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Evaluation of *Aureobasidium pullulans* as potential biocontrol agent against strawberry diseases

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Thesis submitted to fulfil requirement to get diploma of the European Erasmus + Plant Health Master

Subject of thesis

Evaluation of *Aureobasidium pullulans* as potential biocontrol agent against strawberry diseases

By

Maha Jamshaid

Registered School: Montpellier SupAgro

Research Supervisors: Johan A. Stenberg, Ramesh Raju Vetukuri & Mudassir Iqbal

Place of internship: Swedish University of Agricultural Sciences

Department: Plant Protection Biology

Address: Sundsvägen 14B, 230 53 Alnarp

Thesis defense on September 23, 2020 in front of jury composed of

President: Frédéric Marion-Poll

Members: Manu Plantegenest

Serge Kreiter



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List of Abbreviations

<i>A. pullulans</i>	<i>Aureobasidium pullulans</i>
<i>B. cinerea</i>	<i>Botrytis cinerea</i>
<i>C. acutatum</i>	<i>Colletotrichum acutatum</i>
DPI	Days Post-Inoculation
EPPO	European and Mediterranean Plant Protection Organization
PDA	Potato Dextrose Agar
<i>P. cactorum</i>	<i>Phytophthora cactorum</i>

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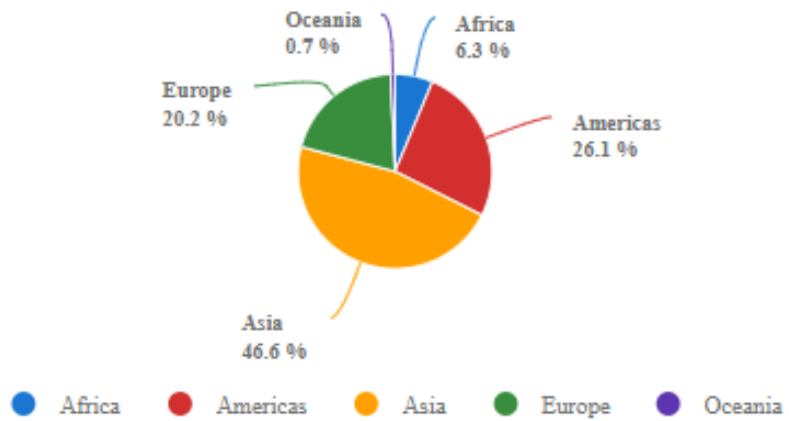


Figure 1: Illustration of strawberry cultivation in different geographical regions

Copy Right: The figure is adopted from FAO agency
(<http://www.fao.org/faostat/en/#data/QC/visualize>) accessed on September 9, 2020.

1. Introduction

Garden strawberry, (*Fragaria × ananassa* Duch.) is high-value fruit and widely distributed all over the world. Because of high nutritional value, containing vitamins, minerals, potassium and folate contents, it is appreciated among all continents and gives great benefits to a human diet providing micro and macronutrients and antioxidants (Bianco et al. 2009). This crop is gaining popularity and its production is steadily increasing globally (Essghaier et al. 2009). According to FAOSTAT (2018), an area under production of strawberry is 372,361 ha and cultivation is 8,337,099 tons whereas in Europe production is comprised of 20.2% (Figure 1). In Sweden, strawberry is leading berry crop and its cultivated area and yield corresponded to almost 2,007 ha and 15,640 tons respectively (FAOSTAT 2018). In line with its economic importance in many countries, serious financial loss may arise due to many biotic and abiotic factors that damage to quantity and quality of the fruits and reduce the yield. Strawberry is attacked by many phytopathogens including fungi, bacteria, viruses and nematodes at different developmental stages of plants and their response to pathogens varies depending on their different genotype. Among all pathogens, phytopathogenic fungi are responsible for 80% of the plant diseases in agriculture (Shuping et al. 2017). Garden strawberry is affected by more than 50 different genera however, *Botrytis*, *Colletotrichum*, *Phytophthora* are considered to be the economically most important ones and they are included in the European and Mediterranean Plant Protection Organization (EPPO), A2 list (EPPO 2004). Controlling of fungal diseases is an immediate need in the agricultural industry and current strategies are largely based on different genetic resistance and synthetic fungicides, which has many obvious concerns related to its excessive and frequent use, negative environmental impact and decreasing susceptibility among pathogens. Therefore, there is an interest in the development of alternative methods that can provide effective control while giving the least consequences to human health and the environment. The present research was based on investigating the inhibitory potential of the biocontrol fungus *Aureobasidium pullulans* against three fungal pathogens.

1.2 Grey mould disease

Botrytis cinerea Pers. is an ascomycetes (perfect stage *Botryotinia fuckeliana*), and is a global pathogen causing more than \$10 billion annual economic loss of fruits, vegetables and ornamental crops (Hua et al. 2018). The disease is prevalent in all continents but mainly in cool, temperate, and warm-temperate zones (Jarvis 1977). They act as saprophytes in the absence of hosts and

remain dormant under unfavourable conditions. Conidia from neighbouring infected plants are dispersed by air and water splashes, which facilitate primary infection and enter the plants through any kind of injury or natural opening and invade to bottom of the receptacle, here mycelium remains quiescent until ripening of fruits or can easily penetrate open flowers (Bristow et al. 1986). Once environmental conditions are favourable, i.e. when the temperature is around 25°C with long periods of high wetness, they start causing rotting in fruits, flowers and leaves. If favourable condition continuous, the fungus keep on sporulating, giving rise to secondary inoculum. Due to its polycyclic nature, it is difficult to control although methods like a choice of cultivar, moderate use of nitrogen fertilisation, straw mulching, adequate spacing, sanitation and reduced leaf wetness can decrease the incidence of *B. cinerea* (Legard et al. 2000). The only effective control is using of synthetic fungicides on strawberry during the flowering stage. However, it is recently reported that excessive use of pesticides can lead to the development of resistance in *B. cinerea* isolates in a very short time (Leroch et al. 2013) and be a severe threat to commercial success because of their classification as high-risk pathogens (Petrasch et al. 2019).

1.3 Anthracnose disease

Anthracnose is an important disease of strawberry, incited by the two fungal species *Colletotrichum acutatum* Simmonds, and *Colletotrichum gloeosporioides* Penz. *Colletotrichum* belongs to Ascomycetes. *C. acutatum* is the second most destructive pathogen in strawberry cultivation after *B. cinerea* (Baroncelli et al. 2015) and causes yield loss of over 50% (Howard et al. 1992). It was first reported in California in 1983 and later on spread on broader geographical regions (Smith 2008). Conidia of *C. acutatum* can survive in soil and dead plant material, disseminated through air/water splashes and germinate appressorium after landing on plant surface where the infection period starts. The biotrophic phase remains symptomless for short time 1-3 days and later on enter into the necrotrophic phase that lead to pathogenic lesion and plant death (Cannon et al. 2012). The optimum temperature for the development of anthracnose is 25 to 30°C. Strawberry anthracnose can be associated in all parts of plants: in nursery lesion formation on petioles, stolons are commonly found (Freeman et al. 2001) whereas in the field flower blight and root rot are usually found (Howard et al. 1992). No chemical control is effective in the control of this disease, although cultural techniques like drip irrigation, and tunnel farming can considerably prevent dispersion of inoculum (Madden et al. 1997).

1.4 Root rot

Root disease caused by *Phytophthora cactorum* (Lebert and Cohn) hampers the production of cultivated strawberry. It is a hemibiotrophic oomycete, a soil-borne pathogen that occurs sporadically but it has the potential to damage crop leading to up to 50% yield loss (Ellis et al. 1998). Characteristic symptoms include wilting of plants and brown necrosis of vascular tissues of the crown and sometimes lead to complete death of the crop. It forms sexual oospores which are a primary source of inoculum that remains persistent in the soil for several years, which makes its management difficult. When conditions are favourable oospores germinate to produce sporangia which release the oospores giving rise to sporangia which produce zoospores. Zoospores are the asexual motile stage and they are chemotactically attracted by roots where they attach to the surface and penetrate the root epidermis. After that, they start developing haustoria to acquire nutrients from the root for their growth and sporulation (Nellist et al. 2019). The most conventional methods to control the disease is by use of fungicides and cultural methods (Madden et al. 1991). Good soil drainage helps in mitigating disease besides, fosetyl-Al, and phosphate-based products could also prevent disease (Eikemo et al. 2003). These measures only partly limit the disease, but complete control of the pathogen is rarely achieved.

1.5 *Aureobasidium pullulans* as a biocontrol agent

Strawberry disease management is normally reliant on fungicides and due to many rising concerns of resistant pathogen isolates by excessive use of fungicides, non-authorization of fungicides (Howard 1990) residual effect on the environment as well as on human being have lead in increasing investigation on alternative measures. Biological control is defined as the use of living organisms to control pathogens and this could be a promising substitute of chemical products (Wisniewski and Wilson 1992). Biological control is normally a rational and safe crop management practice that has been used on a commercial scale against many plant diseases (Handelsman and Stabb 1996; Whipps 2001). Biological control agents have several different modes of action including direct or indirect antagonisms to control plant pathogens. The mechanisms involved include antibiosis, parasitism, competition and induced resistance (Pal and Gardener 2006; Blakeman and Fokkema 1982). In recent times, among many microorganisms, yeast or yeast-like fungi, in particular, are promising biocontrol agents in controlling post-harvest pathogens because they create competition for space and nutrients and

induce host defence reaction (Droby et al. 1994). They are highly acceptable for consumers because they rarely produce antibiotics. The yeast-like fungus *A. pullulans* de Bary. belongs to the ascomycetes and is found in the family Dothideales (De Hoog and McGinnis 1987). It is a polymorphic species, ubiquitous, and occur naturally in the phyllosphere and carposphere of plants (e.g. apple, grape, cucumber, green beans, and cabbage, strawberry) it is known as a black yeast due to melanin production. It has a wide list of habitats (Domasch et al. 1980) and is mostly present in the temperate zone. During last few decades, *A. pullulans* has been described as an efficient biocontrol agent against fungal diseases (Di Francesco et al. 2020). It is also registered as a plant protection products such as Botector (Bio-Ferm, Tulln, Austria) prepared from a mixture of two isolates of *A. pullulans* (*A. pullulans* DSM14940 and *A. pullulans* DSM14941) available commercially against the post-harvest disease of grapes and strawberry (Romanazzi et al, 2016).

This study was conducted to determine the inhibitory potential of four different isolates of *A. pullulans* against three fungal pathogens: *B. cinerea*, *C. acutatum* and *P. cactorum* during *in vitro* as well as *in vivo* experiments. The research aimed to evaluate different approaches to identify the optimization method for application of locally isolated biological control isolate (AP-SLU6) on strawberry against three fungal pathogens and assess their validity by making a comparison between detached leaf and fruit assays during *in vitro* and *in vivo* experiments. Knowledge about their inhibitory effects will help to recognize the potential of biocontrol and pathogenicity of diseases in its presence and to identify suitable timing for its application in greenhouse conditions.

The research was based on two hypotheses;

1. Different isolates of *A. pullulans* (AP-30044, AP-30273, AP-53383 and AP-SLU6) can inhibit the activity of three fungal pathogens of strawberry i.e. *B. cinerea*, *C. acutatum* and *P. cactorum* during *in vitro* conditions.
2. Spray application of the locally isolated isolates AP-SLU6 can reduce the development of grey mould, anthracnose, and root rot under greenhouse conditions.

Results of this study suggest that *A. pullulans* can significantly inhibit the mycelial growth of the fungal pathogens employed in co-culturing; and also reduce the incidence of post-harvest diseases on strawberry plants under greenhouse conditions.

2. Materials and methods

2.1 Plant material

Two different strawberry (*Fragaria × ananassa*) cultivars, Ostara and Honeoye, were purchased from Plantagen (Lund and Malmö, Sweden). Strawberry fruits (cv. Honeoye) used in this experiment were directly picked from Borgeby Jordgubbar farm, Bjärred, Sweden.

2.2 Fungal isolates and maintenance conditions

Four *A. pullulans* isolates (AP-30044, AP-30273, AP-53383 and AP-SLU6), *B. cinerea* isolate B05, *C. acutatum* isolate SAO-1 and *P. cactorum* isolate RV4 were maintained on potato dextrose agar (PDA) medium at 25 °C.

2.3 Inoculum preparation

The conidia were harvested from a 4-weeks old culture of *A. pullulans*, isolate AP-SLU-6. *B. cinerea*, *C. acutatum* and *P. cactorum* were maintained on PDA plates at 25 °C by adding 6-8 ml of sterile water to the culture and followed by scraping the surface of the mycelium with a spreader to release the conidia. Conidia were counted using a haemocytometer (Hausser Scientific, Horsham, PA) under a light microscope (Laborlux 12 Leitz, Germany).

2.4 In vitro antagonistic assays

2.4.1 Fungal confrontation assay

The *in vitro* antagonistic ability of *A. pullulans* isolates against *B. cinerea*, *C. acutatum* and *P. cactorum* was determined by performing a dual-plate confrontation assay. A 9-cm PDA plate was inoculated with a 15- mm-diameter mycelial agar plug of an *A. pullulans* isolates on one side of the plate. After 7 days of incubation at 25 °C, an agar plug of *B. cinerea* was inoculated at an equal distance on the opposite side of the plate to compensate the growth difference while *C. acutatum* and *P. cactorum* were inoculated at the same day and mycelial growth was measured daily for up to 5 days post-inoculation (DPI). The assay was performed using six biological replicates and the growth rate was measured by the following formula;

Percentage reduction in mycelium growth = (Control – Treatment) / Control × 100

2.4.2 Detached fruit assay against *B. cinerea*

Strawberry fruits (cv. Honeoye) at different stages were harvested from the field and brought to the laboratory. Injuries to the fruits were made close to their neck. The conidia concentration of *A. pullulans* isolate AP-SLU6 and *B. cinerea* was maintained 15×10^5 /mL followed by inoculation of 15 μ L to the injured neck of each fruit, then sealed in the plastic tray and incubated at 22 °C and relative humidity of 70% for 10 days. The disease severity was scored at ten DPI as described previously (Adikaram et al, 2002). Four treatments were included: (1) fruits + *Bc* only, (2) fruits + AP-SLU6 only, (3) fruits + *Bc* + AP-SLU 6 (combine treatment), (4) fruits + *Bc* + AP-SLU6 (pre-application). In the third treatment, a mixture of the biocontrol and the pathogenic fungi was applied at the same time while treatment four was pre-treated with *A. pullulans* AP-SLU6 and followed by inoculation of *B. cinerea* after 48 hrs of incubation. The assay was performed on ten biological replicates of each treatment.

2.4.3 Detached leaf assay against *C. acutatum*

Strawberry leaves (cv. Ostara) were detached from 24 different plants and brought to the laboratory where two injuries were made underneath of leaves with a sharp needle. The conidia concentration of *A. pullulans* isolate AP-SLU6 and *C. acutatum* was maintained 1.5×10^6 /mL and subsequently inoculated by applying 20 μ L to the injured surface of each leaf and incubated at 25 °C, relative humidity 90 %. The lesion diameter was measured by ImageJ software (version 1.52p) at 10 DPI. Five treatments were included: (1) leaves + water, (2) leaves + *Ca* only, (3) leaves + AP-SLU6 only, (4) leaves + *Ca* + AP-SLU6 (combine treatment), (5) leaves + *Ca* + AP-SLU6 (pre-application). Pre-treatment with *A. pullulans* AP-SLU6 was applied after 24 hr incubation followed by inoculation of *C. acutatum* while in the combined treatment, a mixture of the biocontrol and the pathogenic fungus was applied at the same time. The assay was performed on six biological replicates of each treatment.

2.4.4 Detached leaf assay against *P. cactorum*

Strawberry leaves (cv. Ostara) were detached and injuries were made as described in the previous paragraph. The conidia concentration of the *A. pullulans* isolate AP-SLU6 and *P. cactorum* was maintained at 1.5×10^5 /mL followed by inoculation of 20 μ L to the injured surface of each leaf and then incubated at 22 °C, relative humidity 90 %, for one week. The lesion diameter was

measured at seven DPI by ImageJ software (version 1.52p). Five treatments were included: (1) leaves + water only, (2) leaves + *Pc* only, (3) leaves + AP-SLU6 only, (4) leaves+ *Pc* + SLU-6 (combine treatment), (5) leaves + *Pc* + SLU6 (pre-application). In the fourth treatment, a mixture of the biocontrol and the pathogenic fungus was applied at the same time while treatment fifth was pre-treated with *A. pullulans* AP-SLU6 and followed by inoculation of *P. cactorum* after 24 hr of incubation. The assay was performed on six biological replicates of each treatment.

2.5 In vivo biocontrol assays

2.5.1 Biocontrol of grey mould disease

A grey mould disease assay on strawberry (cv. Honeoye) plants was performed in a greenhouse in a complete randomised design. Plastic pots were filled with 150 g of soil and planted with strawberry plants and maintained under 20 to 22 °C. The same conidial concentration as used in the detached leaf assay against *B. cinerea*. The prepared formulations of *A. pullulans* isolate AP-SLU6 was sprayed on flowers and fruits every after seven days during the three weeks of the experiment. The assay was performed on five treatments; (1) plant + water only, (2) plant + *Bc*, (3) plant + AP-SLU6 only, (4) plant + *Bc* + AP-SLU6 (combine treatment), (5) plant + *Bc* + AP-SLU-6 (pre- application). Six biological replicates were used for each treatment. Pre-treatment was performed with AP-SLU6 followed by inoculation of *B. cinerea* after 48 hr of incubation while in combine treatment, a mixture of the biocontrol and the pathogenic fungus was applied at the same time. The disease severity was scored after one month of harvesting the experiment by using scale 0 = no fungal growth, 1 = fungal growth only on the margin of lesion, 2 = even but slight fungal growth all over, and 3 = dense fungal growth all over (Adikaram et al. 2002).

2.5.2 Biocontrol of anthracnose disease

An anthracnose disease assay on strawberry (cv. Honeoye) was performed in a greenhouse in a complete randomised design. Plastic pots were filled with 150 g of soil and planted with strawberry plants and maintained under 23 ± 2 °C. The same conidial concentration used as during detached leaf assay against *C. acutatum*. The prepared formulations of *A. pullulans* isolate AP-SLU6 was sprayed on leaves after seven days interval over the three-weeks course of the experiment. Five treatments were included: (1) plants + water only, (2) plants + *Ca*, (3) plants + AP-SLU6, (4) plants + *Ca* + AP-SLU6 (combine treatment), (5) plants + *Ca* + AP-SLU6 (pre-

application). Six biological replicates were used for each treatment. In combined treatment, a mixture of the biocontrol and the pathogenic fungus was applied at the same time while pre-treatment with AP-SLU6 followed by inoculation of *C. acutatum* after 48 hr of incubation. Scoring was done after one month with the following disease rating scale: 0 = healthy, 1 = < 3 mm lesion, 2 = 3 - 10 mm lesion, 3 = > 10 - 20 mm lesion, 4 = > 20 mm, 5 = young leaves dead, 6 = whole plant dead (Smith and Black 1987).

2.5.3 Biocontrol of root rot disease

A root rot disease assay on strawberry (cv. Honeoye) plants was performed under a semi-greenhouse setting using a complete randomised design. The strawberry plant roots were removed from plastic pots and washed with tap water and subsequently placed in glass jars which were filled with almost 150 mL of tap water. The same conidial concentration used as during detached leaf assay against *P. cactorum*. The assay was performed using five treatments: (1) plant + water only (2) plant + *Pc* only, (3) plant + AP-SLU6 only, (4) plant + *Pc* + AP-SLU6 (combine treatment) (5) plant + *Pc* + AP-SLU6 (pre-application). Six biological replicates were used for each treatment. In the combined treatment, a mixture of the biocontrol and the pathogenic fungus was applied at the same time while pre-treated with SLU-6 followed by inoculation of *P. cactorum* after 24 hr of incubation. Afterwards, the roots were removed from the jars and placed in plastic pots which were filled with 150 g of soil and stored in a greenhouse under 23 ± 2 °C for 4 weeks. The disease scoring was performed after one month with scale 0 - 20% = 1, 21 - 40% = 2, 41 - 60% = 3, 61 - 80% = 3, 61 - 80% = 4, 81 - 100% = 5 (Samuelsson 2019).

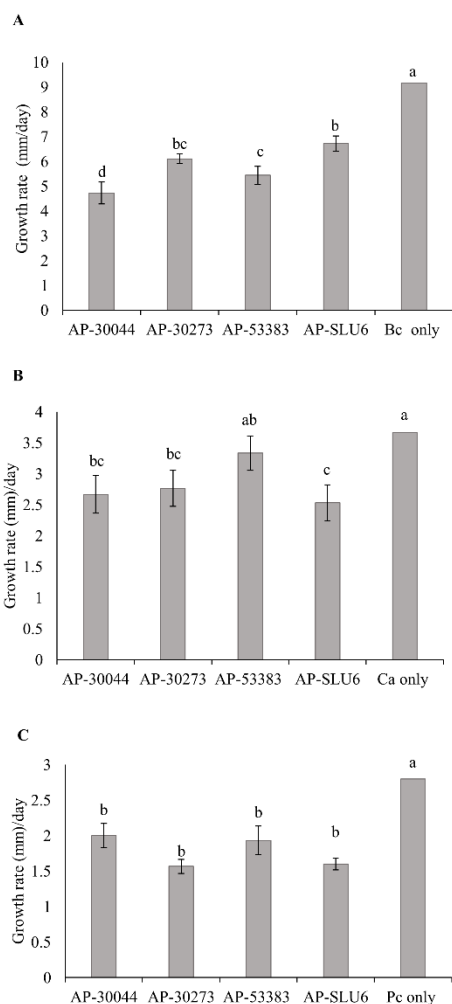


Figure 2: Results of antagonism tests including four different *Aureobasidium pullulans* isolates (AP-30044, AP-30273, AP-53383 and AP-SLU-6) against *Botrytis cinerea*, *Colletotrichum acutatum*, *Phytophthora cactorum* on potato dextrose agar (PDA) Petri plates. A, B, and C shows the growth rate (mm) of *B. cinerea*, *C. acutatum*, *P. cactorum* during confrontation with the four *A. pullulans* isolates as well as alone (control). Agar plates of *B. cinerea*, *C. acutatum*, *P. cactorum* were inoculated on opposite sides of 9-cm diameter Petri plates and the cultures were incubated at 25°C. The growth rate (mm) of *B. cinerea* (A) was recorded at 3 DPI while the growth rates of *Colletotrichum acutatum* (B) and *Phytophthora cactorum* (C) were measured at 5 DPI. The error bars are based on six biological replicates. Different letters represent statistically significant differences ($P \leq 0.05$) between treatments as determined by Fisher's least significant difference method.

3. Results

3.1 *In vitro* antagonism assay against fungal pathogens

The antagonistic ability of the four *A. pullulans* isolates was tested against three fungal pathogens in dual-plate confrontation assays. *B. cinerea* and *C. acutatum* showed significantly reduced growth rate ($P \leq 0.05$) during confrontation with *A. pullulans* isolates compared with their growth rates when grown alone (control) at 3 and 5 DPI respectively (Figure 2A-B). The AP-30044 isolate reduced growth with 48 % (Table 1) of *B. cinerea* followed by AP-53383 compared with control treatment while AP-3027 and AP-SLU6 were equally effective in reducing growth of *B. cinerea* (Figure 2A). However, in the case of *C. acutatum*, the AP-30044, AP-30273 and AP-SLU6 isolates showed equal ability in reducing the growth while AP-53383 showed no significant difference compared with control treatment (Figure 2B). Similarly, *P. cactorum* also showed significantly reduced growth rate ($P \leq 0.05$) when challenged with *A. pullulans* isolates compared with control treatment at 5 DPI (Figure 2C). All *A. pullulans* isolates equally reduced the growth 42% (Table 1) of *P. cactorum* as compared with control treatment (Figure 2C).

Table 1: Percentage inhibition of mycelial growth of fungal pathogens

Treatments	Percentage reduction in mycelial growth of <i>B. cinerea</i>	Percentage reduction in mycelial growth of <i>C. acutatum</i>	Percentage reduction in mycelial growth of <i>P. cactorum</i>
AP-30044	48	27	28
AP-30273	33	24	44
AP-53383	40	9	30
AP-SLU6	26	30	42

Percentage inhibition of mycelial growth of fungal pathogens in presence of *Aureobasidium pullulans* isolates calculated by formula, percentage of reduction in mycelial growth = $\frac{\text{Control} - \text{Treatment}}{\text{Control}} \times 100$. This table demonstrate that AP-30044 showed maximum inhibition against growth of *Bc* followed by AP-SLU6 against *Ca* and AP-30273 against *Pc* i.e. 48%, 30% and 44% respectively. *Bc* = *Botrytis cinerea*, *Ca* = *Colletotrichum acutatum*, *Pc* = *Phytophthora cactorum*.

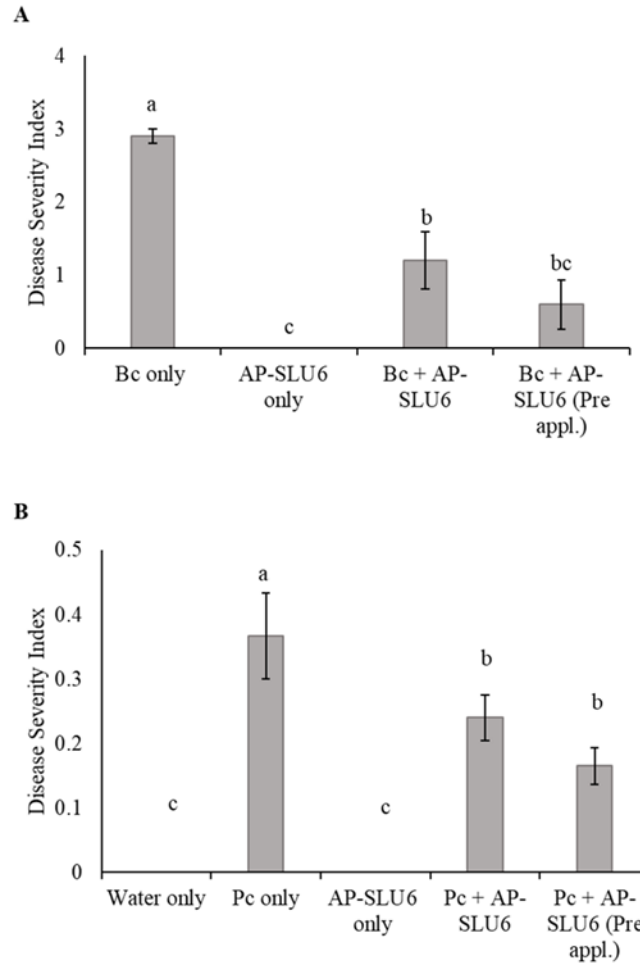


Figure 3: Disease severity of detached strawberry fruits was scored according to disease rating scale as described previously (Adikaram et al. 2002). Briefly, 0 = no fungal Growth, 1= fungal growth on margins of lesion, 2 = even but slightly fungal growth all over, 3 = dense fungal growth all over. Standard error was based on 10 biological replicates. Different letters represent a statistically significant difference ($P \leq 0.05$) between treatments as determined by Fisher's least significant difference method. A. *Bc* = *Botrytis cinerea*, AP-SLU-6 = *Aureobasidium pullulans*. Necrotic lesion size of detached leaf assay was measured at 7 DPI using ImageJ software (version 1.52p). Briefly, 0 - 20% = 1, 21 - 40% = 2, 41 - 60% = 3, 61 - 80% = 4, 81 - 100% = 5 (Samuelsson 2019). Standard error was based on 6 biological replicates. Different letters represent a statistically significant difference ($P \leq 0.05$) between treatments as determined by Fisher's least significant difference method. B. *Pc*= *Phytophthora cactorum*, AP-SLU6 = *Aureobasidium pullulans*.

3.2 Detached fruit assay against *B. cinerea*

B. cinerea on wounded strawberry fruits showed significantly reduced growth ($P \leq 0.05$) when applied with the AP-SLU6 isolate in combination with *B. cinerea* inoculum as well as in pre-treated fruits with AP-SLU6 followed by *B. cinerea* application compared with the growth of *B. cinerea* when applied alone (control). However, no significant difference was found between fruits pre-treated with AP-SLU6 followed by *B. cinerea* as compared to when AP-SLU6 was applied alone (Figure 3A).

3.3 Detached leaf assay against *C. acutatum*

The assay was performed to determine the antagonistic activity of AP-SLU6 against *C. acutatum*, however, no infection was observed on leaves treated with *C. acutatum* at 10 DPI. Therefore, failed to determine the antagonistic ability against *C. acutatum*.

3.4 Detached leaf assay against *P. cactorum*

The necrotic lesion size on strawberry leaves was significantly reduced ($P \leq 0.05$) in treatment where AP-SLU6 was applied in any form of combination either combined or pre-treated application compared with the control treatment where only *P. cactorum* was applied (Figure 3B). However, no significant difference was observed when AP-SLU6 applied alone or water treatment. The necrotic lesions size was measured at 7 DPI.

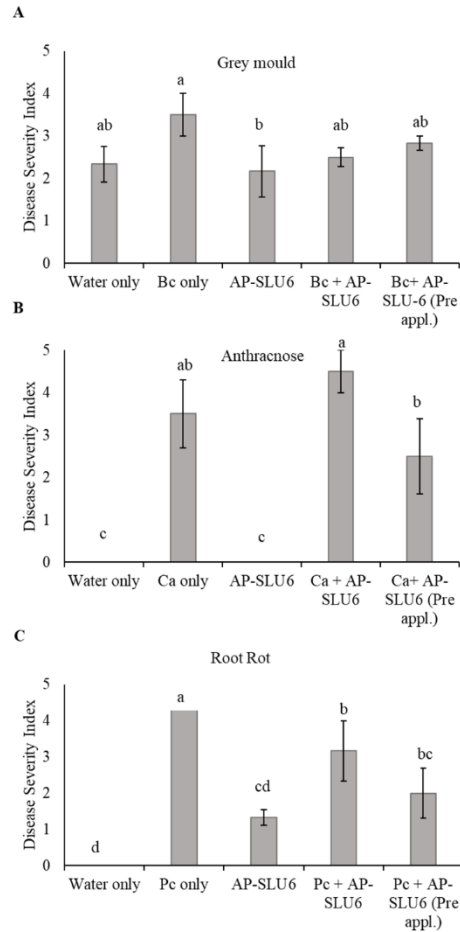


Figure 4: Effect of application of APSLU-6 against grey mould, anthracnose and root rot A, B, and C. Application of APSLU-6 on grey mould of diseases on potted strawberry plants cv. Honeoye was assessed by scale 0 = no fungal growth, 1= fungal growth on margins of lesion, 3= even but slightly fungal growth all over, 4 = dense fungal growth all over (Adikaram et al. 2002) as shown in A. However, scale followed in assessing anthracnose in potted strawberry plants cv. Ostara was 0 = healthy, 1= < 3 mm lesion, 2 = 3 - 10 mm lesion, 3 = >10 – 20 mm lesion, 4 = >20 mm, 5= young leaves dead, 6 = whole plant dead (Smith and Black, 1987) as shown in B. In case of root rot of potted strawberry plants cv. Ostara disease severity was measured by scale 0 - 20% = 1, 21 - 40% = 2, 41 - 60% = 3, 61 - 80% = 4, 81 - 100% = 5 in C (Samuelsson 2019) . Disease severity was investigated by spray application of different treatments of AP-SLU6 applied in potted strawberry plants after one month and analysed by one way ANOVA along with Fisher pairwise comparison method. Different letters represent statistically significant differences ($P \leq 0.05$) between treatments as determined by Fisher's test

3.4 Biocontrol of grey mould

Spray application of AP-SLU6 in any form of combination either combined or pre-treated application followed by *B. cinerea* reduced the disease severity of the grey mould disease compared with the control treatment. However, this reduction was not statistically different (Figure 4A). Only AP-SLU6 application under natural condition significantly ($P \leq 0.05$) enhanced the biocontrol efficacy against grey mould disease compared with control treatment where only *B. cinerea* was applied (Figure 4A).

3.5 Biocontrol of anthracnose disease

Spray application of AP-SLU6 in pre-treated application followed by *C. acutatum* reduced the disease severity of the anthracnose disease compared with control treatment where only *C. acutatum* was applied, however, this reduction was not statistically different (Figure 4B). Only AP-SLU6 and water application alone under natural condition significantly ($P \leq 0.05$) enhance the biocontrol efficacy against anthracnose disease compared with control treatment where only *C. acutatum* was applied (Figure 4B).

3.6 Biocontrol of root rot disease

Application of AP-SLU6 in any form of combination either combined or pre-treated application followed by *P. cactorum* significantly ($P \leq 0.05$) reduced the disease severity (approximately 60%) of the root rot disease compared with control treatment where only *P. cactorum* was applied. (Figure 4C).

4. Discussion

The current study was designed to investigate the biocontrol potential of *A. pullulans* against fungal pathogens which are involved in causing the post-harvest diseases of strawberry. The *in vitro* antagonistic assays were carried out to examine the direct interaction of biocontrol fungus *A. pullulans* against *B. cinerea*, *C. acutatum* and *P. cactorum* on Petri plates during confrontation assays. All four isolates of *A. pullulans* significantly reduced the growth of *B. cinerea*, *C. acutatum* and *P. cactorum* while no obvious difference was observed among *A. pullulans* isolates. The mechanism behind the observed inhibitory impact on mycelia growth is not known, but was possibly generated by the production of volatile compounds, lipids, enzymes or antifungal secondary metabolites. It was demonstrated before that *A. pullulans* can degrade *B. cinerea* cell walls by secreting chitinase and protease in potato (Chen et al. 2018). Besides, *A. pullulans* has been shown to secrete volatile compounds that inhibits the growth of *C. acutatum* (Di Francesco et al, 2015). Moreover, *A. pullulans* is capable of competing for nutrients which could be attributed to antagonist action as *B. cinerea* required a huge amount of nutrients for its growth and development of infection in host (Blackman and Brodie 1977). The exact mechanisms underlying the results in this work should be further investigated in follow-up studies.

A. pullulans is well adapted to live in the phyllosphere of plants and has also been isolated from strawberry fruits (Buhagiar and Barnett 1971; Lima et al 1997). Therefore, it is interesting to study the inhibitory potential of naturally occurring *A. pullulans* isolates on strawberries. In the present study, inoculation on detached leaves and fruit with pathogens allowed assessment of disease in the presence of biocontrol agents without demolishing plants and also reduced the time between inoculation and disease estimation. The impact of the pre-treated and combined application of the two pathogens *B. cinerea* and *P. cactorum* with AP-SLU6 showed similar patterns in controlling the growth of fungal mycelium in detached fruit assay and necrotic lesion in detached leaf assay, but no difference was observed between combined or pre-treated application. The results of the present study is in agreement with the previous findings by Adikaram et al. (2002) where pre-treatment and combined application of *A. pullulans* suppressed the growth of grey mould on wounded sites of green fruits. Similarly, work with other biocontrol agents (e.g. bacterial strains like *Pseudomonas fluorescens*) showed inhibitory effects on the growth of *P. cactorum* on detached strawberry leaves mainly due to the production of metabolites and siderophore (Agusti et al. 2011).

Whereas, experiment performed of detached leaf assay against anthracnose, no symptoms were observed and it is postulated that there is a need to optimize spore concentration for introducing infection of anthracnose.

This study further showed that spray application of AP-SLU6 isolate either used as pre-treated or combined with pathogen on strawberry plants delayed the development of grey mould. Research conducted by Di Francesco et al. (2020), that *A. pullulans* has potential to reduce incidence of grey mould in tomato during *in vivo* assay. Moreover, it is also shown in previous research that *A. pullulans* has potential to control *B. cinerea* on strawberry and also rotting on cherry fruits, grapes and kiwi fruits (Ippolito et al. 1997; Schena et al. 1999). According to Jersch et al. (1989), reduction in the disease was attributed to quiescent pathogen and green fruits as an extract of green fruits contain an antifungal compound called proanthocyanidins and its higher concentration in receptacle reduce colonization of *B. cinerea* that might help in controlling grey mould biocontrol will be more effective when applied in intermediate time on strawberry.

Plants treated with only water were placed in the same chamber developed an infection of grey mould and possible explanation could be that this pathogen spread through air and water splashes therefore, it can be disseminated to water treated plants.

Spray application of AP-SLU6 isolate in pre-treatment decreased the development of anthracnose. These findings are similar to Mari et al. (2012), who showed that two other *A. pullulans* isolates reduced the growth of infection of *C. acutatum* when applied on the wound as pre-treatment on apples. Whereas, in performed experiment, combined application showed an increase of disease when compared to control and I speculated that the enhancement of disease might be due to timing of application during different growth stages of plants. Interestingly, strawberry plants treated with *C. acutatum* was placed in the same chamber with plants having an infection of the grey mould but they did not show any symptoms of *B. cinerea* and this finding is complement to (Tomas-Grau et al. 2020) that axenic culture filtrate of *C. acutatum* act as barrier to the growth of *B. cinerea* in strawberry plants which is due to production of autofluorescent compounds.

In this study, strawberry plants were dipped in *A. pullulans* solution to make direct exposure and attachment on roots and to have a minimal environmental impact. This selected AP-SLU6 isolate showed inhibition of root rot in both treatments either in pre or in combined application when compared to plants treated with *P. cactorum* that were dead. Only AP-SLU6 application on roots of strawberry plants showed browning and it is hard to differentiate that it is due to the age of plants or non-target effects of biocontrol when introduced into soil. The potential of biocontrol agent which is discussed in this work showed positive results against all three fungal pathogens during *in vitro* and *in vivo* under greenhouse conditions.

5. Conclusions

B. cinerea, *C. acutatum*, *P. cactorum* are well-known pathogens of strawberry and there is a strong global interest in controlling these pathogens using biocontrol. This research was performed using different isolates of *A. pullulans*, a potential biocontrol agent against these three fungal pathogens. The tested *A. pullulans* isolates had a significant disease suppression during *in vitro* assays. Introduction of biocontrol in the phyllosphere of strawberries under greenhouse conditions is very complex. However, a locally isolated isolate of *A. pullulans* AP-SLU6 controlled incidence of grey mould and root rot *in vivo* assays. To conclude, the present thesis shows that the biocontrol fungus *A. pullulans* can inhibit the growth of fungal pathogens and these properties will be excellent tools in fungicide-free strawberry production.

6. Future perspectives

Biological control has earned increasing popularity over chemical control, especially during the last decades. *A. pullulans* has been documented in the literature for its safe application in biotechnology and the environment (Bozoudi and Tsaltas 2018). The present research investigated its biocontrol potential, but there is a need for further analysis on some other aspects. Future studies requires characterization of the compounds secreted by different *A. pullulans* isolates to know the exact mechanism of inhibition of fungal growth. Biocontrol agents do not necessarily rely on a single mode of action but based on a series of multiple mechanisms. Therefore, exploitation of different mechanisms of *A. pullulans* is needed, and will aid in the commercialization of biocontrol agents. *A. pullulans* have high genetic variability therefore, morphological characteristics are not sufficient to check variability among closely related isolates. Molecular analysis (DNA/RNA level and proteins, including phylogenetic) is required to know about genetic complexity.

Introduction of biocontrol agents in the phyllosphere is complex because of its dynamic environment and unstable habitat. Inconsistent conditions make biocontrol agents challenging to establish in plants. Hence, deep analysis of the interactions of *A. pullulans* with non-targeted microorganisms, its methods of application, concentrations of inoculum, improved formulations, should be considered in future studies.

Finally, as different isolates of *A. pullulans* show different results, and perhaps different modes of action, there is a need to test combined applications of different isolates. Such combined application could potentially increase disease suppression. Moreover, to investigate the complete vision of the complex interactions of biocontrol with resident microflora, multi-omics need to be explored

7. References

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Thesis Title: Evaluation of *Aureobasidium pullulans* as potential biocontrol agent against strawberry diseases

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Résumé

L'utilisation d'agents de lutte biologique pour lutter contre les maladies fongiques est une approche prometteuse dans la culture des fraises. *Aureobasidium pullulans*, un champignon de type levure décrit comme antagoniste de divers agents pathogènes post-récolte. J'ai mené des expériences pour étudier l'efficacité et la différence dans l'activité antagoniste de quatre isolats différents d'*A. Pullulans* (AP-30044, AP-30273, AP-53383 et AP-SLU6) contre des pathogènes fongiques du fraisier d'importance économique, à savoir *B. cinerea*, *C. acutatum* et *P. cactorum* dans des conditions *in vitro* et *in vivo*. Le résultat de la présente étude suggère que lors des tests de confrontation sur des boîtes de Pétri, les isolats d'*A. Pullulans* inhibent la croissance mycélienne des trois pathogènes fongiques. Les inoculations de plaies de fruits / feuilles traitées avec *B. cinerea* et *P. cactorum* ont également été supprimées par l'application de l'isolat AP-SLU6 d'*A. Pullulans* isolé localement. L'application par pulvérisation de l'isolat AP-SLU6 *in vivo* a réduit l'incidence de la moisissure grise et de l'antracnose, tandis que dans le cas de la pourriture des racines, une réduction significative a été observée. Cette étude indique donc qu'*A. Pullulans* peut être considéré comme un agent de lutte biologique potentiel pour lutter contre ces agents pathogènes dans le fraisier de jardin.

Mots-clés: *Aureobasidium pullulans*, *Fragaria × ananassa*, anthracnose, pourriture grise, pourriture des racines.

Abstract

Utilization of biocontrol agents to control fungal diseases is a promising approach in strawberry cultivation. *Aureobasidium pullulans*, a yeast-like fungus described as antagonists of various post-harvest pathogens. I conducted experiments to investigate the efficacy and difference in antagonistic activity of four different isolates of *A. pullulans* (AP-30044, AP-30273, AP-53383, and AP-SLU6) against economically important strawberry fungal pathogens, i.e. *B. cinerea*, *C. acutatum* and *P. cactorum* during *in vitro* and *in vivo* conditions. The result of the present study suggests that during confrontation assays on Petri plates *A. pullulans* isolates inhibit the mycelial growth of all three fungal pathogens. Wound inoculations of fruit/leaf treated with *B. cinerea* and *P. cactorum* were also suppressed by application of the locally isolated *A. pullulans* isolate AP-SLU6. Spray application of the AP-SLU6 isolate *in vivo* reduced the incidence of grey mould and anthracnose whereas; in the case of root rot significant reduction was observed. This study thus indicates that *A. pullulans* can be regarded as a potential biocontrol agent to control these pathogens in garden strawberry.

Keywords: Anthracnose, *Aureobasidium pullulans*, *Fragaria × ananassa*, Grey mould, Root rot