Sveriges lantbruksuniversitet
Swedish University of Agricultural Sciences

Evaluation of bee-vectored *Aureobasidium pullulans* for biocontrol of grey mould in strawberry

Matilda Jutzeler

Degree Project • 30 hec
Plant Biology for Sustainable Production - Master's Programme
Alnarp 2021

Evaluation of bee-vectored *Aureobasidium pullulans* for biocontrol of grey mould in strawberry

Utvärdering av *Aureobasidium pullulans* för biologisk bekämpning av gråmögel hos jordgubbar med humlor som vektorer.

Matilda Jutzeler

Supervisor: Johan A. Stenberg, SLU, Department of Plant Protection Biology

Co-supervisor: Mudassir Iqbal, SLU, Department of Plant Protection Biology

Examiner: Ramesh R. Vetukuri, SLU, Department of Plant Breeding

Credits: 30 hec
Project level: A2E, master's thesis
Course Title: Independent Project in Biology, A2E
Course Code: EX0856
Subject: Biology

Programme: Plant Biology for Sustainable Production – Master's Programme

Place of Publication: Alnarp
Year of Publication: 2021
Cover Art: Matilda Jutzeler
Online Publication:

Keywords: ***Aureobasidium pullulans, biocontrol, Bombus terrestris, Botrytis cinerea, entomovectoring, fungal disease, strawberry***

SLU, Swedish University of Agricultural Sciences
Faculty of Landscape Architecture, Horticulture and Crop Production Science
Department of Plant Breeding

Publishing and archiving

Approved students' theses at SLU are published electronically. As a student, you have the copyright to your own work and need to approve the electronic publishing. If you check the box for **YES**, the full text (pdf file) and metadata will be visible and searchable online. If you check the box for **NO**, only the metadata and the abstract will be visible and searchable online. Nevertheless, when the document is uploaded it will still be archived as a digital file.

If you are more than one author you all need to agree on a decision. Read about SLU's publishing agreement here: https://www.slu.se/en/subweb/library/publish-and-analyse/register-and-publish/agreement-for-publishing/.

☒ YES, I/we hereby give permission to publish the present thesis in accordance with the SLU agreement regarding the transfer of the right to publish a work.

☐ NO, I/we do not give permission to publish the present work. The work will still be archived and its metadata and abstract will be visible and searchable.

Abstract

The fungal pathogen *Botrytis cinerea* causes grey mould in strawberry (*Fragaria x ananassa*) inflicting great yield loss and significant economic loss. To date, spraying with chemical fungicides is the primary management practice to control this disease. Concerns regarding resistance development of the pathogen, high treatment cost and environmental issues have led to the search for alternative methods. This study investigates if the biocontrol fungus *Aureobasidium pullulans* (isolate AP-SLU6) could be used as a biocontrol agent vectored by buff-tailed bumblebee*,* to suppress grey mould in strawberry under greenhouse conditions. To examine if *A. pullulans* affect bumblebees negatively, trials measuring the flight activity and the hive weight was performed. To test if the bees can carry and deposit the biocontrol agent to strawberry flowers, samples of flowers and bumblebees were collected and examined. Finally, the scoring of grey mould infection on fruits was performed pre-and postharvest. Bee activity and hive weight was not significantly affected by the carried fungal biocontrol agent. The results further showed that bumblebees successfully vectored the biocontrol agent to flowers and significantly reduced the severity of grey mould infected fruits during postharvest storage, thus leading to improved shelf life. This study concludes that *A. pullulans* used as a biocontrol agent vectored by bumblebees, can be an efficient method for controlling postharvest grey mould in strawberry, leading to improved plant health and reduced dependence on synthetic fungicides.

Popular scientific summary

Grey mould infection in strawberries can be reduced by letting bumblebees carry a new beneficial fungus from Alnarp to the strawberry flowers. This was found through an experiment where bumblebee-hives were equipped with a powder formulation containing the beneficial fungus and then letting the bees - loaded with the fungus - visit strawberry flowers. Later examinations of the ripened fruits showed that there were less infection and also a decelerated development of grey mould in strawberry fruits developed from flowers that had been visited by bees carrying the beneficial fungus.

The findings are important since grey mould is a devastating disease which inflicts high yield loss and economic loss for strawberry growers, and there is a need for novel strategies to combat this disease. Other methods to control grey mould include chemical fungicides, which are undesirable from both social and environmental perspectives. Therefore, these findings are of importance since they show that there could be alternative ways to combat grey mould, which could reduce the need of chemical fungicides.

Content

Introduction

Strawberry production and *Botrytis cinerea* infection

Strawberry (*Fragaria x ananassa*) is a valuable horticultural commodity in many countries. However, the strawberry production is threatened by the necrotrophic fungus *Botrytis cinerea,* which causes grey mould and consequently significant yield loss (Hokkanen et al. 2015). Completely resistant cultivars do not exist and therefore improved control management is needed (Petrasch et al. 2019).

Botrytis cinerea persists as resting bodies in the infested field and inoculum survive in the decaying crops. Spores can be spread through wind or water and the primary infection occurs at the flowering stage where the pathogen enters either through the sepals, petals or stamens. *Botrytis cinerea* thrives in humid environments and develops hyphae that grow into the receptacle (Petrasch et al. 2019). The infection leads to a ripened fruit with lesions of grey-brown spores, which cause significant yield loss (Mommaerts et al. 2011). *Botrytis cinerea* can also infect fruits during postharvest storage of fruits and this is due to a symptomless quiescent phase in unripe fruits. Fruits showing no symptoms at harvest can, therefore, develop symptoms, leading to non-marketable fruits or reduced shelf life (Petrasch et al. 2019).

Since grey mould is one of the most common diseases worldwide, which causes significant economic losses in the strawberry market, there have been several methods trying to control this disease over the years (Petrasch et al. 2019). For instance, cultural control using techniques that reduce the relative humidity and adopt strict crop hygiene to avoid grey mould infection. One of the major methods is to control the disease using various chemical fungicides. Another, more recently developed, method to control grey mould is the use of biological control agents. Biocontrol is of importance and is gaining more attention, since chemical fungicides are undesirable due to rapid evolution of resistance and from both social and environmental perspectives (Adikaram et al. 2002).

Control of *B. cinerea* by chemical fungicides

Conventional growers have throughout the years tried to control grey mould using chemical fungicides to reduce yield losses. Spraying crops with chemical fungicides is to date still the main way to control grey mould (Mommaerts et al. 2011). This protection strategy against

grey mould, have led to fungicides losing their ability to control the disease, due to evolution of resistance in the pathogen. Resistance is a major problem since once it arises, resistance is heritable and the pathogen can no longer be controlled by that fungicide, which also results in increased control cost (Brent & Hollomon 2007).

It has also been shown that some fungicides can reduce pollen germination and hence alter the fruit formation leading to a reduced yield (Khanizadeh & Buszard 1987). Another problem is that fungicide residues may remain in harvested fruit. Strawberry represents one of the soft fruit crops with highest frequency of remaining residues from fungicides (Kovacova et al. 2013).

Chemical fungicides are also harmful for the environment since harmful residues maintain in the soil and may leach down to aquatic ecosystems (Kookana et al. 2005). To get rid of fungal diseases, several spraying programs using chemical fungicides in horticultural crops have been developed. Spraying programs often require several applications during a growing season, and the technique is not cost-effective, since it is not precise of the target and a part of the product misses its target, going into the surrounding area. This also leads to a high treatment costs (Hokkanen et al. 2015)

The use of chemical fungicides to control grey mould is, however, still remain an important protection strategy for horticultural crops. However, due to more knowledge about development of resistance and concerns about the impact on environment, there is a need to develop alternative strategies to control grey mould.

Biological control in strawberry production

Biological control in strawberry production is an alternative method to control important pathogens such as *B. cinerea*. Some microbial antagonists naturally occur in the phyllosphere and the use of these as biocontrol agents is more acceptable to consumers, since they leave no harmful residues in the fruits and are considered to be more environmentally friendly than synthetic fungicides (Adikaram et al. 2002). Several different microbial antagonists have been used to suppress grey mould infection in strawberry. Successful suppression of the disease has been shown using e.g *Gliocladium catenulatum* and *Trichoderma* strains and *Aureobasidium pullulans* (Adikaram et al. 2002). These beneficial fungi act as antagonists of the pathogen, involving various mechanisms including antibiosis, competition with the

pathogen for nutrients, space competition, and induction of the host plant's defence, which in turn prevents the pathogen from infecting the plant (Lima et al. 1997).

Aureobasidium pullulans is a yeast-like fungus that is naturally present on the phyllosphere of strawberry. There have been several studies (Lima et al (1997); Bhatt (1962); Adikaram (2002)) showing that *A. pullulans* can be an effective antagonist against *B. cinerea*. This beneficial fungus have been showed to reduce the amount of grey mould infected strawberries when applied at the flowering stage (Lima et al 1997).

Trials using biological control agents against grey mould in strawberry have relied on spraying at the flowering stage (Hokkanen et al. 2015). Blanket spraying is a costly procedure since a huge amount of the product is needed and it has to be applied several times when plants are at the flowering stage. Since flowers develop at different times, it is a challenge to spray at a proper time to prevent grey mould infection. Another potential drawback of spray-application is that it increases the relative humidity, thereby improving the abiotic conditions for grey mould development. Therefore, alternative methods to improve the application of biological control agents is needed (Hokkanen et al. 2015).

Entomovector technology with pollinating insects

Pollinating insects have been used in agriculture for entomovectoring of biological control agents for several years (Velthuis & Van Doorn 2006; Hokkanen 2015). However, this technology is still under development. Pollinating insects as vectors for biocontrol agents has some major benefits, both by means of a more precise application of the biocontrol agent to flowers and at the same time the flowers are pollinated. When crops are flowering, the pollinating insects visit each flower and can thus deliver the biocontrol agent at an optimal time (Hokkanen et al. 2015). In contrast to blanket spraying, this can reduce the amount of product that needs to be applied to the crops and be a more efficient method to control grey mould in strawberry, since it is mainly during flowering that the infection by pathogens occurs. It is also considered a more environmentally friendly method as it reduce the dependence on chemical fungicides and may still be efficient (Hokkanen et al. 2015). Another benefit is that the entomovectoring technology does not increase the humidity around crop, which is beneficial since grey mould thrives in humid conditions (Petrasch et al. 2019).

Bombus terrestris is the most common and widely used commercially reared species used for pollination of strawberry (Mommaerts et al. 2010). Rearing of bumblebees started to develop for providing pollination service (Velthuis & Van Doorn 2006). Bumblebees are compared to honeybees more active in cold, and are less influenced by cloudy days. They also visit a high number of flowers during their foraging and can transfer higher pollen loads (Delaplane & Mayer 2000). A study made by Mommaerts et al. (2011) showed that *B. terrestris* could vector the biocontrol agent *G. catenulatum* that strongly reduced grey mould in strawberry, which led to higher yields.

There is still a need for development of appropriate biocontrol agents to the market that could be used in entomovectoring technology with bumblebees, since there are only a few available (e.g PrestopMix®). This study investigates if buff-tailed bumblebees could be used as vectors for *Aureobasidium pullulans* (isolate AP-SLU6) for the control of grey mould in strawberry. AP-SLU6 is of importance to evaluate as a potentially new biocontrol agent against grey mould, and it could potentially contribute to controlling several diseases, including grey mould (Jamshaid 2020). However, it is not known whether AP-SLU6 is compatible with entomovectoring technology. Also, there is a need for testing bumblebee activity and health when exposed to biocontrol agents such as *A. pullulans* which this study examines, since there is a risk that the vectors could be negatively affected by biocontrol agents (Kevan 2008). This study contributes to the further development of the entomovectoring technology and examination of a potentially new biocontrol agent.

The results of this study show that bumblebees successfully vectored the biocontrol agent *A. pullulans* to strawberry flowers and significantly reduced the severity of grey mould infected fruits during postharvest storage. Also, bumblebee activity was not significant affected by the biocontrol agent which suggests that bumblebees are appropriate for entomovectoring of *A. pullulans* (AP-SLU6).

Aim of study and research questions

The aim of this project was to examine if *A. pullulans* isolate AP-SLU6, could be used as a biocontrol agent vectored by *B. terrestris* to suppress *B. cinerea* in strawberry under greenhouse conditions. The research questions were as follows:

1) Are bee activity and hive performance negatively affected by *A. pullulans* (AP-SLU6)?

2) Can *A. pullulans* (AP-SLU6) be transferred and deposited to strawberry flowers by bumblebees?

3) Does application of *A. pullulans* (AP-SLU6) to strawberry flowers suppress *B. cinerea* under greenhouse conditions?

Materials and methods

Maintenance of experimental organisms

Bumblebees

For the purpose of this study, Biobest (Biobest group NV, Westerlo, Belgium) provided Flying Doctors® hives containing colonies of *B. terrestris* (buff-tailed bumblebee). These hives contain a queen, and about 50-70 workers. The hives also contain an integrated dispenser for biopesticides, which the bees pass through when they exit the hive. The exit holes were possible to close, such that bees only could fly in and not out of the hive again. This function can thus be used to switch off or restart bee-mediated treatments at any time. Every second day, the bees were fed with sterilized dried extra pollen grains on top of the hives (Figure 1), to make sure they got enough pollen. The temperature in the greenhouses where the hives were placed was maintained at 22 °C.

Figure 1 Left: Bumblebee flying out from hive, passing the dispenser with *Aureobasidium pullulans* based formulation or control formulation. Right: Bumble bees feeding on pollen grains on top of the hive

Plants

In total, 110 strawberry plants (cultivar Sonata) were purchased from Olssons frö AB and planted in 1.5 L pots containing fully fertilized soil (Exklusiv Blom & Plantjord, Emmaljunga) mixed with leca and silicon clay. These plants flowered after 3 weeks and were used in the experiment. To prevent arthropod pests to establish on the plants, predatory mites *Amblyseius swirskii* were released into the pots. After planting, the plants were watered regularly as needed (Figure 2).

Figure 2 *Strawberry plants planted and manually watered by showering from above to create a humid environment suitable for grey mould development.*

Bumblebee hives and strawberry plants were placed in separate greenhouses at different parts of campus Alnarp at the Swedish University of Agricultural sciences, where also the different trials were carried out. The purpose of separating the bees from the plants was to prevent bees from visiting strawberry flowers in-between the experimental treatments. In the different chambers, the temperature was set to 22 °C degrees.

Formulations with *A. pullulans*, wheat-based control and Prestop®Mix

Three different formulations were prepared for the experiments. First, a treatment with Prestop® Mix was included to compare the new strain AP-SLU6 (*A. pullulans* formulation) with an already existing biological product on the market. Spray inoculation treatments were included to compare the effect of bumblebees as vectors versus spraying with biocontrol

agent containing liquid *A. pullulans*. Spray treatments was performed once per week for 3 weeks, following instructions of Botector™ (2017) product user manual (Bio-ferm) which is a biotechnical fungicide based on two strains of *A. pullulans*. This product is used by growers for spray treatment to control grey mould, but is not available on the Swedish market. Also, a wheat bran-based formulation was prepared which worked as a control.

Prestop®Mix

Prestop®Mix (Verdera Oy, Finland) is a biological product for biocontrol of fungal pathogens, such as *B. cinerea* in strawberry and raspberry. It contains dried mycelium and spores of *Gliocladium catenulatum* strain J1446 (10^{7}-10^{9} colony forming unit (CFU)/g) which is a naturally occurring soil fungus. A standard package of this product was purchased from a supplier and stored according to the instruction until the start of the experiment.

Preparation of *A. pullulans* powder formulation

Aureobasidium pullulans strain AP-SLU6 was isolated from a woodland strawberry plant growing at SLU Campus Alnarp in southern Sweden. The wheat bran-based formulations of *A. pullulans* and control (without *A. pullulans*) were prepared following Iqbal et al. (2017) and Jensen et al. (2000). Serial dilution technique was used to determine the spore viability in the *A. pullulans* formulation. 1 g of the formulation was mixed with 10 mL of sterile water together with one drop of Tween 20 in 50 mL sterile falcon tube followed by vigorously vortexed, serially diluted to 10^{-7}, and streaked on potato dextrose agar (PDA) plates. The viable conidial concentration was measured based on the colony-forming unit (CFU) on Petri plates.

Preparation of *A. pullulans* and *B. cinerea* liquid formulations

Aureobasidium pullulans and *B. cinerea* cultures were grown on PDA Petri dishes for 2-weeks at 25 °C. The conidia were harvested by adding 5-7 ml of sterile water to the fungal culture, followed by scraping the surface of the mycelium with a spreader. The concentration of the conidia was determined using a haemocytometer (Hausser 114 Scientific, Horsham, PA) under a light microscope (Laborlux12 Leitz, Germany). The working conidial concentration 5×10^{6} and 5×10^{5} was maintained for *A. pullulans* and *B. cinerea* respectively.

Botrytis cinerea inoculation was prepared for spraying plants 48 hours after treatment with bee-transmitted and spray transmitted biocontrol agents. This was performed to determine that all plants in the different treatments were exposed to the pathogen. Two inoculations with *B. cinerea* to the plants was performed during the experiments with ten days between applications.

Experiment 1: Effects of *A. pullulans* on bee performance and formulation vitality

At the day of their arrival, 16 beehives were placed in a single greenhouse with 2 meter distance between the hives (Figure 3). The hives were checked to ensure that the sugar solution bottles opened in the hives and that the gate valves where the bumble bees fly in and out, were positioned correctly. The hives were then left in the greenhouse for 30 minutes before opening, following product instructions for Flying Doctors®. Bees were fed with one 15 ml spoon of pollen grains from Biobest (Biobest group, NV) and hives were left opened during the night.

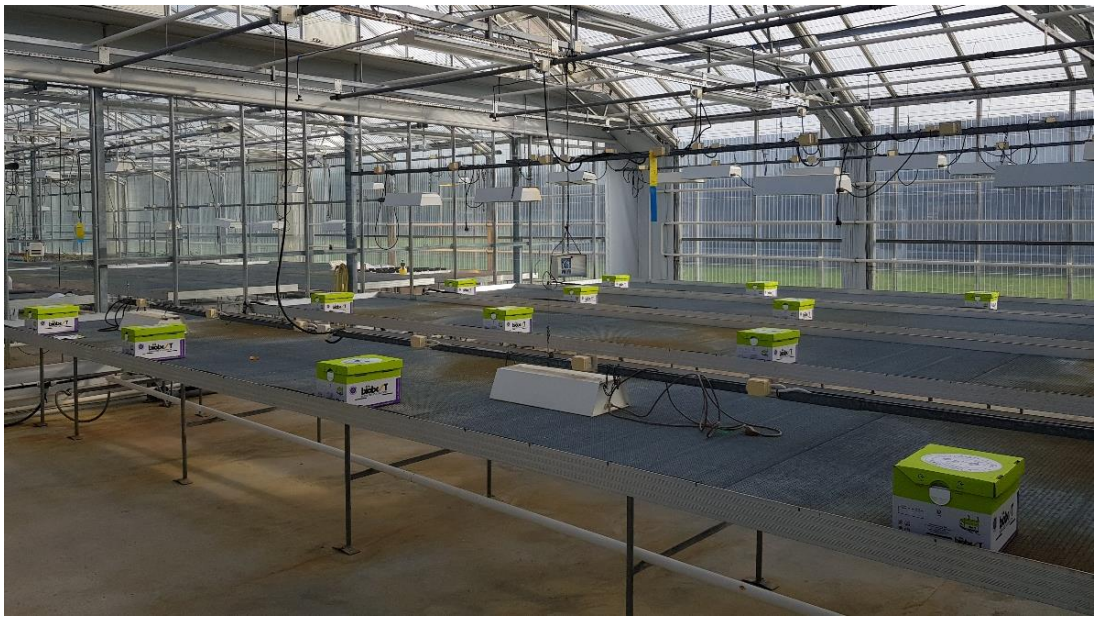

Figure 3 Bee performance experiment with sixteen hives placed in a greenhouse

As the performance (flight activity) of hives may vary among hives, I first scored the flight activity to be able to later divide hives into two homogenous groups before the experimental treatments. The numbers of bumblebee workers flying in and out of each hive were thus counted everyday over a period of 10 minutes per hive. Their activity was measured daily over four days. Hives were then sorted into two groups with as similar mean flight activity as possible. These groups of hives were later used for the two experimental treatments with *A.*

pullulans (formulation with the biocontrol agent) and control (formulation without the biocontrol agent) with eight hives in each experimental group.

Following the sorting of the hives into two groups, to check the hive performance in the different treatments groups, hives were first weighed (without the dispensers) to estimate the start weight before trials started, by removing the plastic hive box inside the cardboard box and placed on a scale (Figure 4, right). This was done in the morning when all bumblebees were inside the hive, since they were locked in the day before. This was done on day 0 before the experimental start and then twice per week during the experiment. A high hive weight can be interpreted as correlating with bigger/more larvae, higher honey production and better performance.

Figure 4 Left: Weighing of dispenser filled with formulation. Right: Weighing of beehive

After weighing, six grams of formulation (with *A. pullulans* or control) were added to each dispenser, after which the experiment started (Figure 5).

To investigate how much formulation that was exported from the dispensers by the bees, the weight of the formulation was scored on day 1 and then day 4 of each week until the end of the experiment (Figure 4, left). Every Monday and Thursday, the dispenser was refilled with new formulation up to six g in each dispenser as long as the old formulation showed critical vitality (defined as the number of colony forming units in the formulation was higher than 1.0×10^8 per gram (see below for description). This process was repeated for five weeks.

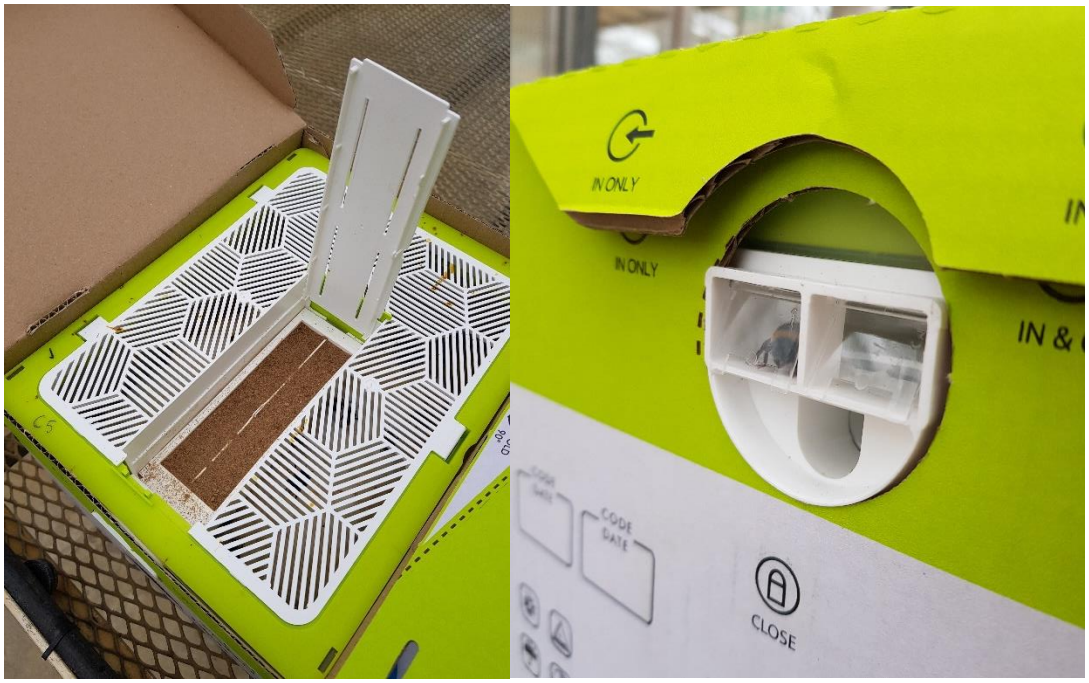

Figure 5 Left: Dispenser filled with formulation. Right: Bumble bee in dispenser about to exit hive

To monitor the vitality (mentioned above) of the formulation in the dispensers over time, a 1 gram sample of *A. pullulans* formulation was taken from the hives the first day and after 10 and 17 days in the dispenser to measure the CFU (colony forming unit), to examine if there were a possible decrease in spores in the formulation. After 17 days, the formulation did not show critical vitality (less than 1.0×10^8 per gram) any longer. Old formulations in the dispensers were thus discarded, and dispensers refilled with 6 g of all-new formulation.

Following application of the two different formulations and release of bumblebees, the hives were left open in the greenhouse for about 2-3 hours for bees to get used to the formulation in the dispenser. Then, the flight activity, number of bees that flies in and out of the hive, was counted for each hive during 10 minutes in the afternoon to examine a possible difference in flight activity between control and *A. pullulans* treated hives. The counting was made from some distance to the hive to not disturb the bumblebees. This procedure was performed twice per week for 4 weeks.

Experiment 2: Quantifying *A. pullulans* spores on bumblebees and flowers

To test if the bees can load *A. pullulans* formulation from the dispensers and vector it to the flowers, six new hives were used for further trials. These hives were placed in a separate greenhouse with identical abiotic conditions as described for the previous experiment. To examine if spores were loaded to the bumble bees, they were allowed to walk through the

hives with dispensers filled with 5 ml of *A. pullulans* formulation and were then captured in a falcon tube upon exit from the hive (Figure 6). This was also done with bumblebees in hives with control-formulation and Prestop®Mix to observe if there was any cross-contamination between bees from different hives, since the hives with different formulations were placed together in the same greenhouse. In total, 24 bumblebees were collected with eight bumblebees from each treatment hive. Bees were stored in a freezer for 30 min to kill them and subsequently washed with 5 mL of sterile water in a falcon tube and kept stirring for 30-min to detach the attached conidia of *A. pullulans*. Thereafter, serial dilution technique was used to determine the spore viability in the suspension of *A. pullulans* and streaked on PDA plates. The viable conidial concentration was measured based on the CFU on Petri plates after 48 hr.

Figure 6 Left: Bumblebee captured in falcon tube. Right: Bumblebee with *A. pullulans* formulation attached to body.

To examine the number of spores on strawberry flowers delivered by bumblebees, sampling of flowers was performed two days after bee transmitted *A. pullulans* formulation since spores had germinated at this time. Eight flowers from random strawberry plants treated with bee transmitted *A. pullulans* formulation were cut with a scissor and collected on Petri dishes. Flower organs were then placed on PDA plates for the detection of *A. pullulans*. Plates were then incubated for 8 days and observed to check formation of colonies and observe *A. pullulans* using a stereomicroscope (Figure 7).

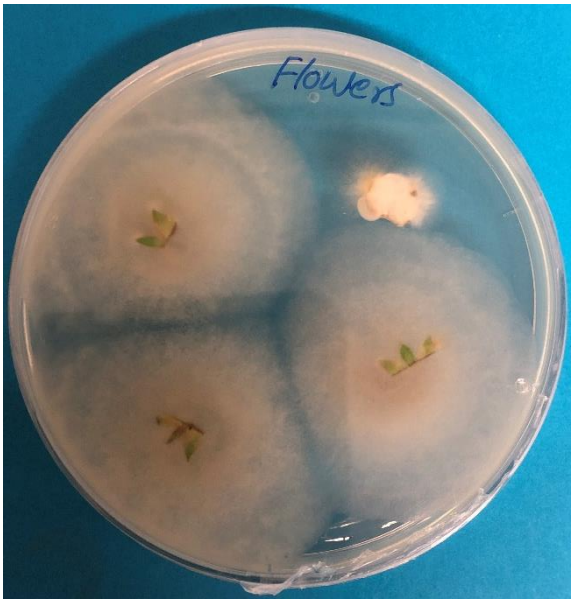

Figure 7. *Aureobasidium pullulans* from strawberry flower sample growing in a Petri dish

Experiment 3: Application of *A. pullulans* to strawberry flowers to suppress *B. cinerea* under greenhouse conditions – and comparisons with other treatments

Experimental design

Plants of the cultivar Sonata used for the biocontrol trials were placed in one of the isolated greenhouses at SLU, Alnarp, as described earlier. The abiotic settings of the greenhouse and the preparation of the plants are described above. Six new beehives where placed in another chamber where they were separated from plants. All strawberry flowers that were already open were cut off before experimental start, and the plants were then covered with a fleece-cover to make sure they contained fresh nectar and were not visited by any insect before the pollination experiment started.

For each of the six treatments, fifteen experimental plants were used. Thus, all-in-all, 90 plants were used. Each plant was randomly assigned to one of the six different treatments to examine the effect of *A. pullulans* as a biocontrol agent to suppress *B. cinerea* in strawberry:

Treatment T1. Bee transmitted wheat bran based Control formulation (no biocontrol agent)

Treatment T2. Bee transmitted wheat bran based *A. pullulans* formulation

Treatment T3. Bee transmitted Prestop Mix ®

Treatment T4. Control (no formulation, and no bee pollination)

Treatment T5. Liquid (water) *A. pullulans* formulation applied by spraying

Treatment T6. Liquid control (only water) applied by spraying

Bumblebees as vectors for biocontrol agent

Since treatments T1-T3 were performed in the same greenhouse, the different treatments were temporally separated each day to not mix the bees and risk contamination of the different biocontrol agents to the strawberry flowers. Two hives were used per treatment and in total, six hives were thus used for treatments T1-T3.

Plants designated for each treatment were moved to the experimental chamber where the beehives were placed during one hour per day during which their designated treatment was activated. Plants were placed with about 2 meters distance from the hives. Following that, one 15 ml spoon of formulation was added to the dispensers in each two hives that were used for the first treatment, always starting with the Control Treatment T1.

Bumblebee hives designated for Control treatment 1 were then opened by opening the exit holes in the dispenser and the bumblebees were able to fly out of the hive to visit the experimental plants designated for this treatment. Plants were exposed to visitation by the bees for one hour. By observing the bees and plants during this time, it was ensured that all open flowers were visited by the bees (Figure 8).

The daily treatments with bumblebees always lasted for one hour. Thus, after one hour the exit holes of the hives were closed whereas the entrance was still open, making it possible for the bumblebees to return to the hives but not able to fly out again. At the same time the plants were removed from the experimental greenhouse and placed in a separate greenhouse to make sure that there were no risk for the plants to be visited by further pollinators.

This procedure was then repeated for treatment T2-T3, but in T2 adding the biocontrol agent formulation with *A. pullulans* and in T3 adding Prestop Mix ® to the hives. Before next treatment was started, there was a waiting time for one hour to make sure no bumblebees where still out foraging in the chamber from the previous treatment. Trials were always performed in the morning or early afternoon when bees were as most active during a period

of the last week of October until middle of November in total exposing plants for bumblebee vectors nine times. Trials then ended when only a few buds remained on the plants. These remaining buds were cut off and aborted from the experiment.

Figure 8 Bumblebees delivering formulation when pollinating strawberry flowers

Spray treatment and *B. cinerea* inoculation

Plants for treatments T4-T6 were placed in an isolated greenhouse where no pollinators were present. Plants in T4 worked as control and were not exposed to either pollinators nor spray treatment. Preparation of liquid *A. pullulans* formulation was performed for treatment 5 (T5) as described earlier. To make the conidia from *A. pullulans* adhere to the flowers, Tween®20 which is a non-ionic detergent was added to the solution. The liquid *A. pullulans* formulation was poured over to a spray bottle and each plant designated to treatment 5 (T5) was sprayed four times with the spray bottle. Plants in T6 worked as a spray control and flowers and buds was sprayed once per week with water only in the same way as liquid *A. pullulans* treatment.

Scoring grey mould symptoms at harvest and postharvest

Fruits from all treatments were harvested when they were fully ripened, which takes place around 4-5 weeks after flowering. To quantify grey mould infection and the efficiency of the different treatments, strawberry fruits were scored for grey mould symptoms. At the day of

harvesting, fruits were scored for grey mould symptoms with a scoring system from 0 to 4 (Figure 9). Fruits scored as 0 had no grey mould symptoms at all. All-in-all, 137 fruits (between 21 and 37 per treatment) were produced from the experimental plants. All these fruits were sampled for grey mould scoring.

Figure 9 Scoring system of grey mould infection showing appearance of severity of the disease

Following harvest and scoring, fruits were placed in plastic sales boxes (BK Pac) with one fruit in each separate box. They were then incubated in a cold room at 3 °C (Figure 10) to measure the effect of the treatments on strawberry shelf life. All incubated fruits were scored every day for one week of the appearance and development of grey mould symptoms.

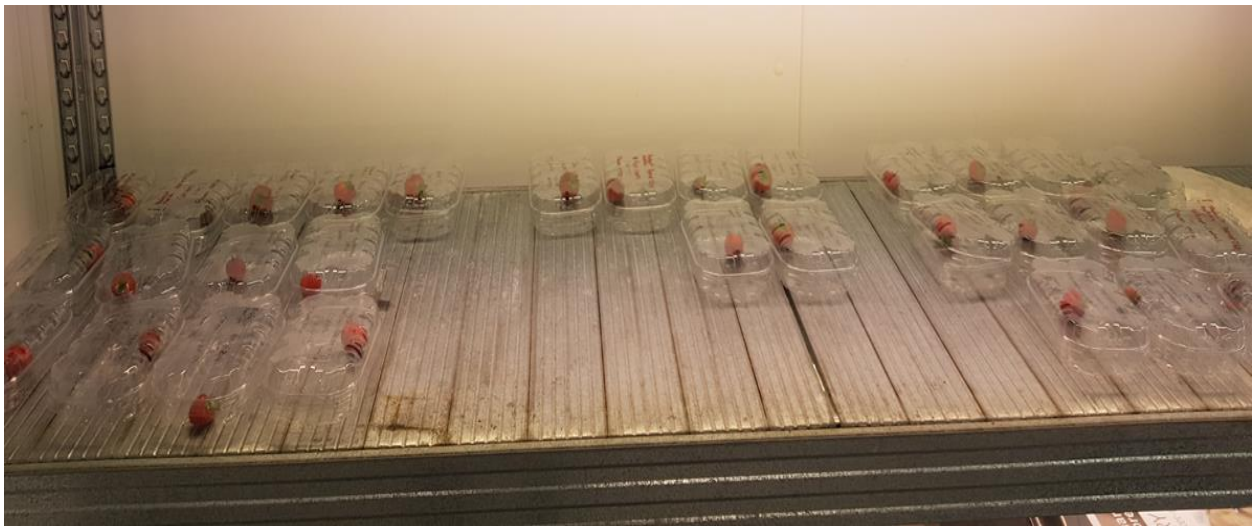

Figure 10 Strawberries in separate sales boxes incubated in a cold room

Statistical analyses

To examine difference in flight activity between *A. pullulans* and control hives in experiment 1, general linear model (GLM) analyses were performed. Statistical analyses using Shapiro-Wilk tests confirmed that all data were normally distributed. The same analyses were made for hive weight. To examine scoring of grey mould infection in different treatments pre- and postharvest in experiment 3, a pairwise test using post hoc Tukey test was performed. All

tests were performed using R 4.0.0 environment for statistical computing (R Core Team, 2020)

Results

Experiment 1: Effects of *A. pullulans* on bee performance

Scoring of in/out flights

The beehives that were divided into two treatment groups (*A. pullulans* and control) showed no significant difference in flight activity before the bee performance experiment started ($P = 0.427$). Results showed no significant difference ($P > 0.05$, Table 1) in number of in/out-flights none of the scoring times between *A. pullulans*- and control hives during experiment. Which suggests that the biocontrol agent *A. pullulans* did not affect the foraging activity of the bumblebees. However, although not significant, the last two scoring events showed a trend in increasing difference between *A. pullulans*- and control hives, with a higher number of in/out-flights in control hives ($P = 0.266$ and $P = 0.119$) (Table 1 & Figure 11) during the last two scoring events. The increasing numbers of in/out-flights during the last scoring events is expected, since the colony will contain a higher amount of bumblebee workers at this time when larvae has become adult.

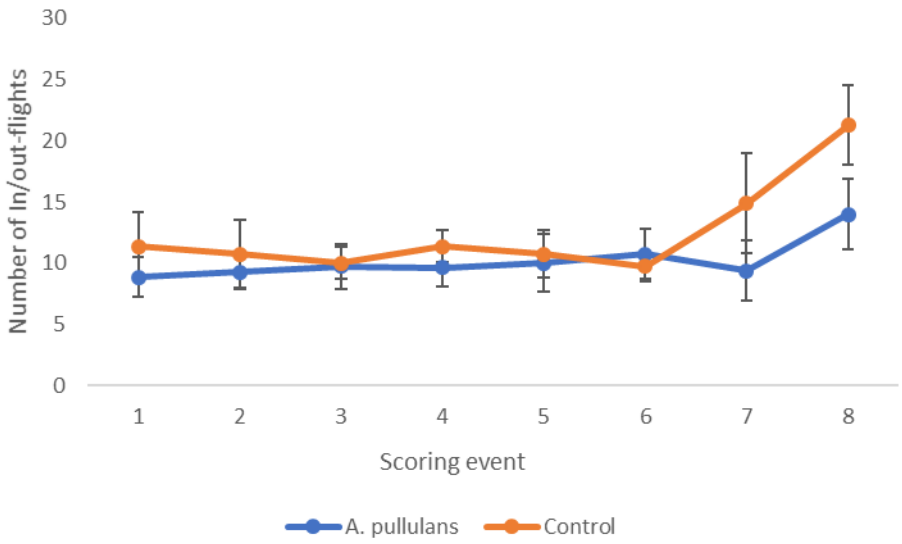

Figure 11 Number of in/out-flights by bumblebee workers in eight *A. pullulans* and eight control hives over a period of 4 weeks. Error bars indicate SE based on eight replicate hives per treatment. Each data point represents a mean number of in and out flights of the eight hives in the treatment.

Table 1 The results of the generalized linear model analyses differences in flight activity between hives exposed to *A. pullulans* versus control. The results show no significant difference between *A. pullulans* and control hives at any of the scoring events.

Scoring event	Dependent variable	Treatments	t-value	p-value	df
1	In/Out-flights	*A. pullulans* vs. Control	-0.776	0.450	7
2	In/Out-flights	*A. pullulans* vs. Control	-0.493	0.629	7
3	In/Out-flights	*A. pullulans* vs. Control	-0.111	0.913	7
4	In/Out-flights	*A. pullulans* vs. Control	-0.872	0.398	7
5	In/Out-flights	*A. pullulans* vs. Control	-0.247	0.808	7
6	In/Out-flights	*A. pullulans* vs. Control	0.418	0.683	7
7	In/Out-flights	*A. pullulans* vs. Control	-1.157	0.266	7
8	In/Out-flights	*A. pullulans* vs. Control	-1.661	0.119	7

Hive performance – Hive weight

Before the experiment started, hives in *A. pullulans* and control groups were weighed (1st scoring event, Figure 12). From start, there was a very small, but still statistically significant, difference in hive weight between the two treatment groups ($P = 0.039$), with a higher mean weight of hives that were going to be treated with *A. pullulans* formulation. At the second time of weighing, when the formulations had been in dispensers for 3 days, there was still a significantly difference between the two treatment groups, but slightly less different ($P = 0.047$) (Table 2). Remaining times of scoring events showed no significant difference between *A. pullulans*- and control treatment ($P > 0.05$). However, there is a trend showing less difference in mean weight between the different treatment groups last times of scoring, with control hives catching up in weight on *A. pullulans* treated hives. However, the mean weight is not significantly different between groups (Table 2 & Figure 12)

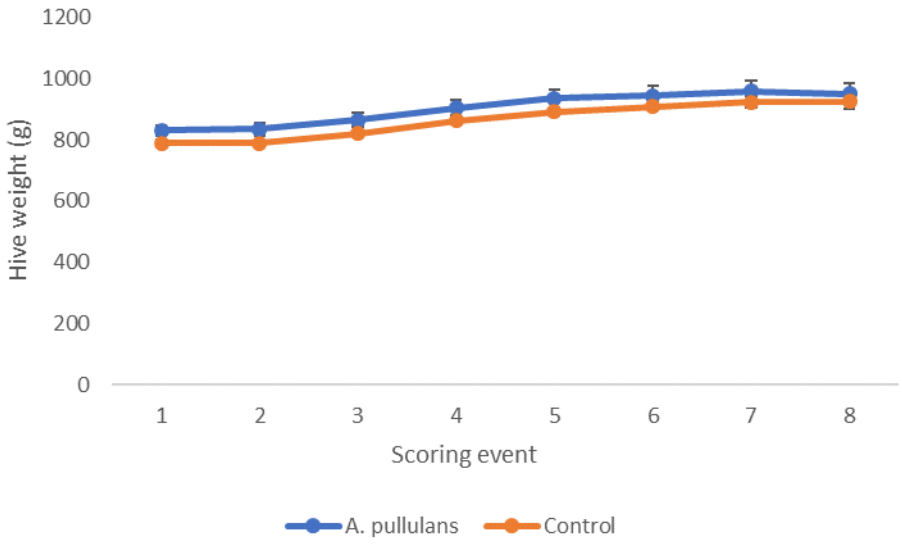

Figure 112 Mean weight of eight *A. pullulans* (blue) and eight control (orange) hives over a scoring period of 4 weeks. Error bars indicate SE based on eight replicate hives per treatment. Each scoring event represents the mean weight of all hives in the treatment.

Table 2 The results of the generalized linear models testing for differences in weight between *A. pullulans* and control hives. Showing a statistically significant difference between the treatments from start and until the second scoring event.

Scoring event	Dependent variable	Treatments	t-value	p-value	df
1	Weight	*A. pullulans* vs. Control	2.265	0.039 *	7
2	Weight	*A. pullulans* vs. Control	2.181	0.046 *	7
3	Weight	*A. pullulans* vs. Control	1.80	0.093	7
4	Weight	*A. pullulans* vs. Control	1.579	0.137	7
5	Weight	*A. pullulans* vs. Control	1.375	0.191	7
6	Weight	*A. pullulans* vs. Control	1.091	0.294	7
7	Weight	*A. pullulans* vs. Control	0.921	0.373	7
8	Weight	*A. pullulans* vs. Control	0.623	0.543	7

Amount of formulation carried from dispensers by bumblebees

To examine how much formulation the bumblebees delivered from the dispenser over time, formulations was weighed and refilled twice per week. In control treatment, an average of 2.65 g disappeared from the dispensers during the 4 week period and in *A. pullulans* treated hives 2.97 g was disappeared. These results show that slightly more *A. pullulans* formulation was delivered from the dispensers each week compared to control-formulation, however, results show no significant difference ($P = 0.289$).

Vitality of *A. pullulans* formulation

From start, the CFU in *A. pullulans* fresh formulations were 7.5×10^8 spores/g and after 10 days in dispenser the CFU was 1.1×10^8 spores/g. Another sample was taken of 17-days old formulation from one hive which showed a CFU of 1×10^7 spores/g (ten million). This showed that the colony forming unit of the formulation decreased over time when applied to dispenser.

Experiment 2: *A. pullulans* spores on bumblebees and flowers

Number of *A. pullulans* and *G. catenulatum* spores on bumble bees

The CFU (colony forming unit) of *A. pullulans* and *G. catenulatum* (Prestop®Mix) was counted in bumblebee samples from the different treatment hives (T2 and T3), to examine and compare the number of spores that got attached to the bumblebees when walking through the dispenser. There was a slight difference between the CFU/ml of the different samples, with a higher number CFU/ml in bumblebee samples from T3 (*G. catenulatum* – Prestop®Mix*),* but this was not significantly different ($P = 0.050$, Figure 13). Also, the number of colonies were counted on the plates 48 h after sampling, showing no significantly difference between number of colonies between T2 and T3 samples ($P = 0.050$). This result shows that the *A. pullulans* formulation gets attached to the bumblebees. In samples from control (T1) there were no *A. pullulans* or *G. catenulatum* colonies growing, which demonstrates that no contamination between hives were present.

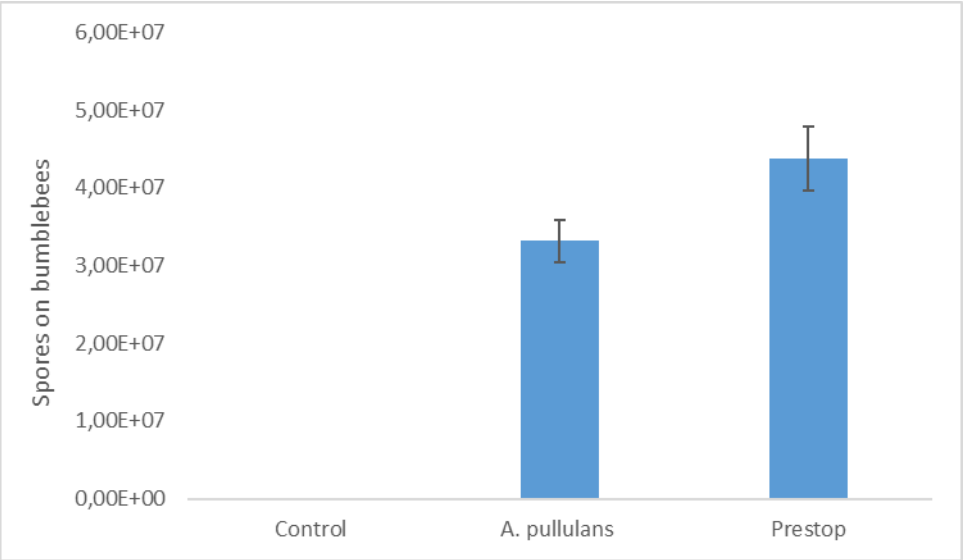

Figure 13 Mean number of spores attached to the eight bumblebee replicates from the different treatments with *A. pullulans* and Prestop. Error bars indicate SE based on eight bumblebee replicates. Showing that spores from the formulations get attached to the bumblebees and that there is no significant difference between numbers of attached spores in *A .pullulans* and Prestop treatment.

A. pullulans spores on flowers

To examine if bumblebees successfully vectored *A. pullulans* spores to flowers, sampling of flowers from treatment 2 (T2) was performed. Results showed that colonies of *A. pullulans* was growing in all petri dishes with flower samples, which indicates that the bumblebees successfully delivered the biocontrol agent to the flowers (Figure 14).

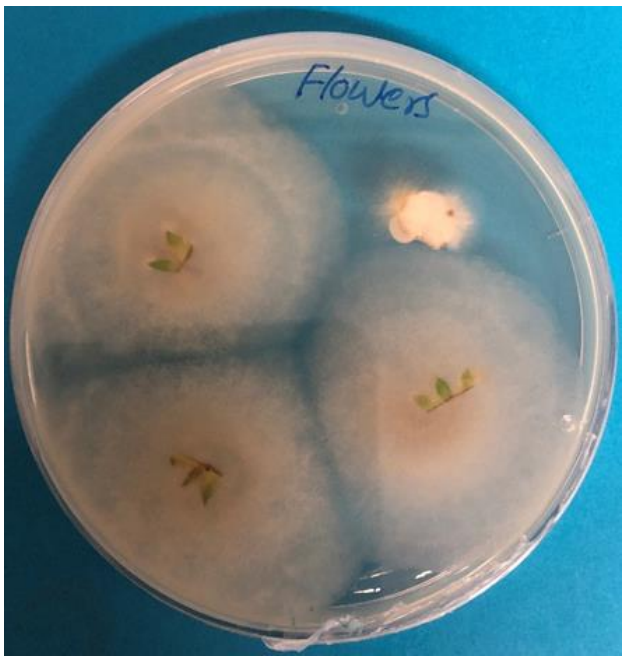

Figure 14 Sample of strawberry flower in petri dish showing *A. pullulans* colonies growing

Experiment 3: Application of *A. pullulans* to strawberry flowers to suppress *B. cinerea* under greenhouse conditions

Scoring of grey mould infection

Fruits from plants treated with the biocontrol agents *A. pullulans* and Prestop®Mix, showed a trend in lower grey mould infection than in control treatments at scoring day 0, i.e. at the day of harvesting (Figure 15). However, this difference was not significantly different (Table 4).

After 7 days postharvest the results show a higher rate of grey mould symptoms in control treatments (Figure 15). Importantly, fruits from the *A. pullulans* treatment vectored by bumblebees showed a significantly lower rate of grey mould infection compared to all control treatments (Table 5; Figure 15).

Comparing the three different biocontrol treatments (with bee-vectored *A. pullulans*, liquid *A. pullulans*, and bee-vectored Prestop®Mix) fruits from *A. pullulans* vectored by bumblebees shows a trend to be least infected by grey mould. Except this, Prestop®Mix showed a significant difference compared to H_20 Control with less infected fruits in day 7 (Table 5). Fruits from liquid spray treatment with *A. pullulans* show a higher rate of infection at both day 0 and day 7 compared to the bee-vectored *A. pullulans* treatment, and fruits with liquid treated *A. pullulans* show no significant difference compared to control treatments (Figure 15 & Table 4).

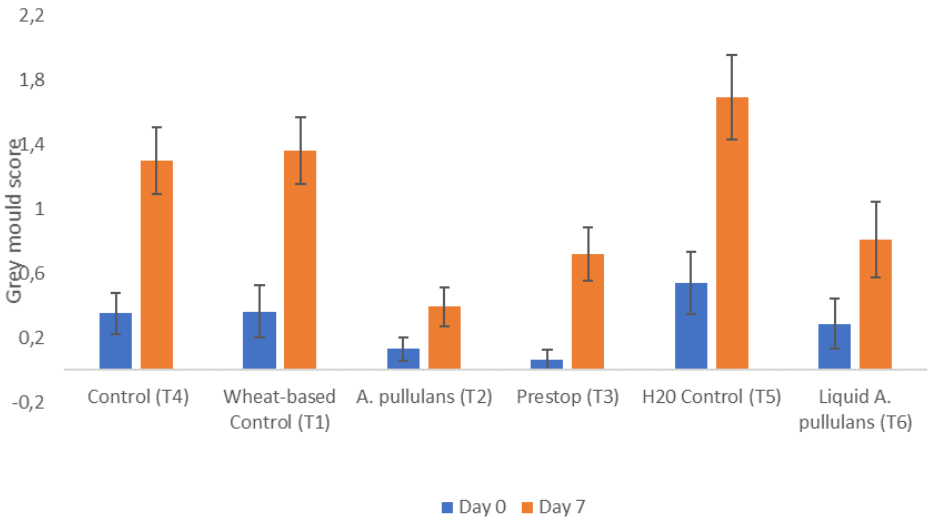

Figure 113 Diagram showing a mean score of grey mould severity in fruits from different treatments T1-T6. Error bars indicate SE. Fruits from bee-vectored *A. pullulans* treatment show the lowest grey mould score seven days postharvest than other treatments.

Table 4 Pairwise posthoc test using the Tukey method for comparing grey mould infection between treatments on day 0. Showing no significant difference between treatments in grey mould severity on scoring day 0.

Scoring Day	Dependent variable	Treatment	t-value	p-value	df
0	Grey mould infection	*A. pullulans vs* Control	-1.173	0.848	158
0	Grey mould infection	*A. pullulans* vs Wheat-based control	-1.121	0.872	158
0	Grey mould infection	*A. pullulans* vs H20 Control	-2.010	0.341	158
0	Grey mould infection	*A. pullulans* vs Prestop	0.351	0.999	158
0	Grey mould infection	*A. pullulans* vs Liquid *A. pullulans*	-0.726	0.978	158
0	Grey mould infection	Prestop vs Control	1.688	0.542	158
0	Grey mould infection	Prestop vs Wheat-based control	-1.572	0.618	158
0	Grey mould infection	Prestop vs H20 Control	2.543	0.118	158
0	Grey mould infection	Prestop vs Liquid *A. pullulans*	-1.121	0.872	158
0	Grey mould infection	Liquid *A. pullulans* vs Control	0.339	0.999	158
0	Grey mould infection	Liquid *A. pullulans* vs Wheat-based control	-0.354	0.999	158
0	Grey mould infection	Liquid *A. pullulans* vs H20 Control	1.215	0.829	158

Table 5 Pairwise posthoc test using the Tukey method for comparing grey mould infection between treatments in day 7. Showing that fruits from bee-vectored *A. pullulans* treatment had significantly lower grey mould scores than control treatments.

Scoring day	Dependent variable	Treatments	t-value	p-value	df
7	Grey mould infection	*A. pullulans* vs Control	-3.143	0.024*	158
7	Grey mould infection	*A. pullulans* vs Wheat-based control	-3.088	0.028*	158
7	Grey mould infection	*A. pullulans* vs H20 Control	-4.187	0.0007*	158
7	Grey mould infection	*A. pullulans* vs Prestop	-1.103	0.879	158
7	Grey mould infection	*A. pullulans* vs Liquid *A. pullulans*	-1.276	0.797	158
7	Grey mould infection	Prestop vs Control	2.208	0.240	158
7	Grey mould infection	Prestop vs Weat-based control	-2.213	0.237	158
7	Grey mould infection	Prestop vs Control	3.397	0.011*	158
7	Grey mould infection	Prestop vs Liquid *A. pullulans*	-0.298	0.999	158
7	Grey mould infection	Liquid *A. pullulans* vs Control	1.645	0.570	158
7	Grey mould infection	Liquid *A. pullulans* vs Wheat-based control	-1.713	0.525	158
7	Grey mould infection	Liquid *A. pullulans* vs H20 Control	2.772	0.067	158

Discussion

This study evaluates a new potential biocontrol agent AP-SLU6 against grey mould in strawberry, that could be used in entomovector technology. It also examines if bumblebee activity and health is potentially negatively affected by this biocontrol agent. This study indicates that *B. terrestris* used for vectoring *A. pullulans,* could be an efficient method to control grey mould in strawberry under greenhouse conditions. Fruits treated with *A. pullulans* vectored by bumblebees showed a significantly lower rate of grey mould infection postharvest than other treatments. This demonstrates that these fruits develop grey mould symptoms at a later stage only, and thus have a longer shelf life, which is desirable due to the higher amount of marketable fruits.

Bombus terrestris workers and hives seem not to be significantly affected by *A .pullulans* according to results from experiment 1. This is important because a high flight activity means that more bumblebees are out foraging and can visit flowers and collect a more considerable amount of pollen and nectar, which results in bigger larvae and colony growth. The

bumblebees also produce honey, which influences the hive weight. A high mortality rate would influence the weight, which would result in lighter *A. pullulans* treated hives, if this formulation had a negative effect on the bumblebees. Since there is no significant difference between the measured characteristics in *A. pullulans* and control hives, the conclusion could be made that bees are at least not strongly negatively affected by *A. pullulans.* This suggest that these Flying doctors® hives are appropriate for entomovectoring of *A. pullulans* formulations.

However, the insignificant trend shown in figure 11, where bumblebee workers' flight activity seems to increase more in control hives than *A. pullulans* hives during the last two scoring events, should be considered. This potential reduction in bee activity in A. pullulans hives also echoes that the hive weight in control treatment increases more than *A. pullulans* treated hives, during the last scoring events. Nevertheless, all bumblebee hives are destined to be replaced by new hives after a certain time when the old colonies die, and if there will be some long-term negative effect of the *A. pullulans* formulation according to the potential trend discovered here, it shouldn't play a significant role in this context.

The results further showed that the vitality of the *A. pullulans* formulation decreased over time and that the CFU differed from new and old formulations. Also, formulations that were in a dispenser for a long time seemed to absorb moisture. This is expected, and most available products on the market are assumed to be replaced approximately every 3rd day. Surprisingly, my results showed that the *A. pullulans* formulation's vitality did not drop below the critical threshold until day 17. However, if strawberry growers should use bumblebee hives for vectoring of *A. pullulans*, a more frequent refill with new fresh formulations would be optimal.

Results from experiment 2 show that *A. pullulans* formulation can be transferred and deposited to strawberry flowers by bumblebees. In every flower sample, *A. pullulans* colonies were growing and on every bumblebee spores were present. Though, bumblebees were collected just when exiting the hives, they may have lost some spores during their flight and may not deliver the same number of spores to the flowers. This would be interesting to further investigate and analyze by calculating the number of spores on strawberry flowers.

Results from experiment 3 show that in contrast to entomovectoring, flowers sprayed with a liquid solution of *A. pullulans* showed no statistically significant reduction in grey mould infected post-harvested fruits. This strengthens the theory that pollinators used as vectors can be a more efficient method in controlling grey mould in strawberry. This could be explained by the more precise delivery of the biocontrol agent to the flowers at a proper time, resulting in longer shelf life of the fruits (Hokkanen et al. 2015). Another explanation to a higher amount of infected fruits in *A. pullulans* liquid spray treatment, could be that the *B. cinerea* thrives in humid conditions and at every spray occasion, the flowers will experience a humid environment (Mommaerts et al. 2011). That theory also supports why fruits in H_2O control treatment had the highest infected fruits during harvest and seven days postharvest.

According to the results, the *A. pullulans* strain AP-SLU-6 used in this experiment seems to be slightly more efficient against postharvest grey mould symptoms when vectored by bumblebees than Prestop®Mix. Even though a higher number of *G. catenulatum* spores than *A. pullulans* spores were found on bumblebees from Prestop®Mix , the lower number of *A. pullulans* spores still had a significant effect in reducing grey mould. This supports earlier studies by Lima et al. (1997) and Adikaram (2002), that *A. pullulans* effectively control grey mold in strawberry – although their studies focused on other strains. Since bumblebees are large and active during cold and cloudy days and do visit a high amount of flowers, this could further strengthen the method of using these as vectors for biocontrol agents (Delaplane & Mayer 2000).

Some results' reliability is impacted by the sample size and the limited time frame for this experiment. For experiment 1, the limited amount of time could impact the results, since scoring data only exist for four weeks and data was not obtained from the whole lifetime of the beehives. There is a limited sample size of fruits in experiment 3 that could influence the results and to confirm these results, the experiment needs to be repeated with a higher number of replicate plants. This experiment could be extended and further studied by letting several different strawberry growers use Flying Doctors® hives in strawberry greenhouses where bumblebee hives can stay open all the time. Further studies should also consider using other strawberry varieties than Sonata, since there could be a variation in disease

incidence among cultivars (Petrasch et al. 2019). Also, because different strawberry cultivars vary in their attractiveness of bees (Mommaerts et al. 2011).

Further research is needed to establish these results and examine the efficacy of *A. pullulans* vectored by bumblebees in relation to other application methods and other biocontrol agents and synthetic fungicides. Nevertheless, this study demonstrates that there are alternative methods for controlling postharvest grey mould in strawberry that could be more efficient and can minimize the use of chemical fungicides.

Acknowledgements

I would like to thank my supervisor Johan A. Stenberg for allowing me to implement this study. Thank you for all support and feedback both during the practical experiments and in the process of writing my thesis! I would also like to thank my co-supervisor Mudassir Iqbal for helping me with the fungal part of this experiment and all the practical help. I am very grateful for this, thank you! I would also like to thank Adam Flöhr for helping me with the statistical models in this project. Without all of your help, I could not have completed this thesis!

Soraya França and Felix Wäckers at Biobest are gratefully acknowledged for providing useful input on the design of the experiments and generously donating Flying Doctors® hives making this experiment possible. Thank you!

References

Adikaram, N.K.B., Joyce, D.C. & Terryc, L.A. (2002) *Biocontrol activity and induced resistance as a possible mode of action for Aureobasidium pullulans against grey mould of strawberry fruit.* Australasian Plant Pathology **31,** 223–229. https://doi.org/10.1071/AP02017

Bhatt. DD & Vaughan. EK (1962) *Preliminary investigations on biological control of grey mould (Botrytis cinerea) in strawberries*. Plant Disease Reporter 46, 342–345.

Botector (2017) *Botector™ Product User Manual Against grey mold.* Tulln: Bio-ferm. [2020-11-02]

Brent K. & Hollomon D. (2007) *Fungicide resistance in crop pathogens: How can it be managed?* Bristol: Crop life international

Delaplane, K.S & Mayer, D.F (2000) *Crop pollination by bees.* New York: ProQuest Ebook Central, https://ebookcentral.proquest.com/lib/slub-ebooks/detail.action?docID=292063.

Hokkanen, H., Heikki & Menzler-Hokkanen, I., & Lahdenpera, M. (2015). *Managing Bees for Delivering Biological Control Agents and Improved Pollination in Berry and Fruit Cultivation.* Sustainable Agriculture Research. Vol 4.

Iqbal, M., Dubey M., McEwan K., Menzel U., Franko M., Viketoft M., Jensen D., & Karlsson M. (2017), *Evaluation of Clonostachys rosea for Control of Plant-Parasitic Nematodes in Soil and in Roots of Carrot and Wheat*. Phytopathology Vol, 108, 52-59.

Jamshaid, M. (2020) *Evaluation of Aureobasidium pullulans as potential biocontrol agent against strawberry diseases.* Swedish university of agricultural sciences. Montpellier SupAgro.

Jensen, B., Knudsen, I.M. & Jensen, D.F. (2000) *Biological Seed Treatment of Cereals with Fresh and Long-term Stored Formulations of Clonostachys rosea: Biocontrol Efficacy Against Fusarium culmorum .* European Journal of Plant Pathology **106,** 233–242 (2000). https://doi.org/10.1023/A:1008794626600

Kevan, P. Kapongo, J.P., Almazarawi, M. & Shipp, L. (2008) Honeybees bumblebees and biocontrol. *Bee pollination in Agricultural ecosystems*. Oxford press 65-76. DOI: 10.1093/acprof:oso/9780195316957.003.00

Khanizadeh, S. &Buszard, D. (1987). *Effects of the fungicides captan and Easout on strawberry fruit development.* Adv. Strawberry Prod. Vol.6, 27–31.

Kookana, R. & Correll, R. & M., Rosalind. (, 2005). *Pesticide Impact Rating Index – A Pesticide Risk Indicator for Water Quality*. Water, Air, & Soil Pollution: Focus. 5. 45-65. 10.1007/s11267-005-7397-7.

Kovacova, J., Hrbek, V., Kloutvorova, J., Kocourek, V., Drabova, L., & Hajslova, J. (2013) *Assessment of pesticide residues in strawberries grown under various treatment regimes*, Food Additives & Contaminants: Part A, 30:12, 2123-2135. 10.1080/19440049.2013.850537

Lima, G., Ippolito, A., Nigro, F. & Salerno, M. (1997) *Effectiveness of Aureobasidium pullulans and Candida oleophila against postharvest strawberry rots.* Postharvest biology and technology. Vol.10, 169-178. https://doi.org/10.1016/S0925-5214(96)01302-6

Mommaerts, V., Put, K. & Smagghe, G. (2011), *Bombus terrestris as pollinator-and-vector to suppress Botrytis cinerea in greenhouse strawberry*. Pest. Manag. Sci., 67: 1069-1075. https://doi.org/10.1002/ps.2147

Petrasch, S., Knapp, S.J., van Kan, J.A.L. & Blanco-Ulate, B. (2019), *Grey mould of strawberry, a devastating disease caused by the ubiquitous necrotrophic fungal pathogen Botrytis cinerea*. Molecular Plant Pathology, 20: 877-892. https://doi.org/10.1111/mpp.12794

R Core Team, 2020. *R: A Language and Environment for Statistical Computing*. R Foundation for Statistical Computing, Vienna. https://www.r-project.org.

Velthuis, H. & Doorn, A. (2006). *A century of advances in bumblebee domestication and the economic and environmental aspects of its commercialization for pollination*. http://dx.doi.org/10.1051/apido:2006019. Vol. 37.