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Bee-Vectored *Aureobasidium pullulans* for Biological Control of Gray Mold in Strawberry

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ABSTRACT

Gray mold caused by *Botrytis cinerea* is a common postharvest disease in strawberries, reducing shelf life considerably. We investigated the potential of the yeast-like biocontrol fungus *Aureobasidium pullulans* (AP-SLU6) vectored by bumblebees (*Bombus terrestris*) in the Flying Doctors® system to inhibit the pathogen and increase the shelf life of harvested strawberries (cultivar Sonata). Using bumblebees as vectors of various biocontrol agents is becoming increasingly popular, but any potentially negative effects on bee performance have been understudied. Our results show that, over the 4-week period of the trial, the performance and activity of the bees were not negatively affected by *A. pullulans*. The bees successfully picked up the powder formulation; then, they carried and deposited it on the flowers. The vectoring of the biocontrol agent significantly reduced gray mold development on the harvested fruits by 45% and increased shelf life by 100% in comparison with control treatments. This suggests that the biocontrol fungus applied during flowering successfully reduced *Botrytis* infection and thus, effectively protected the fruits from gray mold. In addition, the bee-vectored application of the biocontrol agent was found to be significantly more effective than spray application because the latter may temporarily increase humidity around the flower, thereby creating a suitable environment for the pathogen to thrive. In summary, our study demonstrates that *A. pullulans* vectored by bumblebees can decrease gray mold infection and improve the shelf life of strawberries without adversely affecting the bees, thus providing a basis for the sustainable and efficient control of gray mold on strawberry.

Keywords: *Aureobasidium pullulans*, biological control, *Bombus terrestris*, *Botrytis cinerea*, entomovectoring, *Fragaria* × *ananassa*, garden strawberry

Gray mold caused by the fungus *Botrytis cinerea* Pers. is one of the most devastating diseases of many crops, including strawberry (*Fragaria* × *ananassa* Duch; Petrasch et al. 2019). Infection starts in the flowers, remaining dormant before developing profusely when the fruits mature. Ultimately, the pathogen causes fruit deterioration accompanied by abundant sporulation of the pathogen (Bristow et al. 1986; Petrasch et al. 2019). In strawberry, the symptoms often appear after harvest, when they significantly reduce the shelf life of the sales boxes (or punnets) of fruit (Hokkanen et al. 2015). Infections are much more severe under humid cultivation conditions (Ries 1995).

Gray mold is typically controlled by spraying synthetic fungicides. However, environmental and political concerns worldwide call for agrochemical pesticides to be replaced by more sustainable methods (Stenberg 2017). Moreover, chemical control frequently results in the evolution of fungicide resistance in the pathogen, making the products less efficient (Diánez et al. 2002; Iqbal et al. 2019, 2021; Leroch et al. 2013; Myresiotis et al. 2007; Weber 2011). In addition, chemical control frequently has further negative side effects, such as reduced pollen viability, which can lead to suboptimal fertilization and consequent fruit deformation (Kovach et al. 2000). For these reasons, it is essential to develop new sustainable approaches to combat gray mold. Biological control is one such promising method that may reduce, and even eliminate, the need for chemical control (Stenberg et al. 2021). However, the availability of efficient and reliable biocontrol organisms is still limited, and application techniques need to be further improved.

The yeast-like fungus *Aureobasidium pullulans* (De Bary) G. Armaud is a biocontrol agent with promising potential to replace agrochemical plant protection products (Iqbal et al. 2021). *A. pullulans* is naturally present in the phyllosphere and carposphere of many fruits and vegetables (Bozoudi and Tsaltas 2018), and is thus an obvious candidate for augmentation biological control. One product (Botector®) already includes a mixture of two *A. pullulans* strains (DSM 14940 and DSM 14941) with potential to control gray mold. However, this product is only effective when applied to flowers. Spraying is therefore costly because a large amount of the product needs to be applied to reach only a small target area of flowers. It also needs to be applied repeatedly throughout the flowering stage because flowers develop sequentially over an extended period; it is therefore difficult to find an optimal window in which to apply a spray to prevent gray mold. Another potential drawback of spray-application is that it increases the relative humidity (RH) around the flower, thereby optimizing the conditions for gray mold development. Alternative methods are thus needed to improve the application and efficacy of biological control agents.

Entomovectoring methods (i.e., using pollinating insect vectors to apply, with high precision, fungal biocontrol agents to flowers) have been used in agriculture for several years (Hokkanen et al. 2015; Velthuis and van Doorn 2006), although this technology is still being developed. Using pollinating insects as vectors for biocontrol agents has some major benefits. First, the biocontrol agent is delivered to the flowers in a targeted manner, reducing product use by 80 to 90% (Hokkanen et al. 2015; van Delm et al. 2015). It also increases the likelihood that the biocontrol agent will reach the focal point of infection (i.e., the flower) soon after flower opening,

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thus providing protection throughout the flowering period. Second, in contrast to spray application, which increases the RH, neither the bees nor the powder affect the abiotic conditions. Third, bees already serve as pollinators in horticultural production; giving them a new role as vectors of biocontrol agents further increases their value as ecosystem service providers.

Previous attempts to employ entomovectoring to combat gray mold include the application of the beneficial fungi *Clonostachys rosea* (previously *Gliocladium roseum*), and *Trichoderma harzianum* to strawberry and raspberry flowers by honeybees and bumblebees (Maccagnani et al. 1999; Mommaerts et al. 2011; Peng et al. 1992; van Delm et al. 2015; Yu and Sutton 1997). *Bombus terrestris* L. is the most common species reared commercially to pollinate strawberries (Mommaerts et al. 2011). Bumblebees are more active in cold conditions and are less affected by cloudy days than honeybees.

In this study, we explore the potential of entomovectoring the new *A. pullulans* strain AP-SLU6 using the Flying Doctors® system to control gray mold and thereby increase the shelf life of harvested strawberries. Iqbal et al. (2021) has shown that although spray application of this strain efficiently inhibited a number of pathogens, it was less effective at controlling gray mold, which thrives under the humid conditions created by spraying. In this study, we tested whether applying *A. pullulans* affects the performance and flying activity of the bees; whether the bumblebees can successfully deliver the *A. pullulans* formulation to strawberry flowers; and whether applying *A. pullulans* using bumblebees can lead to successful biocontrol of gray mold in strawberry under greenhouse conditions and extend the shelf life of harvested strawberries. The results of this study suggest that bumblebees successfully vectored the biocontrol agent to strawberry flowers and significantly reduced the severity of gray mold infection and enhanced the shelf life during postharvest storage. Additionally, bumblebee activity was not negatively affected by the biocontrol agent, demonstrating that bumblebees are compatible with entomovectoring of *A. pullulans*.

MATERIALS AND METHODS

Plant material. Strawberry plantlets (*Fragaria* × *ananassa*, cultivar Sonata) were obtained from Olssons Frö AB (Helsingborg, Sweden) and planted in plastic pots (1.5 liters) filled with fertilized potting soil (Emmaljunga, Vittsjö, Sweden). The plants were grown in a greenhouse at 22 ± 2°C for 3 weeks before being used in the experiment. Predatory mites (*Amblyseius swirskii*) were released to pots to prevent attack by arthropod pests.

Fungal strains, maintenance, and formulation preparation. *A. pullulans* strain AP-SLU6 and *B. cinerea* strain B05.10 were maintained on potato dextrose agar (PDA) medium (Oxoid; Basingstoke, Hampshire, England) at 25°C under dark conditions. Both fungal strains were revived from stock cultures preserved in 20% (wt/vol) glycerol at −80°C.

Wheat-bran based powder formulations of *A. pullulans* and a control (without *A. pullulans*) were prepared as described in the literature (Iqbal et al. 2018; Jensen et al. 2000). A serial dilution technique was used to determine the blastospore viability in the *A. pullulans* formulation. One gram of the formulation was mixed with 10 ml of sterile water together with one drop of Tween 20 in a 50-ml sterile Falcon tube, which was then vigorously vortexed, before being serially diluted to 10^{-7}, and streaked onto PDA petri dishes. The viable conidial concentration was measured based on the number of CFUs on petri dishes. The final blastospore concentration (10^8 CFUs/g) of the formulation was maintained for use in the experiment.

For comparison, we included an existing entomovectored biocontrol product, Prestop® Mix (Verdera, Expoo, Finland), as an additional treatment. Prestop® Mix contains *C. rosea* f. *catenulata* strain J1446 as the active substance. Prestop® Mix was stored at 4°C according to the manufacturer's instructions until the start of the experiment.

Bumblebees. Bumblebees (*B. terrestris*) were used as the vector to transport the fungal formulations to the strawberry flowers using the Flying Doctors® system (Biobest, Westerlo, Belgium). This system comprises a hive (containing one queen and 100 to 110 workers) with an integrated product dispenser. Bumblebees leaving the hive pass through its dispenser containing one of the tested formulations (*A. pullulans*, Prestop® Mix or control formulation) and so, become loaded with one of the formulations upon leaving the respective hive.

Inoculum preparation. *A. pullulans* and *B. cinerea* cultures were grown on PDA petri dishes for 2 weeks at 25°C under dark conditions. The blastospore (*A. pullulans*) and conidia (*B. cinerea*) were harvested by adding 5 to 7 ml of sterile water to the fungal culture, followed by scraping the surface of the agar with a spreader. The conidial concentration was determined using a model 114 hemocytometer (Hausser Scientific, Horsham, PA) under a Laborlux 12 light microscope (Leitz, Wetzlar, Germany). The working *A. pullulans* blastospore and *B. cinerea* conidial concentrations were maintained at 5×10^6 and 5×10^5 per ml, respectively.

Biomonitoring the effect of *A. pullulans* powder formulation on hive weight and flight activity. To test if *A. pullulans* affects hive weight and flight activity, an experiment was set up in a greenhouse at 22 ± 2°C where 16 Flying Doctors® hives were placed 2 m apart from each other. The 16 hives comprised eight biological replicates of two treatments: hives with dispensers loaded with *A. pullulans* powder formulation; and hives with dispensers loaded with a control comprising the identical carrier formulation, but without the biocontrol agent. Both *A. pullulans* and control formulations had the same density.

The hives (excluding sugar bottles) were weighed before the beginning of the experiments to ensure similar starting weights. Then, a spoonful (~8 g) of the allocated formulation was added to each dispenser. The dispensers were refilled with fresh formulations twice per week to maintain their viability. To test for any potential difference that the *A. pullulans* and control formulations might have on the flight activity of the bees, the numbers of bees flying in and out of the respective hives were recorded. This was done during a 15-min period in the morning before the dispensers were refilled with formulation. Both the hive weights and flight activities were recorded at the beginning of the experiment and then twice per week for a period of 4 weeks.

Biomonitoring of *A. pullulans* transportation by bumblebees. To quantify the level of loading with *A. pullulans* after bees had passed through the dispenser, an experiment was performed at 22°C under greenhouse conditions involving three biological replicates of the following three treatments: hives loaded with *A. pullulans* powder formulation; hives loaded with Prestop® Mix; and hives loaded with the control formulation. The dispensers were filled with a spoonful of *A. pullulans*, Prestop® Mix, or control formulation, according to their respective treatments. Eight bees from each treatment (i.e., four bees per hive) were captured in a sterile 50-ml Falcon tube upon departure from their hives. The Falcon tubes were stored in a freezer for 1 h to kill the bees; this freezing treatment does not affect the conidia. The bees were then stirred in 5 ml of sterile water in a Falcon tube for 30 min to detach the conidia from their bodies. Thereafter, a serial dilution technique was used to determine the spore viability from different treatments, by streaking the suspensions on PDA petri dishes and subsequently incubating them at 25°C under dark conditions. The viable conidial concentrations were measured based on the number of CFUs on petri dishes after 48 h.

Estimation of *A. pullulans* CFUs delivered to strawberry flowers. To evaluate the effectiveness of bumblebees in terms of successful delivery of *A. pullulans* to strawberry flowers, a sample of eight strawberry flowers from treatment 3 (*A. pullulans* vectored by bumblebees, see next paragraph) was analyzed. Each flower was excised using a sterilized pair of scissors, kept in a moist plastic bag, and stored at 4°C until processed. Processing involved the

flowers being cut into small pieces with a sterilized knife, and thereafter washed with sterilized water. To estimate the number of CFUs, a serial dilution technique was used, streaking the suspension on PDA petri dishes and incubating it at 25°C under dark conditions. The viable conidial concentrations were measured based on the number of CFUs on the petri dishes after 48 h.

Biocontrol of gray mold using *A. pullulans*. A gray mold assay on strawberry (cultivar Sonata) plants was carried out in two greenhouses using a complete randomized experimental design. Plastic pots were filled with 1,200 g of potting soil (Emmaljunga, Vittsjö, Sweden), planted with strawberry plants and kept at 22°C in a greenhouse. Six treatments were included in the assay: T_1, control formulation vectored by bees; T_2, *A. pullulans* powder formulation vectored by bees; T_3, Prestop® Mix vectored by bees; T_4, control (i.e., no bees or formulations); T_5, spray application of *A. pullulans* liquid formulation; and T_6, spray application of water only. Each treatment comprised 15 biological replicates.

All entomovectoring treatments were performed in Greenhouse A to avoid any cross contamination between entomovectoring treatments and the spray and control treatments. Spray and control applications as well as incubation of all experimental plants, irrespective of treatment, took place in Greenhouse B. The temperature and RH values were identical in both greenhouses. Bumblebees vectored the biocontrol agent (10^8 CFUs/g) to plants in the three entomovectoring treatments (T_1 to T_3), while T_5 and T_6 inoculations were performed with liquid *A. pullulans*, and water only, respectively, using a handheld sprayer.

Two Flying Doctors® hives were allocated per entomovectoring treatment (T_1 to T_3) and dispensers were filled with a spoonful (~8 g) of the formulation according to the designated treatment. Plants for each entomovectoring treatment (T_1 to T_3) were placed in Greenhouse A, where they were exposed to bees for 1 h during the morning. The plants were then transported to Greenhouse B for incubation. The three entomovectoring treatments in Greenhouse A were conducted consecutively, with a 1-h interval between treatments to ensure that no cross contamination between the beehives could take place. The plants in treatments T_1 to T_3 were exposed to the entomovectoring treatments three times per week (every 2 to 3 days); the experiment ran in total for 3 weeks. Inoculation of *B. cinerea* (5×10^5 per ml) was performed 48 h after the *A. pullulans* bee-vectored application.

Plants from treatments T_4 to T_6 were always kept in Greenhouse B where no pollinators were ever present. Plants in treatment 4 (T_4) acted as a control because they were exposed to neither pollinators nor sprayed inoculations. Plants exposed to treatments T_5 and T_6 were sprayed with liquid *A. pullulans* or water, respectively, using handheld sprayers. The prepared liquid formulations of *A. pullulans* (5×10^6 per ml) and *B. cinerea* (5×10^5 per ml) were only sprayed onto the flowers and fruits according to treatments (rather than spraying the whole plants) every 7 days for the 3 weeks of the experiment. Inoculation of *B. cinerea* was performed 48 h after the *A. pullulans* application. The summarized comparison between the bee-vectored *A. pullulans* method and application by spraying is given in Table 1.

Four weeks after the plants were potted, the ripe fruits were harvested and scored for gray mold infection by measuring the density of the mycelial growth as described in the literature (Adikaram et al. 2002; Iqbal et al. 2021). In short, 0 = no fungal growth, 1 = fungal growth only on the margin of the lesion, 2 = even but slight fungal growth all over, 3 = even but moderate fungal growth all over, and 4 = dense fungal growth all over. The disease severity was measured immediately after harvesting. The fruits were then placed individually in plastic sales boxes (punnets) and incubated at 4°C.

Effects of *A. pullulans* on gray mold infection and strawberry shelf life. To determine the effect of the above-mentioned biocontrol treatments (T_1 to T_6) on the shelf life of harvested strawberries, we stored the punnets of fruit at 4°C for 3 weeks. The effect of different treatments on shelf life was evaluated by scoring gray mold infection every day during the incubation period of 3 weeks, following the above-mentioned disease scale. The number of days it took for the fruits to show disease score = 1 was used as a measure of shelf life.

Statistical analyses. Data on bee performance, flight activity, and shelf life were analyzed using analysis of variance with a general linear model approach. Shapiro–Wilk tests confirmed that these data were normally distributed. Pairwise comparisons were performed using Fisher's least significant difference or the Tukey–Kramer method at 95% significance level. Data on gray mold disease scores and the numbers of CFUs attached to bees were not normally distributed; global comparisons were therefore analyzed using nonparametric Kruskal–Wallis tests. Pairwise comparisons were made using Dunn's test with Bonferroni correction for multiple comparisons. All analyses were performed using the open-source statistical software R v.3.2.5 or in Minitab 18.1 (Minitab Inc., State College, PA).

RESULTS

Effects of *A. pullulans* on bee performance and flying activity. Our tests for any effects that *A. pullulans* powder formulation might have on bee performance under greenhouse conditions revealed that all hives gained weight during the experimental period, but there were no significant differences between the *A. pullulans* and control treatments ($F_{1,14} = 2.04$; $P = 0.175$; Fig. 1A). Likewise, no significant difference ($F_{1,14} = 2.43$; $P = 0.141$) was observed between the two treatments on the bees' flying activity (Fig. 1B), suggesting that *A. pullulans* would not negatively affect the foraging activity of the bumblebees.

Vectoring efficiency. Bumblebees exiting the hives carried, on average, 3.31×10^5 and 4.38×10^5 CFUs/bee of *A. pullulans* and *C. rosea* f. *catenulata* (Prestop® Mix), respectively, when departing from the hives (Fig. 2). The difference between *A. pullulans* and Prestop® Mix was not significant ($\chi^2_2 = 17.76$; $P = 0.198$). The two biofungicide inocula were found mainly on the upper parts of the bees' bodies and legs (checked visually under a binocular microscope). As expected, no fungal colony developed from samples collected from bumblebees from the control treatment (Fig. 2), confirming that no cross contamination had occurred between the different treatments.

Establishment of vectored *A. pullulans* on flowers. All flowers sampled from plants that had been visited by bees loaded with *A. pullulans* contained considerable amounts of this biocontrol agent. The numbers of CFU varied between 1.2×10^2 and 2.1×10^3 CFU/flower (Supplementary Fig. S1).

TABLE 1. Comparison between bee-vectored *Aureobasidium pullulans* and spraying application method

Activity	Bee-vectored method	Spraying method
Aureobasidium pullulans formulation	Powder	Liquid
Concentration of *Aureobasidium pullulans*	10^8 CFU/g	5×10^6 CFU/ml
Target point	Flowers and unripe fruits	Flowers and unripe fruits
Aureobasidium pullulans application	Nine times (2- or 3-day intervals)	Three times (7-day intervals)
Botrytis cinerea formulation	Liquid	Liquid
Concentration of *Botrytis cinerea*	5×10^5 CFU/ml	5×10^5 CFU/ml
Botrytis cinerea application	Three times (7-day intervals)	Three times (7-day intervals)
Replicates per treatment	15	15

Biocontrol efficiency. The application of *A. pullulans* in any form provided very good protection against gray mold caused by *B. cinerea*. Plants treated with *A. pullulans* vectored by bumblebees reduced the disease score on freshly harvested fruits (day 0) by 69% as compared with the control treatments; however, this effect was not statistically significant for day 0 (χ^2_5 = 7.6; P = 0.18; Fig. 3).

After 7 days of incubation in punnets, *A. pullulans* and Prestop® Mix, vectored by bees, significantly (χ^2_5 = 25.8; $P \leq 0.01$) lowered the gray mold infection on fruits compared with control treatments (Fig. 3); this reduction corresponded to 73 and 50%, respectively. Moreover, *A. pullulans* was 45% more efficient than Prestop® Mix in inhibiting gray mold development (χ^2_5 = 25.8; $P \leq 0.01$; Fig. 3). Spraying *A. pullulans* directly on plants did not significantly reduce the disease severity, although it lowered the intensity of infection by ~50% (Fig. 3).

Effects of the biocontrol treatments on fruit shelf life. Punnets of strawberries developed from flowers treated with bee-vectored *A. pullulans* showed a significantly ($F_{5,218}$ = 5.68; $P \leq 0.01$) prolonged shelf life (100%) compared with the control treatment. These punnets of fruit displayed the first symptoms of gray mold infection after 10 days on average. By contrast, Prestop® Mix-treated strawberries displayed gray mold symptoms after 8 days, and strawberries from the control treatment already showed symptoms after 5 days (Fig. 4).

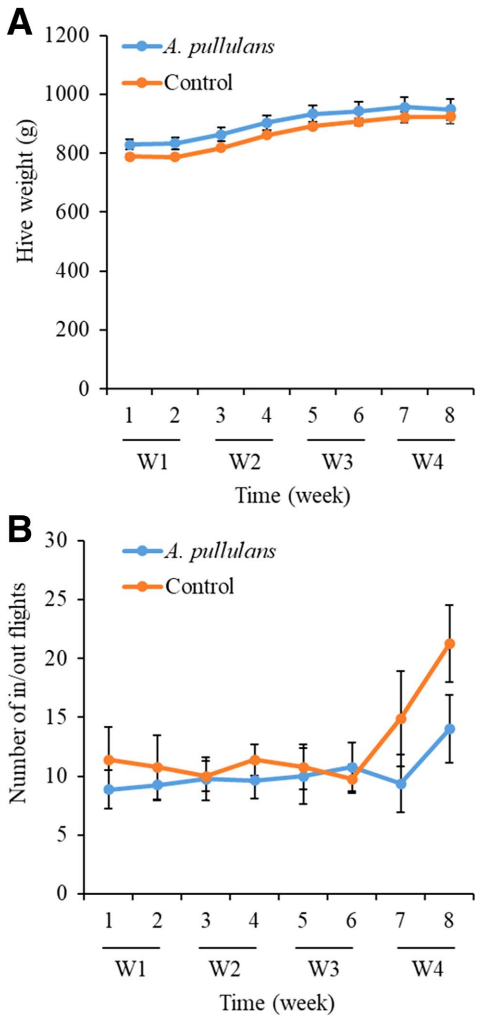

Fig. 1. Effects of *Aureobasidium pullulans* (AP-SLU6) on hive performance and bee flight activity. **A,** Effects of *A. pullulans* on hive weight. **B,** Effects of *A. pullulans* on bee flight activity. Treatments were scored twice per week for a period of 4 weeks. Error bars indicate standard errors based on eight biological replicates.

DISCUSSION

Our study was designed to investigate the potential of the new biocontrol agent, *A. pullulans* (AP-SLU6), to control gray mold in strawberry using traditional spraying and entomovectoring technology. Our results show that *A. pullulans* (AP-SLU6) is indeed an effective biocontrol agent that strongly inhibits gray mold development, regardless of the application technique used, although entomovectoring out-performs spraying. Fruit from plants with bee-vectored *A. pullulans* showed a significantly lower rate of gray mold infection during postharvest compared with other treatments. Moreover, these fruits developed gray mold symptoms at a later stage, and had double the shelf life when cold-stored in punnets.

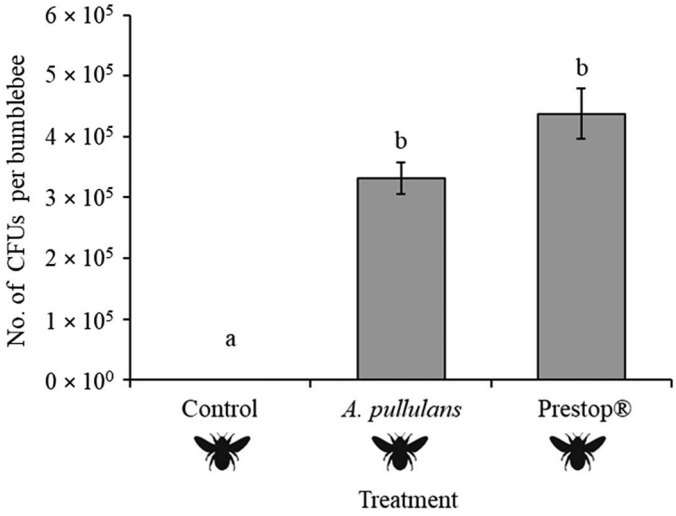

Fig. 2. Mean numbers of CFUs of *Aureobasidium pullulans* (AP-SLU6) on bumblebees sampled upon departure from their hives. The control hives were not loaded with any biocontrol agent. Error bars indicate standard errors based on eight biological replicates. Different letters indicate statistically significant differences ($P \leq 0.05$) between treatments as determined by Dunn's test with Bonferroni correction.

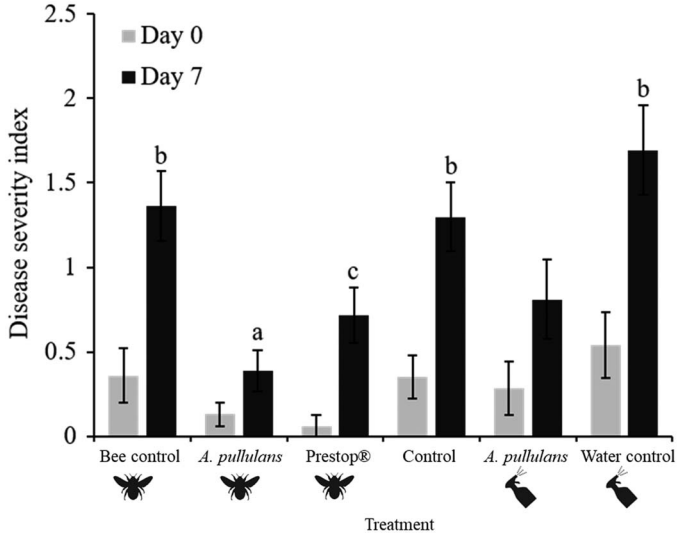

Fig. 3. Effects of *Aureobasidium pullulans* (AP-SLU6) and other treatments on gray mold development on strawberry. Harvested fruits were incubated at 4°C, and disease scores were recorded on freshly harvested fruits (day 0) and 7 days after harvest. Error bars indicate standard errors based on 15 biological replicates from separate plants. Different letters indicate statistically significant differences ($P \leq 0.05$) between treatments as determined by Dunn's test with Bonferroni correction. In brief, 0 = no fungal growth, 1 = fungal growth only on the margin of the lesion, 2 = even but slight fungal growth all over, 3 = even but moderate fungal growth all over, and 4 = dense fungal growth all over (Adikaram et al. 2002; Iqbal et al. 2021).

One challenge with entomovectoring has been to develop a formulation that sticks to the bees when they pass through the dispenser when leaving the hive, yet does not stick so strongly that it can't be unloaded when visiting a flower. Our results show that the bumblebees picked up the *A. pullulans* formulation (3.31×10^5 CFUs/bee) very well as they exited the hives. The blastospore densities on the bees in our study are similar to those reported for honeybees and bumblebees vectoring *Trichoderma* and *Clonostachys*, respectively (Maccagnani et al. 1999; Yu and Sutton 1997). The blastospore densities of *A. pullulans* recovered on the bee-treated flowers were high (3×10^3 CFUs/flower), even though some of the CFUs may have been washed away during watering of the plants, suggesting that bee-vectoring is efficient. The densities of *A. pullulans* CFUs recovered from the flowers in this study are similar to those of *Trichoderma* on bee-visited flowers as found by Kovach et al. (2000), Shafir et al. (2006), and Muthoni Macharia et al. (2020).

Another potential challenge with entomovectoring is that the microbial biocontrol agent employed to target the pathogen may have negative side effects on the bees (Karise et al. 2016). As very few previous studies have focused on bee performance, such potential side effects are almost unexplored (for studies involving *Trichoderma* and *Clonostachys*, see Brownold et al. 2005, Kovach et al. 2000, and Mommaerts et al. 2011). In our study we found that *A. pullulans* did not significantly influence bee performance or flying activity when compared with the control treatment, suggesting that *A. pullulans* is relatively (if not completely) harmless to bumblebees.

Our greenhouse trials show that the application of *A. pullulans* in any form inhibits gray mold development on strawberry fruits. This is important because different application techniques may be preferred in different cultivation systems. However, it is evident that the inhibition is more pronounced when *A. pullulans* is delivered to flowers by bumblebees.

Previous studies focusing on *Trichoderma* (Kovach et al. 2000) also found that bee-vectoring can be more efficient than spraying in reducing gray mold development on strawberry. Kovach et al. (2000) and others typically explained the advantage of entomovectoring in terms of bees being more efficient in targeting and depositing the biocontrol agent directly to the flower at a critical time. However, a complementary explanation could be that spraying raises the humidity, thereby making the abiotic conditions more favorable for gray mold development, and counteracting to some extent the effect of the biological control.

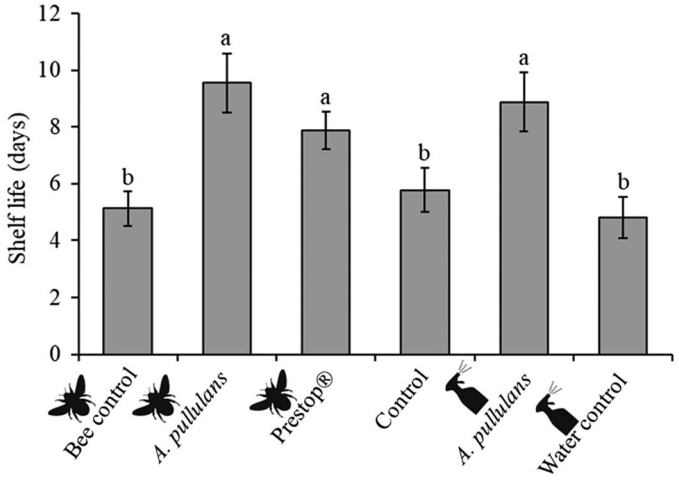

Fig. 4. Effects of *Aureobasidium pullulans* (AP-SLU6) and other treatments on the shelf life of harvested strawberry fruit incubated at 4°C in punnets. Gray mold disease severity was recorded every day for a period of 3 weeks. Error bars indicate standard errors based on 15 biological replicates. Different letters indicate statistically significant differences ($P \leq 0.05$) as determined by Tukey-Kramer tests. In brief, 0 = no fungal growth, 1 = fungal growth only on the margin of the lesion, 2 = even but slight fungal growth all over, 3 = even but moderate fungal growth all over, and 4 = dense fungal growth all over (Adikaram et al. 2002; Iqbal et al. 2021).

Bee-vectored Prestop® Mix was included in this study as an established entomovectored biocontrol product already available on the market. It thus served as a positive control to compare its efficiency in relation to *A. pullulans* (AP-SLU6). Our results show that both *A. pullulans* (AP-SLU6) and Prestop® Mix vectored by bumblebees significantly and strongly reduce gray mold, but that *A. pullulans* is ~45% more efficient, suggesting that *A. pullulans* may have an advantage over *Clonostachys* as an active substance.

Although the severity of disease (measured as disease scores) is an important and established method to evaluate microbial biocontrol agents, it is not necessarily the most useful measure for farmers or retailers. Hokkanen et al. (2008) developed a sales box or punnet test in which the number of days until the first symptoms are visible, is used to evaluate biocontrol efficiency. This measure of shelf life is indeed more useful and practical as strawberry punnets are unmarketable as soon as any gray mold symptom is visible by eye, no matter how small the symptoms may be. It is evident from our results that the shelf life of greenhouse-grown strawberry fruits is prolonged when *A. pullulans* is delivered by bumblebees rather than by spray application.

In summary, we have demonstrated that *A. pullulans* (AP-SLU6) vectored by bumblebees can decrease gray mold substantially, leading to an improved shelf life of strawberry punnets by almost 100%. Thus, *A. pullulans* AP-SLU6 seems to be a potent biocontrol agent against gray mold, especially when it is applied by bees, which it does not adversely affect, rather than by sprayer. Making new potent biocontrol agents available for further development is important because few sustainable methods currently exist to combat gray mold.

LITERATURE CITED

Adikaram, N. K., Joyce, D. C., and Terry, L. A. 2002. Biocontrol activity and induced resistance as a possible mode of action for *Aureobasidium pullulans* against grey mould of strawberry fruit. Austral. Plant Pathol. 31: 223-229.

Bozoudi, D., and Tsaltas, D. 2018. The multiple and versatile roles of *Aureobasidium pullulans* in the vitivinicultural sector. Fermentation (Basel) 4:85.

Bristow, P., McNicol, R., and Williamson, B. 1986. Infection of strawberry flowers by *Botrytis cinerea* and its relevance to grey mould development. Ann. Appl. Biol. 109:545-554.

Brownold, E., Flanders, S., and Kovach, J. 2005. The Effect of *Trichoderma harzianum* on Honey Bee Survival. Xerces Society, Portland, OR. www.xerces.org

Diánez, F., Santos, M., Blanco, R., and Tello, J. 2002. Fungicide resistance in *Botrytis cinerea* isolates from strawberry crops in Huelva (southwestern Spain). Phytoparasitica 30:529-534.

Hokkanen, H. M., Menzler-Hokkanen, I., and Lahdenpera, M.-L. 2015. Managing bees for delivering biological control agents and improved pollination in berry and fruit cultivation. Sustain. Agric. Res. 4:89-102.

Hokkanen, H., Menzler-Hokkanen, I., Mustalahti, A.-M., Koivisto, I., and Korhonen, K. 2008. Precision biological control is also effective in rainy summer [*Biologinen täsmätorjunta tehosi sadekesänäkin*]. Puutarha Kauppa 12:14-15.

Iqbal, M., Dubey, M., Broberg, A., Viketoft, M., Jensen, D. F., and Karlsson, M. 2019. Deletion of the nonribosomal peptide synthetase gene *nps1* in the fungus *Clonostachys rosea* attenuates antagonism and biocontrol of plant pathogenic *Fusarium* and nematodes. Phytopathology 109:1698-1709.

Iqbal, M., Dubey, M., McEwan, K., Menzel, U., Franko, M. A., Viketoft, M., Jensen, D. F., and Karlsson, M. 2018. Evaluation of *Clonostachys rosea* for control of plant-parasitic nematodes in soil and in roots of carrot and wheat. Phytopathology 108:52-59.

Iqbal, M., Jamshaid, M., Zahid, M. A., Andreasson, E., Vetukuri, R. A., and Stenberg, J. A. 2021. Biological control of strawberry crown rot, root rot and grey mould by the beneficial fungus *Aureobasidium pullulans*. BioControl 66:535-545.

Jensen, B., Knudsen, I. M., and Jensen, D. F. 2000. Biological seed treatment of cereals with fresh and long-term stored formulations of *Clonostachys rosea*: Biocontrol efficacy against *Fusarium culmorum*. Eur. J. Plant Pathol. 106:233-242.

Karise, R., Mulja, R., Smagghe, G., Kaart, T., Kuusik, A., Dreyersdorff, G., Williams, I. H., and Mänd, M. 2016. Sublethal effects of kaolin and the biopesticides Prestop-Mix and BotaniGard on metabolic rate, water loss and longevity in bumble bees (*Bombus terrestris*). J. Pest Sci. 89:171-178.

Kovach, J., Petzoldt, R., and Harman, G. E. 2000. Use of honey bees and bumble bees to disseminate *Trichoderma harzianum* 1295-22 to strawberries for *Botrytis* control. Biol. Control 18:235-242.

Leroch, M., Plesken, C., Weber, R. W., Kauff, F., Scalliet, G., and Hahn, M. 2013. Gray mold populations in German strawberry fields are resistant to multiple fungicides and dominated by a novel clade closely related to *Botrytis cinerea*. Appl. Environ. Microbiol. 79:159-167.

Maccagnani, B., Mocioni, M., Gullino, M., and Ladurner, E. 1999. Application of *Trichoderma harzianum* by using *Apis mellifera* as a vector for the control of grey mould of strawberry: First results. IOBC Bull. 22:161-164.

Mommaerts, V., Put, K., and Smagghe, G. 2011. *Bombus terrestris* as pollinator-and-vector to suppress *Botrytis cinerea* in greenhouse strawberry. Pest Manag. Sci. 67:1069-1075.

Muthoni Macharia, J., Wanjiku Gikungu, M., Karanja, R., and Okoth, S. 2020. Managed bees as pollinators and vectors of bio control agent against grey mold disease in strawberry plantations. Afr. J. Agric. Res. 16:1674-1680.

Myresiotis, C., Karaoglanidis, G., and Tzavella-Klonari, K. 2007. Resistance of *Botrytis cinerea* isolates from vegetable crops to anilinopyrimidine, phenylpyrrole, hydroxyanilide, benzimidazole, and dicarboximide fungicides. Plant Dis. 91:407-413.

Peng, G., Sutton, J. C., and Kevan, P. 1992. Effectiveness of honey bees for applying the biocontrol agent *Gliocladium roseum* to strawberry flowers to suppress *Botrytis cinerea*. Can. J. Plant Pathol. 14:117-129.

Petrasch, S., Knapp, S. J., van Kan, J. A., and Blanco-Ulate, B. 2019. Grey mould of strawberry, a devastating disease caused by the ubiquitous necrotrophic fungal pathogen *Botrytis cinerea*. Mol. Plant Pathol. 20:877-892.

Ries, S. M. 1995. RPD No. 704 – Gray Mold of Strawberry. Integrated Pest Management, University of Illinois Extension, University of Illinois, Champaign, IL. http://ipm.illinois.edu/diseases/series700/rpd704/

Shafir, S., Dag, A., Bilu, A., Abu-Toamy, M., and Elad, Y. 2006. Honey bee dispersal of the biocontrol agent *Trichoderma harzianum* T39: Effectiveness in suppressing *Botrytis cinerea* on strawberry under field conditions. Eur. J. Plant Pathol. 116:119-128.

Stenberg, J. A. 2017. A conceptual framework for integrated pest management. Trends Plant Sci. 22:759-769.

Stenberg, J. A., Sundh, I., Becher, P. G., Björkman, C., Dubey, M., Egan, P. A., Friberg, H., Gil, J. F., Jensen, D. F., Jonsson, M., Karlsson, M., Khalil, S., Ninkovic, V., Rehermann del Rio, G., Vetukuri, R. R., and Viketoft, M. 2021. When is it biological control? A framework of definitions, mechanisms, and classifications. J. Pest Sci. 94:665-676.

van Delm, T., Van Beneden, S., Mommaerts, V., Melis, P., Stoffels, K., Wäckers, F., and Baets, W. 2015. Control of *Botrytis cinerea* in strawberries with *Gliocladium catenulatum* vectored by bumblebees. J. Berry Res. 5:23-28.

Velthuis, H. H., and Van Doorn, A. 2006. A century of advances in bumblebee domestication and the economic and environmental aspects of its commercialization for pollination. Apidologie (Celle) 37:421-451.

Weber, R. W. 2011. Resistance of *Botrytis cinerea* to multiple fungicides in Northern German small-fruit production. Plant Dis. 95:1263-1269.

Yu, H., and Sutton, J. 1997. Effectiveness of bumblebees and honeybees for delivering inoculum of *Gliocladium roseum* to raspberry flowers to control *Botrytis cinerea*. Biol. Control 10:113-122.