2004, the wet period ended earlier, with monthly rainfall totals from December to March of 105, 212, 70, and 20 mm, respectively. Disease levels decreased by the end of the season, and we did not sample in late March.

Dispersal distance

The variation between the three samples within each subplot was much smaller than the variation between subplots, so we averaged them for a single estimate of the number of *T. harzianum* colonies per flower per subplot. Flowers that were not netted and could be visited by bees had an average ($\pm$SE) of 2.2×10^4 ($\pm 4.8 \times 10^3$) CFU per flower, an order of magnitude more than netted flowers, 1.6×10^3 ($\pm 7.0 \times 10^2$). The variance for the exposed flowers was clearly larger than the variance for the netted flowers; this could make any ANOVA comparison suspect. Therefore, we calculated the difference between the bee-visited and netted treatments for each of the 12 plots that contained both bee-visited and netted flowers. Every single one of these differences was positive. Therefore, without making any distributional assumptions and without having to consider any of the design complexities, one can easily reject a null hypothesis of no difference ($P = 0.00049$).

Due to the higher variance in the bee-carried *T. harzianum* treatment, we performed separate analyses to study the spatial effects. In the netted treatment, there were no significant effects of distance ($F_{1,7} = 0.0$, NS) and transect ($F_{3,7} = 0.2$, NS). In the bee-carried *T. harzianum* treatment, the effect of distance was not significant ($F_{1,11} = 1.3$, NS), but the effect of transect revealed an interesting trend ($F_{3,11} = 2.7$, $P = 0.095$). Visual inspection suggested that *T. harzianum* populations were greater in the NE transects (Figure 4); this was confirmed by a *t*-test comparing the two SW and the two NE transects ($t_{1,14} = 2.96$, $P = 0.010$).

Discussion

Honey bees were effective in dispensing *T. harzianum* to control grey mould in strawberry in a commercial field. We used the 'Triwaks' dispenser type, which we have recently shown can dispense high levels of *T. harzianum* inoculum consistently over the day (Bilu et al., 2004). The mean *T. harzianum* CFU carried on the body of a bee leaving the hive ranged between 1.5×10^5 and 3.9×10^4, from 1 to 10 h after loading the dispenser, respectively (Bilu et al., 2004). The amount that a bee deposits on a flower per visit is not known, but must be considerably less than that found on the bee's body upon leaving the hive. Inoculum may be lost as the bee flies towards the flowers, and the amount delivered per flower is diluted by the many flowers that a bee visits per trip. Free (1968) reported that nectar and pollen collectors visited a mean of 23.5 and 37 flowers per trip, respectively. We found that flowers exposed to bee visitations, had an average of 2.2×10^4 *T. harzianum* CFU per flower. This amount must have accumulated over many bee visits to the same flower, possibly over several days that the strawberry flower is open. The flower handling behaviour of honey bees, in particular, leads to efficient deposition of pollen (and presumably inoculum) from the bee's body onto the strawberry flower (Free, 1968; Chagnon et al., 1993).

Strawberry flowers are typically visited many times. In some cultivars, a single flower rarely receives more than six bee visits, which suffice for optimal pollination (Chagnon et al., 1989), while in other cases flowers receive more visits, with up to 11 (Kakutani et al., 1993) or 25 (Skrebtsova, 1957) visits leading to optimal pollination and high-grade fruit. The foraging behaviour of bees is affected by several variables, including the relative

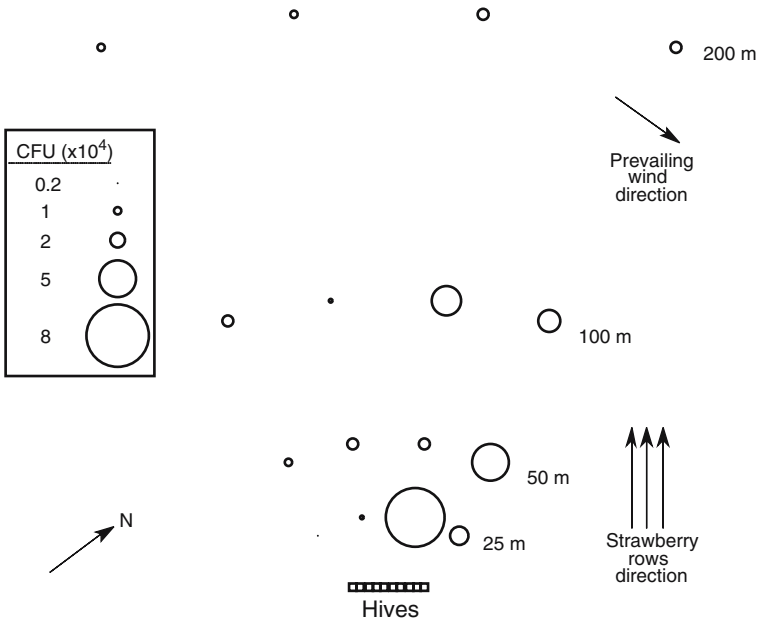

Figure 4. Map of the strawberry field in the 2003–2004 season showing mean *Trichoderma harzianum* population density per flower in bee-visited plots, at each of four plots at each of four distances, from bee hives equipped with Triwaks dispensers. Ten hives were aligned perpendicularly to the direction of strawberry rows. Prevailing winds were from the west.

profitability of nectar rewards of the strawberry cultivar in relation to competing flora (Abrol, 1992). In our study, bee colony density was relatively high, in order to achieve saturation pollination and a high percent of large, well-formed fruits (Delaplane and Mayer, 2000).

Effective levels of *T. harzianum* are in the order of 10^4 CFU per flower (Yigal Elad, unpubl.). We found such levels even at 200 m from the hives. There was, however, a spatial pattern to the density of *T. harzianum* delivered to flowers across the field, with higher density in the north-eastern transects. This may be due to microclimatic conditions, such as wind (the prevailing winds were from the west), or minor elevation gradients affecting humidity and soil wetness, and consequently the attractiveness of flowers. For example, strawberry varieties that produce richer nectar receive more bee visits (Abrol, 1992). The orientation of the rows in an agricultural field relative to the hives may also affect dispersal patterns, with greater distances of dispersal achieved by bees flying along rows than across rows (Ferrari, 1990). This tendency, however, cannot explain the spatial pattern of *T. harzianum* in our study. We also found some *T. harzianum* on flowers that were netted, albeit at a density of an order of magnitude less than in bee-visited flowers. Some of these may be naturally occurring, but it is likely that propagules drifted through the nets from the high foraging activity of *T. harzianum*-loaded bees in the field. Such drift was also suggested to have occurred in the studies of Peng et al. (1992) and of Kovach et al. (2000). It was also shown in cucumber that *T. harzianum* established significant populations in untreated control plants when this biocontrol population was sprayed in the treatment plots (Elad et al., 1993).

We did not count the number of fruits that were produced in the experimental plots, but we obtained yield data from a nearby similar commercial strawberry field from 2002–2003. The mean number of fruits in a 5 m plot increased from 177 in January, to 405 in February, and 668 in March. Based on these yields, the percentage of symptomatic fruits was stable over the season in the control group; the percentages for January, February and March were 31%, 39%, and 39%, respectively. In the spray group the percentages were 21%, 20%, and 28%, respectively, and for the two bee treatments combined, they were 15%, 7%, and 29%, respectively. Thus, it appears that

without treatment, the number of symptomatic fruits increases later in the season, but the proportion of symptomatic fruits remains constant. Chemical and bee-carried *T. harzianum* treatments are most effective when the number of symptomatic fruits per plot is at low to medium levels. At the high disease levels towards the end of the season in 2002–2003, neither chemical nor *T. harzianum* treatments were effective. Control by any control agent is generally difficult at high disease pressure (Escande et al., 2002).

Mobility of bees and hence the distribution of disseminated inoculum may vary between confined conditions in the greenhouse and unrestricted flight in the field (Sutton, 1995). Therefore, control of grey mould by honey bee-disseminated *T. harzianum* needs to be evaluated both in the greenhouse (Maccagnani et al., 1999) and in the field (Kovach et al., 2000). In open plots in the field, as in Kovach et al. (2000) and in our study, it is difficult to incorporate a treatment of flowers visited by bees that do not carry the biocontrol inoculum. Thus, we cannot distinguish potential effects on disease of bee visits relative to dissemination of the control agent. It is possible that higher fertilization rates of flowers that are visited by bees result in reduced incidence of the disease. Snetselaar et al. (2001), for example, showed that pollination of maize ears rendered them more resistant to corn smut fungus. Any generalizations between crops and pathogens, however, must be treated with caution, because the effect of pollination on disease resistance is probably affected by the flower infection pathway of the pathogen. On the other hand, bees may also spread the pathogen, thus increasing the incidence of disease. Dedej et al. (2004), for example, showed that honey bee activity increased the incidence of mummy berry disease on blueberry, but when bees were used to disseminate a biocontrol agent, disease incidence was reduced. Thus, a possible increase in disease due to transfer of the pathogen between flowers is more than offset by a reduction in disease due to the dissemination of biocontrol agent, in addition to the advantages of better pollination.

Strawberry is grown in the field in Israel during the winter, when conditions for *B. cinerea* are favourable. Consequently, the incidence of *B. cinerea* is greater than during the summer growing season in temperate regions (Peng et al., 1992; Kovach et al., 2000). In the latter study, the incidence of disease in untreated plots ranged between 0–2.4% in dry years and 9–21% in a wet year. Our results provide further support for the effectiveness of using honey bees to disseminate *T. harzianum* to control grey mould in strawberry, also in environments were *B. cinerea* incidence is moderately high. The length of the growing season also differs between summer, about 1 month, and winter, about 3 months. Our study demonstrates that this control method is also effective during a long growing season. It seems that 10 honey bee colonies can control grey mould in a 6 ha field and effectively disseminate inoculum at least 200 m. The use of bees as disseminators of biological agents is effective in controlling grey mould in an affordable, environment- and consumer-friendly manner. It illustrates an alternative important use of honey bees, in addition to their common use as pollinators of crops.

Acknowledgements

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