Biological control of verticillium stem striping disease on oilseed rape

Fungi as friends and foes

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Swedish University of Agricultural Sciences, SLU
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Agricultural Programme – Soil and Plant Sciences
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Biological control of verticillium stem striping disease on oilseed rape. Fungi as friends and foes.

Biologisk bekämpning av kransmögel orsakad av Verticillium på raps. Svampar som vänner och ovänner.

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Abstract

The cultivation of oilseed rape faces challenges caused by pests and pathogens, and soil-borne pathogens such as *Plasmodiaphora brassicae* (clubroot) are among the main causes of disease. *Verticillium longisporum* is another soil-borne pathogen, which causes the verticillium stem striping disease in Brassica plants, including oilseed rape. In this project the fungal biological control agent *Clonostachys rosea* was used in an attempt to utilize biological control of verticillium stem striping disease in oilseed rape. The main *in planta* experiment was perfomed to investigate this hypothesis, but the results showed no significant effect of *C. rosea* to prevent verticillium stem striping disease in oilseed rape. Three *in vitro* experiments were also performed to study the interactions between these two fungi, *C. rosea* and *V. longisporum,* and showed that *C. rosea* can inhibit the growth of *V. longisporum* in the *in vitro* setting. In summary, *C. rosea* show some potential of being able to control *V. longisporum* in an *in vitro* setting, but none *in planta.*

Keywords: Biological control, Oilseed rape, *Clonostachys rosea*, *Verticillium longisporum*, Verticllium stem striping disease

Sammanfattning

Odlingen av raps står inför utmaningar orsakade av skadedjur och patogener, och jordburna patogener som *Plasmodiaphora brassicae* (klumprotsjuka) är en av de huvudsakliga orsakerna till sjukdom. *Verticillium longisporum* är en annan jordburen patogen som orsakar kransmögel på *Brassica*-växter, inklusive raps. I det här arbetet testades den biologiska bekämpningssvampen *Clonostachys rosea* i ett försök att med hjälp av biologisk bekämpning skydda raps mot kransmögel orsakad av *V. longisporum*. Ett *in planta*-försök utfördes för att testa denna hypotes, men resultatet visade ingen signifikant effekt av *C. rosea* för att bekämpa kransmögel orsakad av *V. longisporum* på raps. Tre mindre *in vitro*-försök utfördes också för att undersöka hur de två svamparna *C. rosea* och *V. longisporum* interagerar med varandra. De visade att *C. rosea* kan hämma *V. longisporums* tillväxt i en *in vitro*-miljö. Sammanfattningsvis visade *C. rosea* en viss potential att kunna bekämpa *V. longisporum* i en *in vitro*-miljö, men uppvisade ingen effekt *in planta*.

Nyckelord: Biologisk bekämpning, Raps, *Clonostachys rosea*, *Verticillium longisporum*, Kransmögel

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1. Introduction

Oilseed rape is one of the most important arable oilseed crops worldwide, second after soybean (Berry et al. 2012). In Sweden, it is the most important one. During the 10 year period 2010-2020 it was annually grown on about 4 percent of Sweden's arable land (SCB 2020). Now the trend seems to inflate, with an increase of 8 percent of total oilseed rape acreage in 2021 from the year before, and an increase of 20 percent in 2022 from the year before. The total acreage of oilseed rape in Sweden 2022 was the highest recorded since 1994 (SCB 2021; SCB 2022a).

However, it is also one of the most chemically controlled crops for pathogenic fungi in Sweden, in 2021 it was the second one after potato. In the crop year 2020/21 almost half (48%) of the oilseed rape crop acreage in Sweden was treated with fungicides (SCB 2022b) to control diseases such as sclerotinia disease, dark leaf spot and light leaf spot (Jordbruksverket 2022a; Jordbruksverket 2022b; Jordbruksverket 2022c). Although chemical control indeed can be a powerful tool to control diseases in crops, there are ecological implications and dangers that need to be considered (Edwards 1993). To minimize our dependence of chemical pesticides is one of today's top priorites in agriculture (Directorate-General for Agriculture and Rural Development 2023). One way to do this is with the implementation of biological control. By adding living agents (organisms or viruses) to the crop we can give it a natural protection against the dangers from pathogens. In other words, by enhancing the competitor population we can suppress the pest populations (Driesche & Bellows 2012).

There are several pathogens that pose a threat to the oilseed rape crop production. One of the pathogens of oilseed rape is *Verticillium longisporum* that was first reported in southern Sweden in 1969 (Kroeker 1970). Belonging to the genus *Verticillium*, *V. longisporum* is an ascomycete, but unlike the closely related *V. dahliae* the host range of *V. longisporum* is more limited and it solely prefer brassicaceous plants. Unlike the common name "verticillium wilt" for *Vertcillium* infections, the preferred name for *Verticillium* infections on oilseed rape is "vertcillium stem striping disease", since it does not cause wilting symptoms in oilseed rape. As of today there is no effective fungicide treatment or fully-resistant cultivars available for *V. longisporum* (Depotter et al. 2016).

Fortunately, *V. longisporum* is not devoid of competition, as *Clonostachys rosea* emerges as a potential antagonist. Also being an ascomycete fungus, *C. rosea* is

described as an ecological generalist and an aggressive mycoparasite with little harmful effect on the plant host. It has already proven to be an effective biological control agent for several other diseases in many different crops, including grey mould (*B. cinerea*) in strawberries and Fusarium head blight (*Fusarium* spp.) along with root- and stem base rot (*Phytophthora* spp. and *Sclerotinia* spp.) in grain crops (Jensen et al. 2021). However, *C. rosea* has not been tested against *V. longisporum.*

To find out whether biological control of verticillium stem striping disease is possible we need to try it out in a proper setting. This suggests a system that simulates the natural conditions wherein the biocontrol agent will be used, hence an *in planta* experiment where both the biocontrol agent, pathogen, plant and its enviroment are present is favourable. The complexity of the biocontrol trait make *in vitro* ("in glass"; composing an abnormal biological context) experiments an inappropriate setting for the screening process, although they can be a formidable appliance in determining details of the interactions between different participants (Köhl 2021). Hence, the *in vitro* experiments of this project were conducted to study the interactions between the two fungi with the three research questions: (1) Can *C. rosea* inhibit the growth of *V. longisporum* when in direct contact? (2) Can the secondary metabolites produced by *C. rosea* inhibit the growth of *V. longisporum*? (3) Can the presence of *C. rosea* in the plant, oilseed rape, inhibit the presence of *V. longisporum*?

The *in planta* experiment was conducted to answer this project's main hypothesis: Can the biocontrol agent *C. rosea* be used to control stem striping disease caused by *V. longisporum* on oilseed rape?

2. Background

2.1 *Brassica napus* (oilseed rape)

Brassica napus, commonly oilseed rape, is a member of the Brassicaceae family with bright-yellow flowers. It is cultivated for its oil-rich seeds, which are used for both human consumtion and animal feed (Williams 2010). It is an important crop globally, especially in temperate regions, with the largest production in Europe followed by Canada, China and India (FAO 2023). The benefical effects of oilseed rape as a break crop in crop rotations are well documented (Angus et al. 1991; Kirkegaard et al. 1994; Angus et al. 2015), especially in cereal dominated cropping systems, which are typical in Sweden (SCB 2020). This is due to its deep taproot that loosens up the soil and its ability to inhibit pests, pathogens and weeds associated with cereal crops, resulting in a higher yield of the following crop (Kirkegaard et al. 1994; Angus et al. 2015).

Unfortunately, the cultivation of oilseed rape faces several challenges. The crop is susceptible to a large number of pests and diseases. Soil-borne pathogens, such as *Plasmodiophora brassicae* (clubroot) and *Verticillium longisporum* (verticillium stem striping disease), are among the main causes of disease. This is problematic since it limits the possibillity of including a high recurrence of oilseed rape in the crop rotation (Zheng et al. 2020).

There is also growing concern about the environmental impact of oilseed rape cultivation, particularly with regard to pesticide use (Wallenhammar et al. 2022). In the crop year 2020/21 almost all (82%) of the oilseed rape crop area in Sweden was treated with weed pesticides, and half (48%) with fungi pesticides (SCB 2022b). To reduce this use of pesticides in the cultivation of oilseed rape, alternative pest management strategies such as adopting integrated pest management (IPM) practices, including the use of biological control, are necessary (Wallenhammar et al. 2022).

2.2 *Verticillium longisporum*

Verticillium longisporum is a soil-borne fungus, as are all *Verticillium* species, which produces resting structures that can stay dormant and viable for more than 10 years (Pegg and Brady 2002). This characteristic allows the pathogen to spread over time and space via various means, including water, farm equipment and human interference. Furthermore, *V. longisporum* is a plant pathogen that evolved through hybridization, the crossing of previously separated species (Inderbitzin et al. 2011).

The disease caused by *V. longisporum* is monocyclic, meaning that there is one infection cycle per crop cycle. Its life cycle (Fig. 1) starts with the germination of dormant microsclerotia (survival units capable of producing infective conidia, a type of asexual reproductive spore, in favorable conditions) in the soil, in response to plant root exudates. The pathogen then grows as hyphae towards the root and enters the plant through open wounds or by direct penetration. Once inside, the fungus grows as hyphae both inter- and intracellularly until it reaches the xylem, the plant's water transport system, and is distributed throughout the plant. Finally, the cycle completes when microsclerotia are produced in the stem and released back into the soil when the plant residue decompose (Depotter et al. 2016).

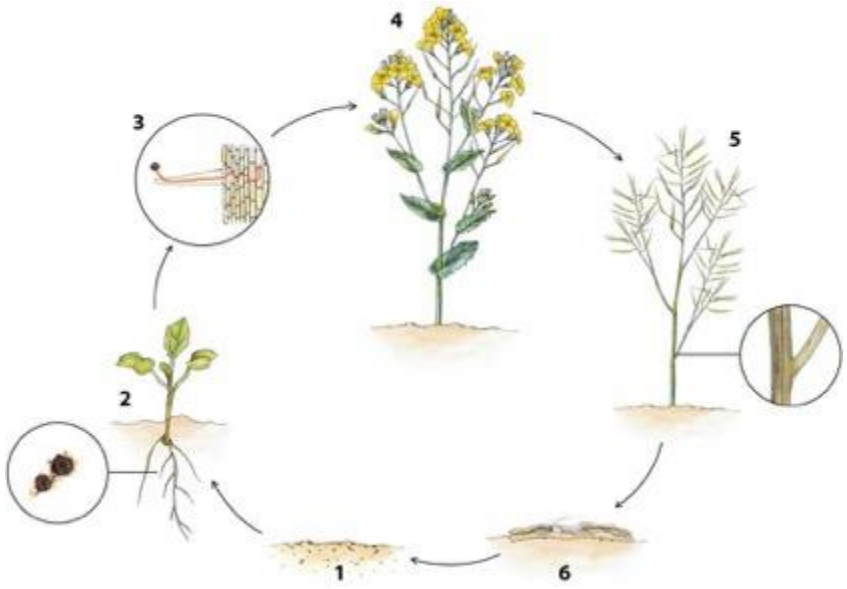

Figure 1. Disease cycle of verticillium stem striping disease on oilseed rape (adapted from Depotter et al. 2016).

Even though there is some size overlap between the conidia of *V. longisporum* and other *Verticillium* species, the conidia of *V. longisporum* are generally longer and the microsclerotia elongated, thus its name (*longisporum* = long spores). However, it is the brassicaceous host-specificity of *V. longisporum* that credits it as its own species (Karapapa et al.1997).

Verticillium stem striping disease is an inconspicuous one, as it is difficult to detect in a growing field. Three symptoms occur; (1) stunted plants, smaller and less developed than expected (Floerl et al. 2008), (2) yellow leaves, and (3) dark unilateral striping on the stem of the plant during the ripening of the crop (Fig. 2) (this is microsclerotia produced by the pathogen in the stem cortex). No other disease symptoms occur during the growing season (Depotter et al. 2016). Even though these symptoms can make the disease difficult to detect in a growing field, the yield and economic losses have been widely noted for a long time in both Sweden and internationally (Heale & Karapapa 1999).

Figure 2. Symptoms of verticillium stem striping disease on oilseed rape (adapted from Depotter et al. 2016).

As mentioned in the introduction, no effective fungicide treatment or resistance cultivars are available for *V. longisporum* (no genuine resistance genes are available). Despite this, host resistance remains the preferred control method for verticillium stem striping disease, which require more screening for resistance sources in oilseed rape and their incorporation in new cultivars (Depotter et al. 2016). Another management strategy is crop rotation, but few studies have investigated its efficacy against *V. longisporum.* Given that the microsclerotia can remain viable and dormant for over 10 years, crop rotation may prove to be a challenging control method (Depotter et al. 2016).

2.3 Biological control

Biological control, or biocontrol, is one half of what is usually refered to as bioprotection. This can be used as an umbrella term to include the two types of plant protection agents; living agents (biological control) and non-living agents (nature-based substances). Consequently, biological control is the use of living agents to combat pest and pathogens. This is done by taking advantage of basic ecological interactions known as symbiotic relationships (like predation, parasitism, pathogenicity, competition, antagonism, etc.; Stenberg et al. 2021).

The practice of biological control has likely been used in some manner for as long as cultivation has existed. It has definitely been used for at least a millennium, as the first record of its use was in China year 304 (Shijiang 1983). Nevertheless, it first became an effective technique similar to today's practice in the 1890s (Bosch et al. 1982). Albeit existing for so long, it has in recent years received a boost of interest as a reasonable alternative to wean off from our chemical control methods (Stenberg et al. 2023).

Biological control consist of two principal approaches; (i) enhancement of resident antagonistic agents in the cropping system (conservation biological control), and (ii) adding of new antagonistic agents to the cropping system (classical and augmentative biological control, depending on wether the agent is to be permenantly or temporarily established; Stenberg et al. 2021).

Conservation biological control, the enhancement of pre-existing natural enemies, is achieved through minimizing harmful practices (e.g. pesticide use and habitat disruption associated with soil management) or incoporating beneficial practices (e.g. supplying food or hosts, as well as shelter and refugium, for natural enemies) (McCravy 2008). For example, sown grass or flower strips in the field can enhance the number of species of natural enemies without enhancing, or even decreasing, pest abundance (Nentwig et al. 1998).

Classical biological control, the introduction of an exotic natural enemy of a pest for permanent establishment and long-term control, comprise many successful examples, such as the suppression of ash whitefly (*Siphoninus phillyreae*) that had spread to 28 states of the United States in 1988. By the introduction of two parasitic wasps (*Encarsia partenopea* from Italy and *E. partenopea* from Israel) in the autumn of 1989 and a predator beetle (*Clitostethus arcuatus* from Israel) in the spring of 1990, the pest's abundance and ability to cause harm or damage was reduced to acceptable levels within 2 years (Bellows et al. 1992).

Augmentative biological control is the temporary introduction of a natural enemy to combat a pest. Among the most effective agents is the genus *Trichoderma* spp, a group of fungi capable of controlling various plant diseases, including root rot, damping off, wilt and fruit rot (Zin & Badaluddin 2020).

More than reducing the environmental impact and our dependence of chemical control, biological control also enables us to combat diseases that we have no

chemical control for (Kevan & Shipp 2011). Overall, biological control is a promising tool of sustainable agriculture, as it aims to reduce the use of chemical pesticides and minimize the ecological impact while maintaining or increasing crop productivity (Stenberg et al. 2023).

2.4 *Clonostachys rosea*

Clonostachys rosea possesses antibiotic traits and is an endophyte, both important traits for a potential biocontrol agent (Köhl 2021). Additionally, it is a necrotrophic mycoparasite, capable of killing a host fungus and deriving the necessary nutrients for its growth from the dead material (Sun et al. 2020). One component of the mycoparasitic ability is the secretion of fungal cell wall-degrading enzymes like chitinases, the activity of which is linked to the biocontrol ability in *Clonostachys* (Jensen et al. 2021). The antibiosis trait of *C. rosea* includes the ability to compete for space and resources, along with defence of these resources through the production of secondary metabolites with antagonistic effect, which further enhances its mycoparasitic ability (Jensen et al. 2021).

As an endophyte, it can be present in the entry points of plant organs when the pathogen arrives, making it primed for mycoparasitic attacks (Saraiva et al. 2015). This harmless yet hostile entry of the endophyte can trigger the plant to activate inducible (temporary) defence responses and in the prolongation induced systemic resistance, making it a more robust defender against future pathogen attacks (Kamou 2020).

A successful biocontrol agent in many different crops, *C. rosea* is used to control a wide range of diseases, for instance Fusarium head blight (*F. graminearum*) in pea and soy bean, black scurf (*Rhizoctonia solani*) in potato and root rot (*F. culmorum*) in cereals (wheat and barley) (Köhl & Ravensberg 2021). It has also been successfully tested as a biocontrol agent against clubroot in oilseed rape by seed coating, at least under controlled conditions (Andersen et al. 2018). Therefore, it is known that *V. longisporum* can infect oilseed rape and that *C. rosea* can be used to control other pathogens in oilseed rape, such as *P. brassicae*. It remains to be determined whether *C. rosea* can effectively control verticillium stem striping disease in oilseed rape.

3. Material and methods

3.1 *In vitro* experiments

3.1.1 Dual cultures

In petri dishes, *C rosea* and *V. longisporum* were grown on two different types of media. Czapek Dox Agar (CDA) (Sigma-Aldrich, Sacramento, CA) is a defined medium with high pH (>7) and sucrose as carbon source. Potato Dextrose Agar (PDA) (Difco, Franklin Lakes, NJ) is a more general purpose media with a lower pH (~5,6) and dextrose as carbon source.

In each medium we had three treatments: (1) solo cultures of *C. rosea*, (2) solo cultures of *V. longisporum*, and (3) dual cultures of *C. rosea* and *V. longisporum.* The experiment was set up with 6 replicates per treatment, and ended up with 6 replicates for *V. longisporum* solo cultures on CDA media and 4 replicates on PDA media. The solo cultures of *C. rosea* ended up with 6 replicates on both media. The dual cultures with both fungi ended up with 4 replicates on CDA media and 5 replicates on PDA media.

Fungi were inoculated on opposite sides with an 5 mm diameter agar plug with actively growing mycelia. Petri dishes were incubated at 25°C in darkness. Measurements of the fungal diameter were made every day from day 5 (to the right in Fig. 3), and their overlapping growth zone from day 9, until day 29.

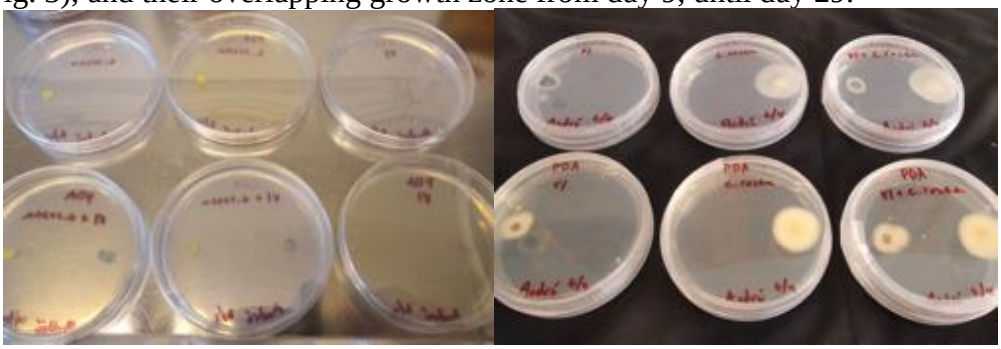

Figure 3. Petri dish experimental design. The six treatments at the time of inoculation (left) and at the start of measuring on day 5 (right).

3.1.2 Growth in liquid culture filtrates

In two different media, Potato Dextrose Broth (PDB, Difco) and Czapek Dox Liquid Media (Czapek, Sigma-Aldrich), *C. rosea* was inoculated with an 5 mm diameter agar plug with actively growing mycelium and allowed to grow for 3, 4 or 5 days on a shaker in 20°C in darkness. The solution was then filtered to remove the mycelia and to keep only the secondary metabolites. In these *C. rosea* culture filtrates, *V. longisporum* was inoculated (Fig. 4) and left to grow for 7 days on a shaker in 20°C.

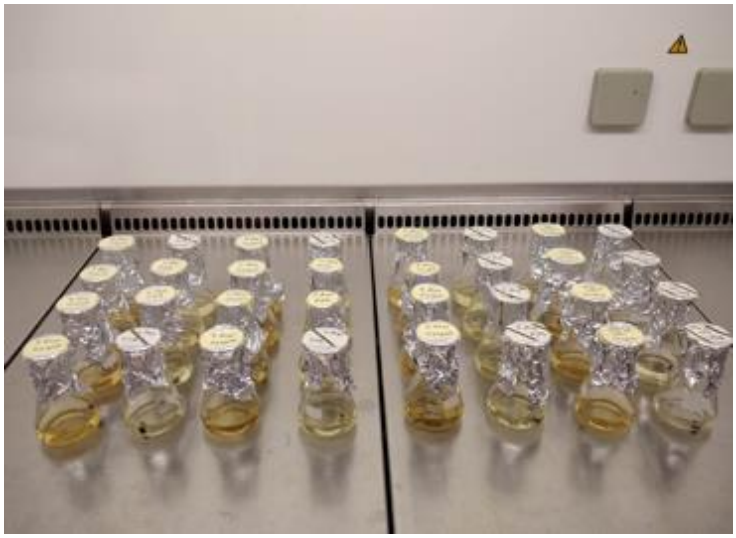

Figure 4. Growth in liquid culture filtrates experimental design. Two different media (PDB and Czapek) and four different treatments per media (C. rosea growth for 3/ 4/ 5 days and without C. rosea, only media). In this picture V. longisporum has just been inoculated.

After this, *V. longisporum* was removed from the media with a tweezer and put on a paper filter to remove any excess media before transferred to Eppendorf tubes and kept in 50°C for approximately 2 hours to dry. Finally, the biomass of *V. longisporum* was weighed. A control treatment with only media and *V. longisporum* (no *C. rosea* involvement) was also included. This experiment included 4 replicates of each treatment, start to finish.

3.1.3 Quantification of pathogen biomass

Surface sterilized oilseed rape seeds were germinated on agar plates (Fig. 5) with half strength Murashige & Skoog medium (Duchefa), supplemented with sucrose.

A conidial suspension of *C. rosea* was prepared by adding distillied autoclaved water to an agar plate with actively growing mycelium and scraping the plate. Conidial concentration was determined with haecytometer and diluted to approximately 10^6 conidia/ml. After five days, the seedling's roots were soaked in

this conidial suspension of *C. rosea* for 10 minutes, or mock-treated with water, and transferred to half MS agar plates without sucrose.

Figure 5. Oilseed rape seedlings germinating (left) and root coating with C. rosea (right).

Five days later the seedlings were inoculated with the pathogen *V. longisporum* using the same method as described for *C. rosea.* Five days post inoculation with *V. longisporum,* the plant stems and root were harvested, surface sterilized with ethanol for 30 seconds and washed with autoclaved water two times. They were then freezed in liquid nitrogen, ground to a fine powder and stored at -70°C.

To extract the DNA, a CTAB-chlorophorm-based protocol was used for DNA extraction. Fungal DNA was quantified using the *V. longisporum GADPH* reference gene and normalized with *B. napus SAND* gene, using the following primers: GADPH, forward sequence: 5'-TCTCGTTGACACCCATACG-3', and reverse sequence: 5'-TCTTACCACCACCGACAAG-3', and SAND, the forward sequence: 5'-GCTGAAGGTGGATTGCGTG-3', and reverse sequence: 5'-ATACCGAGCATACCAGAAACTCC-3'. This experiment was set up with 10 replicates of each treatment, but ended up with 2 replicates for the *V. longisporum* treatment and 3 replicates for the treatment with both fungi.

3.2 *In planta* experiment

Preparation of fungal inoculum

Five days before starting the experiment, both fungi (*C. rosea* and *V. longisporum*) were inoculated on PDA plates (Difco). The biological control strain used in this project (Fig. 6) was the strain IK726 originating from Denmark (Mamarabadi et al. 2007). It is a well-studied strain with good efficacy and biocontrol traits (Köhl & Ravensberg 2021). The pathogen *V. longisporum* isolate vl43-3, isolated from Sweden, was used in this project since it is an aggressive isolate, increasing the chance of infection.

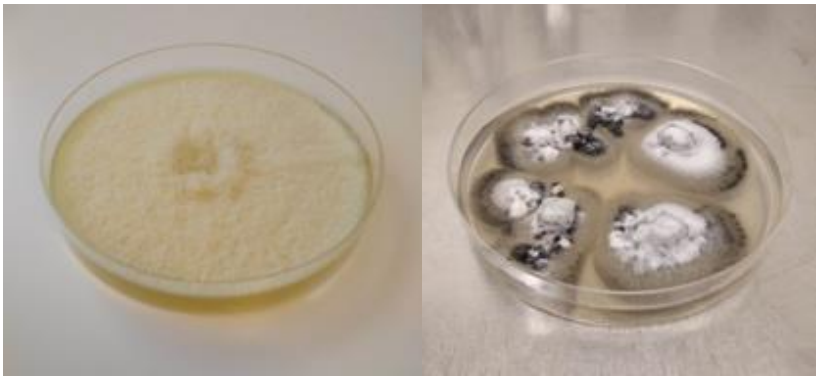

Figure 6. Petri dishes with C. rosea IK726 (left) and V. longisporum vl43-3 (right).

Plant material

The plant material used in this project was oilseed rape (*Brassica napus*), cultivar "Hanna". It is an old cultivar of spring rapeseed that is no longer used in agriculture, but it was chosen as a good screening candidate due to its high susceptibility to this disease, once again increasing our chance of infection.

The seeds were surface sterilized and germinated in petri dishes on wet filter papers (Fig. 7). A total of 250 seeds were used, distributed on 5 petri dishes (50 seeds each), with the aim of attaining 120 viable plants.

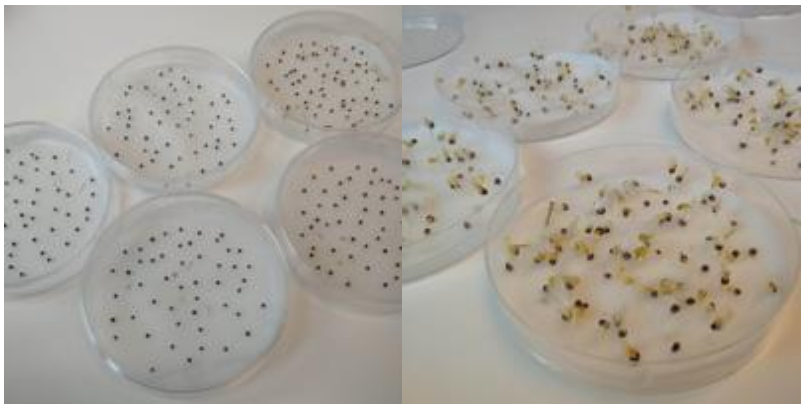

Figure 7. Petri dishes with oilseed rape seeds on wet filter paper, before (left) and after (right) germination.

Application of the biocontrol agent

Four days after germination the seeds were put in either a conidia suspension of *C. rosea* or pure water for approximately 10 minutes. At this point 10 seeds were separated and used for viable count (CFU). Conidia were extracted from the seeds, diluted and spread on agar plates. After five days, the colonies on each plate were counted. The formula "CFU = average number of colonies / 0,1 x dilution factor" disclosed about 100 000 conidia/ seed.

Planting

Immidiately after coating treatment, the seedlings were potted in commercial peat soil (Hasselfors garden, Sweden) (Fig. 8), which contains a high nutrient value and perlite, allowing a good oxygen supply. The pots were put on trays of six and continuously watered with tap water from underneath, not to flush away any fungi.

Figure 8. Greenhouse experimental design with the tray setup (left) and the four treatments (right).

Pathogen inoculation and experimental setup

Twelve days after the pot planting, all plants were removed from the soil, the roots cleaned with water and inoculated either in a conidial suspension of *V. longisporum* (10^6 conidia/ml) or in water as a mock inoculation. After 15 minutes of root coating, the plants were replanted in the pots and put back into the greenhouse (Fig. 9). Four different treatments were used: (1) *C. rosea* only, (2) *V. longisporum* only, (3) *C. rosea* and *V. longisporum*, and (4) control (mock-treated, no fungal inoculation). The experiment was set up with 30 replicates for each treatment, and ended up with 27 for the control treatment, 22 for the *C. rosea* treatment, and 25 for *V. longisporum* and the treatment with both fungi. The greenhouse conditions was about 20°C during the day, 10°C during the night, and 16 hours of light hours per day.

Figure 9. Some sad-looking oilseed rape plants after inoculation with the pathogen.

Plant biomass and disease symptoms evaluation

Four weeks after infection, plants were harvested (Fig. 10), symptoms documented (number of yellow leaves per plant) and the aerial biomass (no roots included) was measured (wet and dry weight).

Figure 10. Forty days old oilseed rape plants, ready to harvest.

3.3 Data analysis

To analyse the data from the *in planta* and the growth in liquid culture filtrates *in vitro* experiments, pairwise comparisons were perfomed on the biomass data using the Student's t test at the 95% significance level, implemented in Microsoft Excel. In the *in planta* experiment, a ratio from the yellow leaves were also calculated as number of yellow leaves per plant and treatment. In the dual growth *in vitro* experiment, the growth was plotted against the number of days in a figure for each media, and split into before and after contact. In the quantification of pathogen biomass *in vitro* experiment a mean of DNA biomass from each treatment was calculated and then compared to get a relative amount of DNA biomass.

4. Results

4.1 *In vitro* experiments

4.1.1 Dual cultures

In two different media (CDA and PDA) and with three treatments per media (*C. rosea*, *V. longisporum*, and both *C. rosea* and *V. longisporum*), both fungi were inoculated. The growth rate was measured and the overlapping growth zone observed.

The growth was very similar for both solo and dual cultures up until the time for contact (Fig. 14 and 15). After this, the growth of both *C. rosea* and *V. longisporum* was inhibited in the dual cultures compared to their growth in the solo ones (Fig. 16 and 17). This was observed in both types of media.

The growth of *C. rosea* solo cultures (Fig. 11) had on CDA media a growth rate of 0.28 cm/day, and on PDA media 0.18 cm/day. It had more white pigment and mycelia grew more thin and longspread on CDA, while it had more yellow pigment and grew more fluffy on PDA.

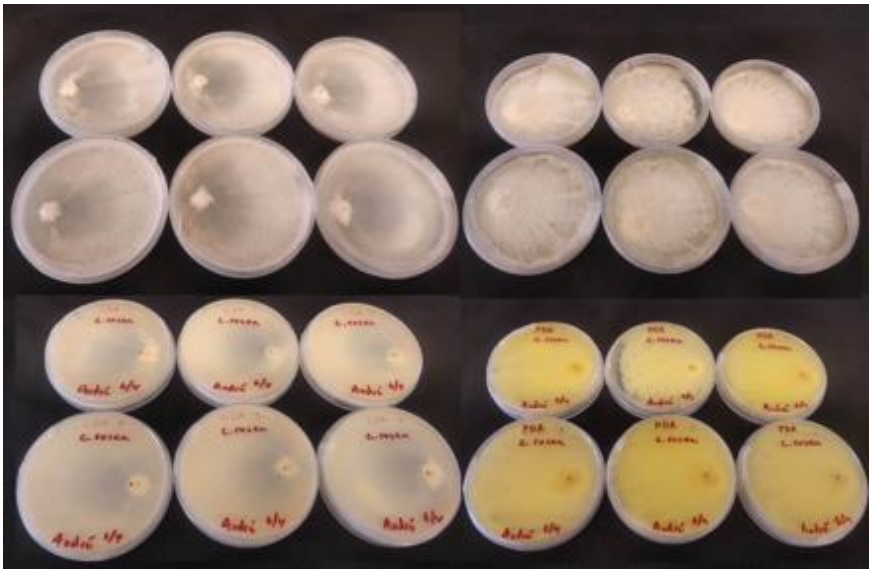

Figure 11. C. rosea solo growth on CDA (left) and PDA (right) media at the end of experiment.

The growth of *V. longisporum* solo cultures (Fig. 12) had on CDA media a growth rate of 0.096 cm/day, and on PDA media 0.11 cm/day. The microsclerotia of *V. longisporum* are black, thus the isolate produced more microsclerotia on PDA media than on CDA.

Figure 12. V. longisporum solo growth on CDA (left) and PDA (right) media at the end of experiment.

In dual cultures, *V. longisporum* had on CDA media a growth rate of 0.09 cm/day before contact and -0.015 cm/day after contact (Fig. 19). On PDA media the growth rate was 0.11 cm/day before contact and it decreased with -0.015 cm/day after contact. The dual cultures growth of *C. rosea* had on CDA media a growth rate of 0.23 cm/day before contact and 0.1 cm/day after contact. On PDA media the growth rate was 0.15 cm/day before contact and 0.11 cm/day after contact.

The overlapping growth zone kept expanding until *C. rosea* covered the whole plate in two of the four replicates on CDA media (the two top plates in the left picture of Fig. 13), in the other two replicates it stopped at 1.2 cm. On PDA media the overlapping growth zone grew to 1.9-2.3 cm, but in all replicates it then decreased with 0.2-0.9 cm until the overgrowth began diminishing completely, leaving only traces of its growth on *V. longisporum.* At the end of the experiment the lytic zone, the seemingly empty space between the fungi, was 0.4-0.6 cm.

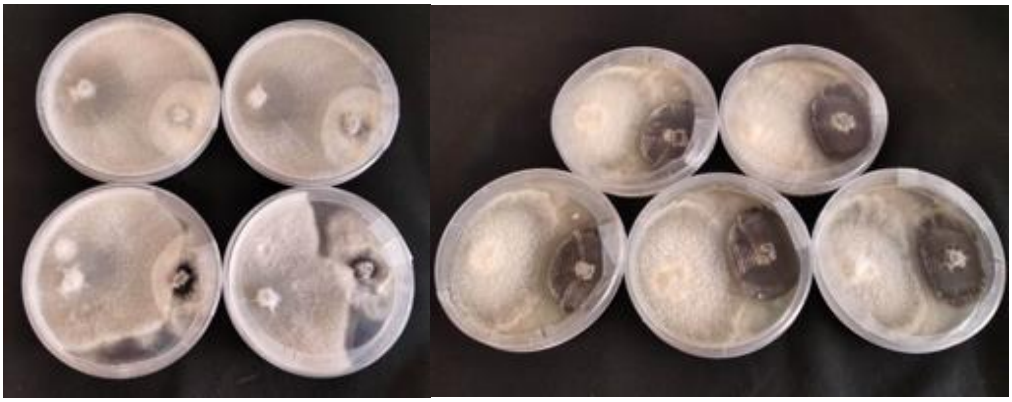

Figure 13. C. rosea and V. longisporum dual growth on CDA (left) and PDA (right) media at the end of experiment.

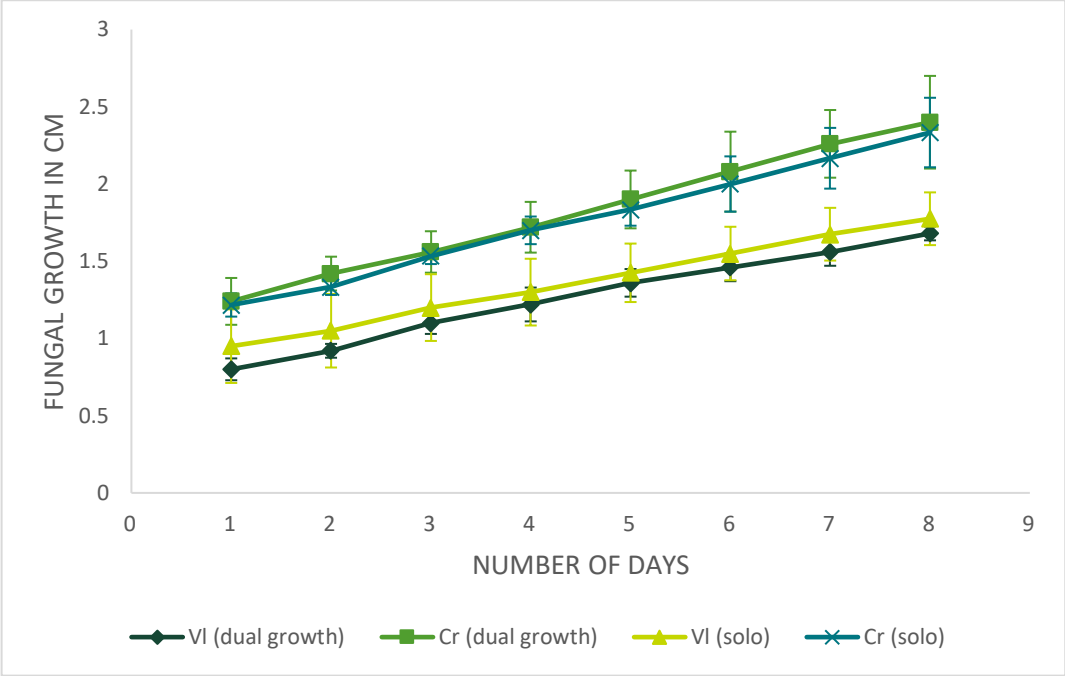

Figure 14. Connected line chart of fungal growth on PDA before contact. V. longisporum and C. rosea, in dual and solo growth. The vertical error bars display the standard deviation.

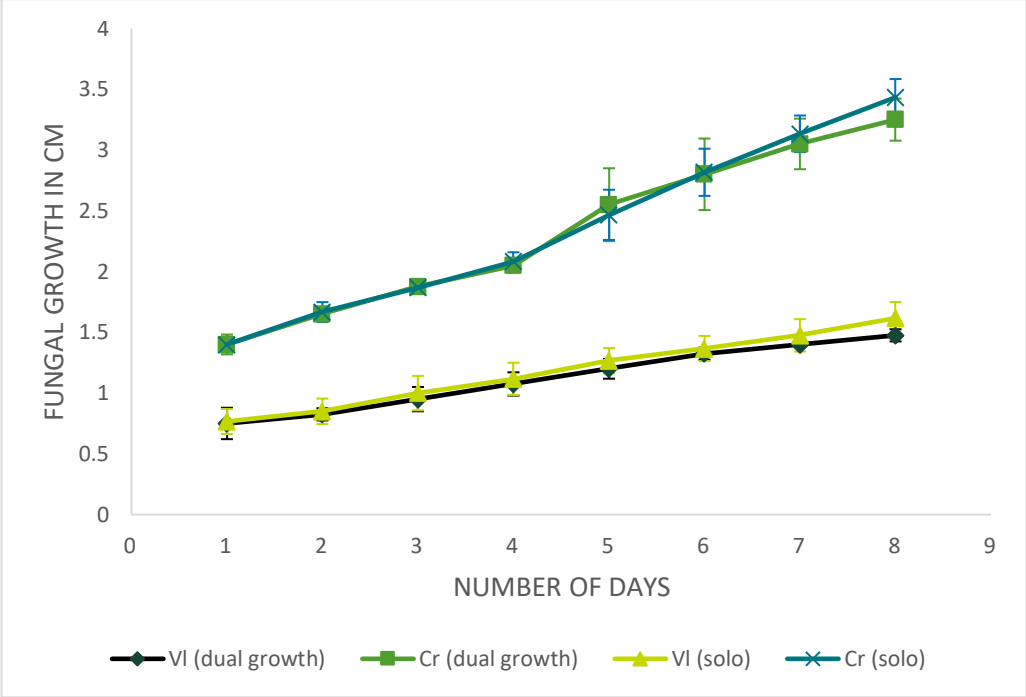

Figure 15. Connected line chart of fungal growth on CDA before contact. V. longisporum and C. rosea, in dual and solo growth. The vertical error bars display the standard deviation.

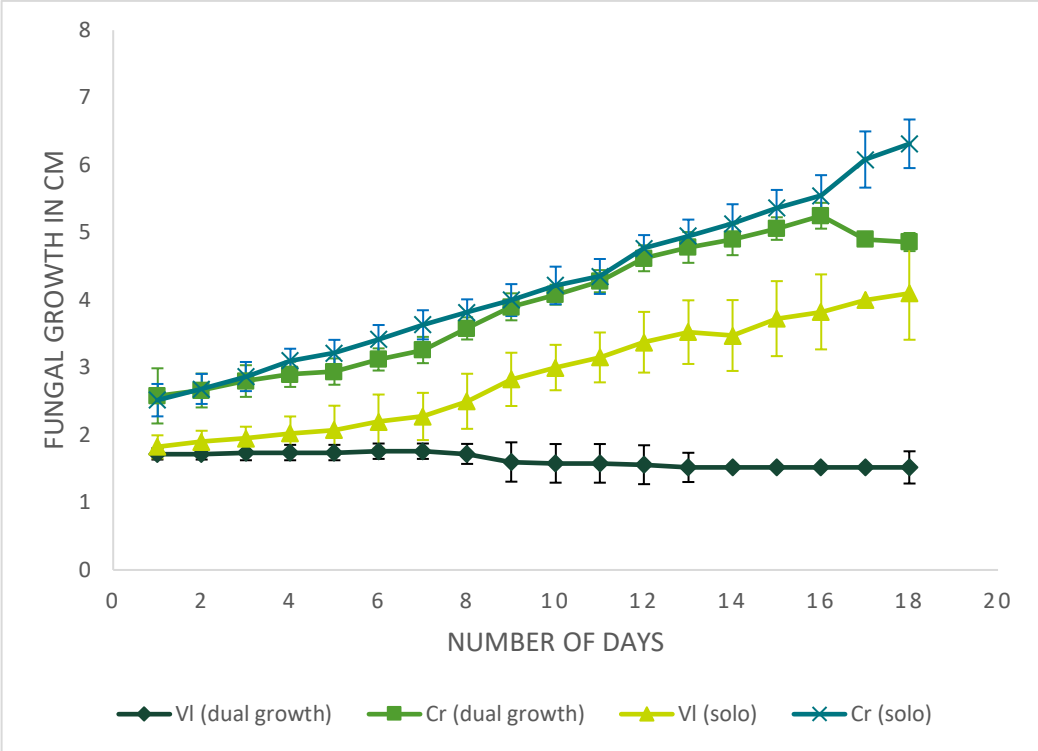

Figure 16. Connected line chart of fungal growth on PDA after contact. V. longisporum and C. rosea, in dual and solo growth. The vertical error bars display the standard deviation.

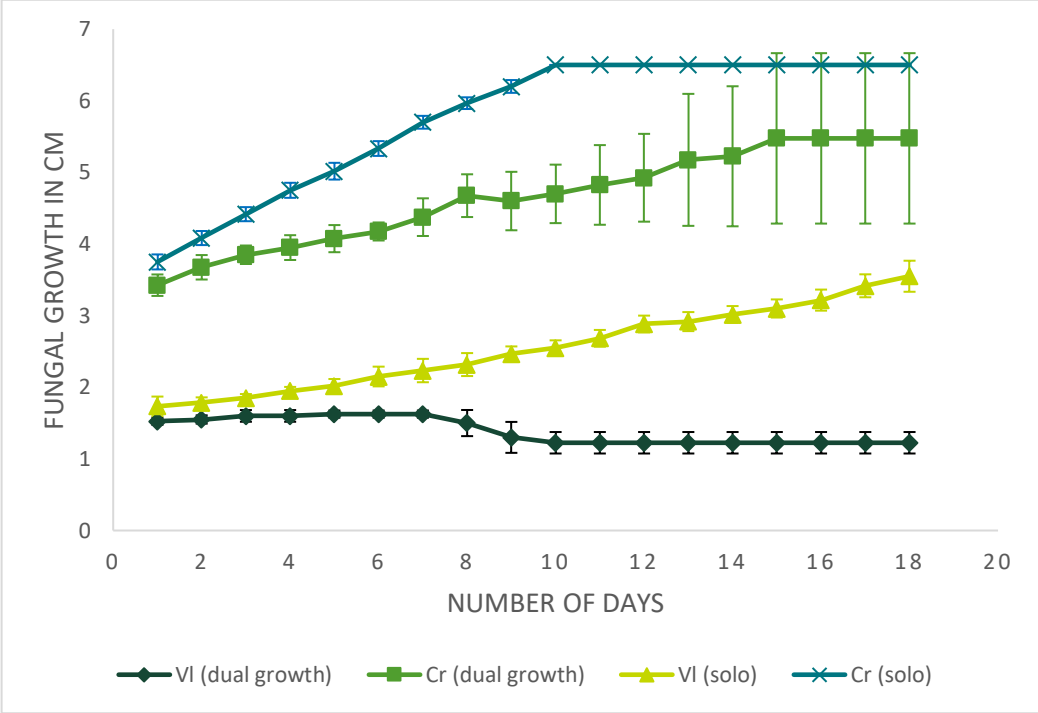

Figure 17. Connected line chart of fungal growth on CDA after contact. V. longisporum and C. rosea, in dual and solo growth. The vertical error bars display the standard deviation.

4.1.2 Growth in liquid culture filtrates

In this experiment *C. rosea* grew for 3, 4 or 5 days in two types of liquid media (Czapek or PDB) to produce and secrete its secondary metabolites. Mycelia was then removed and the liquid filtrate used as media to inoculate the pathogen *V. longisporum*. A control treatment for both type of media (without *C. rosea*) was also included.

The results showed a statistical significant difference ($p < 0,05$) between the control treatment of each media compared with the *C. rosea* treatments. No significant difference between the other treatments was observed (Fig. 18). No comparison between the two types of media was perfomed.

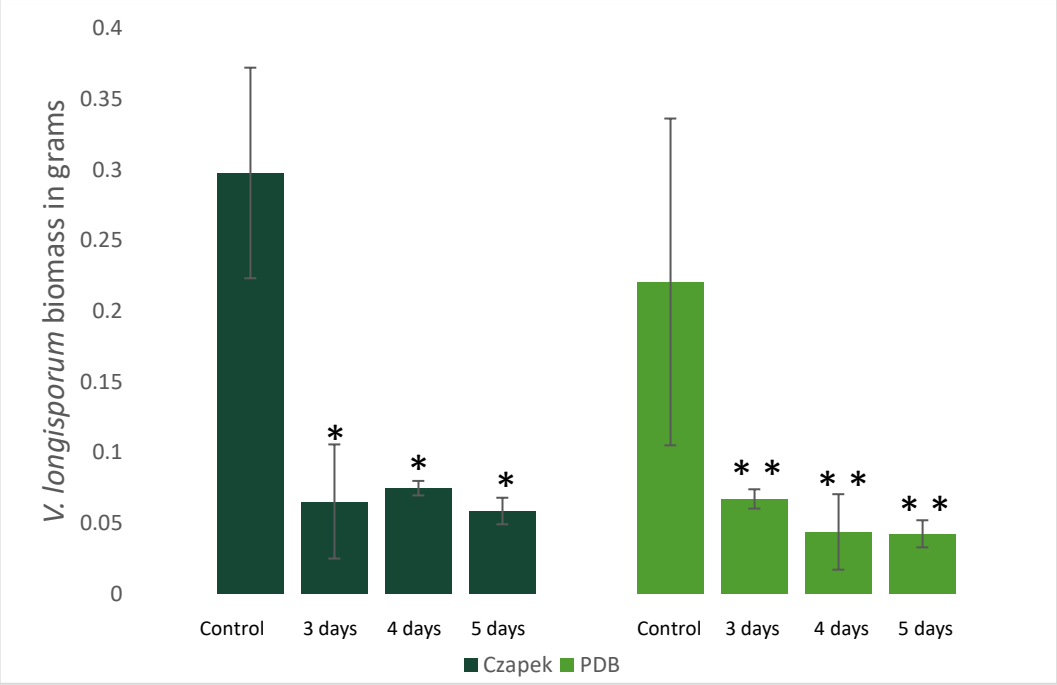

Figure 18. V. longisporum biomass in Czapek (dark green) and PDB (light green) with the four different treatments (control and C. rosea culture filtrate of 3, 4 and 5 days). Mean ± S.D., n=4. T-tests, p-value < 0,05. The asterisks (and **) indicate statistical significance differences between the control of the same medium.*

4.1.3 Quantification of pathogen biomass

Oilseed rape seedlings were grown in petri dishes on ½ MS media with three different treatments: with *V. longisporum*, with both *V. longisporum* and *C. rosea* and a control without any fungal inoculation. The leaves were removed, the remaining plant surface sterilized and from this plant material the oilseed rape and *V. longisporum* DNA was extracted.

The results were inconclusive as not enough biological replicates were attained. For the treatment with *V. longisporum* 2 replicates were attained, and for the treatment with both *V. longisporum* and *C. rosea* 3 replicates. The data (Fig. 19) showed no statistically significant differences in these two treatments, although *V. longisporum* biomass tends to be lower in seedlings where *C. rosea* previously has been inoculated.

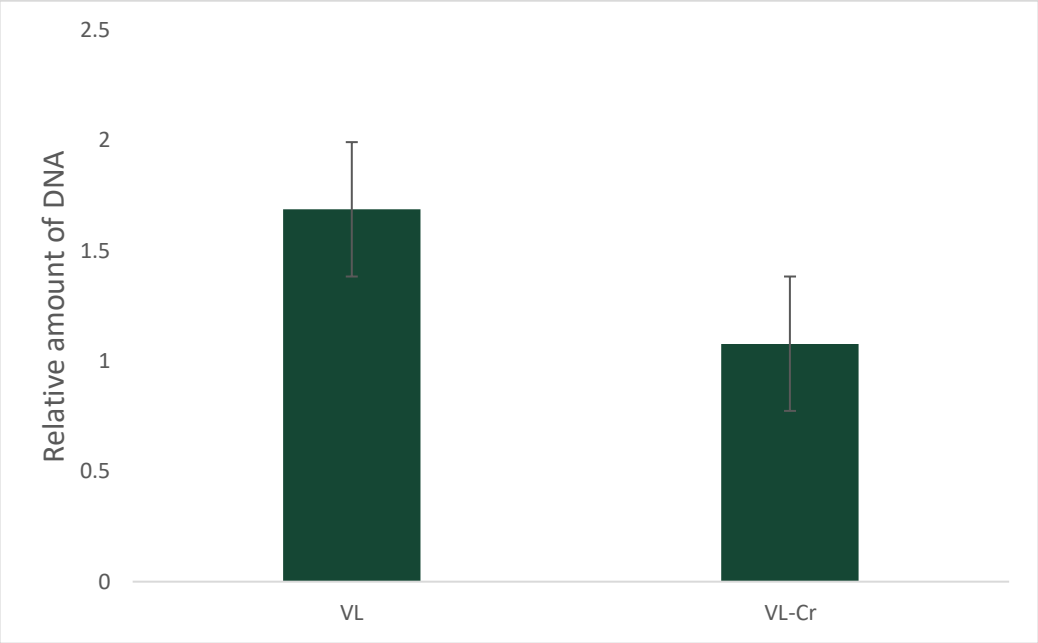

Figure 19. Bar chart of relative amount of V. longisporum DNA, compared between treatment with and without C. rosea. Mean ± S.D., n=2 (VL), n=3 (VL-Cr). T-tests, p-value < 0.05.

4.2 *In planta* experiment

In a greenhouse setting oilseed rape seedlings were inoculated with *V. longisporum* and *C. rosea*. Four weeks after pathogen inoculation symptoms were observed (as yellow leaves per plant) and the dry and wet aerial biomass (no roots included) determined.

The results for the fresh biomass gave the highest weight for the control treatment, followed by the *C. rosea*, the *V. longisporum*, and at last the treatment with both *C. rosea* and *V. longisporum* (Fig. 20). There was a statistical significant difference ($p < 0.05$) between the control and the *V. longisporum* treatment, as well as between the control and the treatment with both *C. rosea* and *V. longisporum*. There was also a statistically significant difference between the treatments with *C. rosea* and those with both *C. rosea* and *V. longisporum*. However, no statistically significant difference between infected plants treated with or without *C. rosea* was observed (Fig. 20).

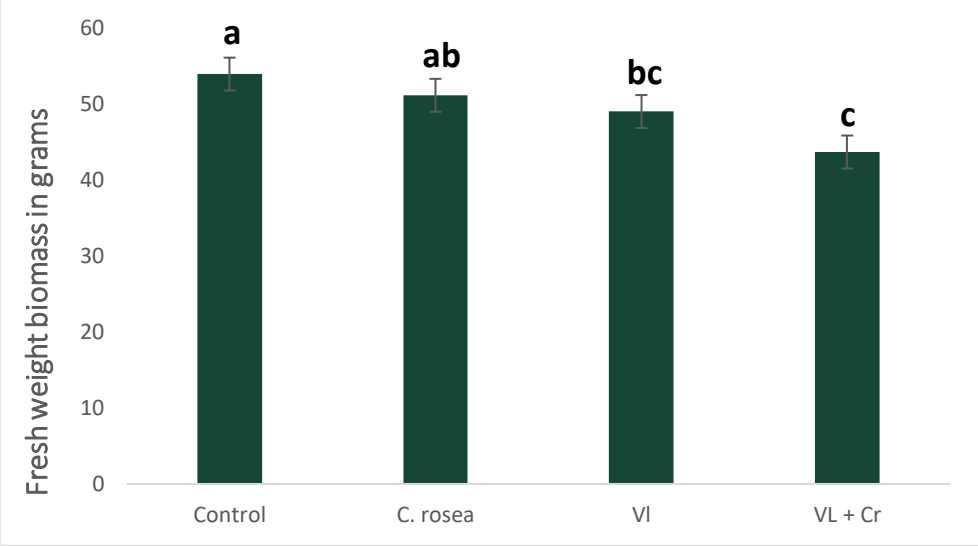

Figure 20. Bar chart of fresh weight biomass for the four treatments. From left: control, C. rosea, V. longisporum (Vl) and both C. rosea and V. longisporum (VL+Cr). Different letters above the bars indicate statistically significant differences between treatments. Mean ± S.D., n=27 (control), n=22 (Vl), n=25 (Vl and Vl + Cr). T-tests, p-value < 0.05.

The results for the dry biomass gave a similar trend as the fresh biomass, with the exception of the *C. rosea* treatment which diverged notably (Fig. 21). The control treatment gave the highest weight, followed by the *V. longisporum*, the treatment with both *C. rosea* and *V. longisporum*, and at last the *C. rosea* treatment (Fig. 21). There was a statistical significant difference ($P < 0.05$) between all treatments except the *V. longisporum* and the treatment with both *C. rosea* and *V. longisporum*.

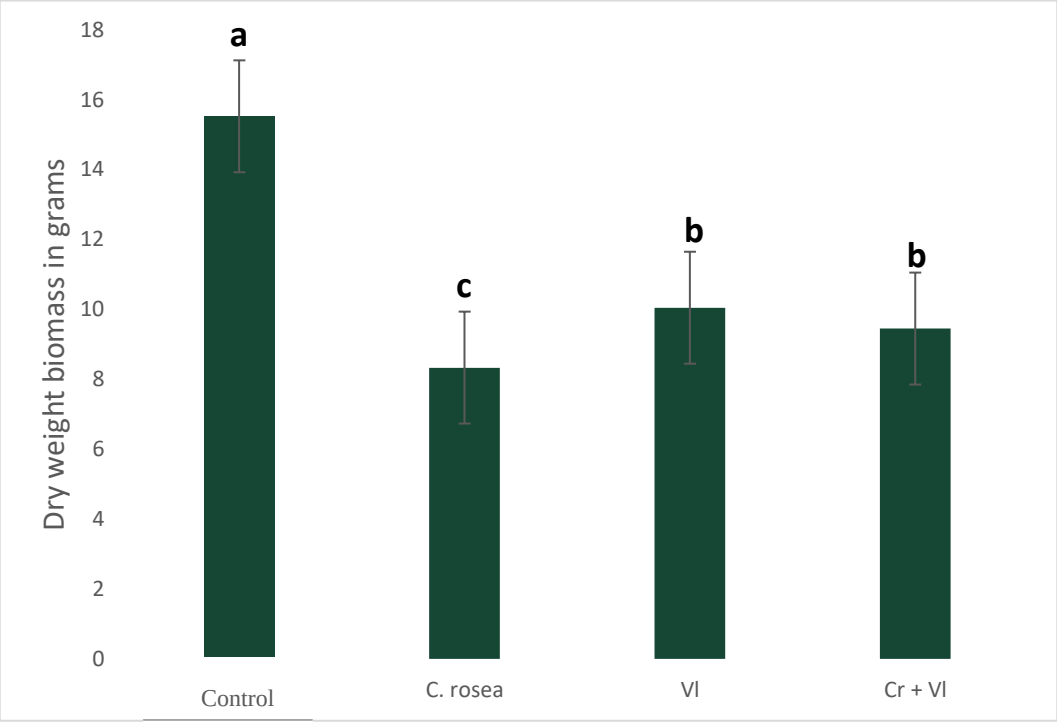

Figure 21. Bar chart of dry plant biomass for the four treatments. From left; control, C. rosea, V. longisporum and both C. rosea + V. longisporum. The letters above the bars display a statistical significant difference between bars without the same letter. Mean ± S.D., n= 27 (control), n=22 (C. rosea), n=25 (Vl and Cr+Vl). T-tests, P < 0.05.

The results for the fresh weight also comply with the visual observation of the plants (Fig. 22 and 23), no clear difference could be seen between the control treatment and *C. rosea* treatment, and no clear difference between the *V. longisporum* and the treatment with both *C. rosea* and *V. longisporum* could be seen. The plant size variation was also bigger in the two treatments with *V. longisporum*, compared to the two treatments without. The two figures (22 and 23) are included to demonstrate this plant size variation.

Figure 22. Picture of oilseed rape from each treatment. From left to right: both C. rosea and V. longisporum, V. longisporum, C. rosea and control treatment.

Figure 23. Picture of oilseed rape from each treatment. From left to right: both C. rosea and V. longisporum, V. longisporum, C. rosea and control treatment.

The results for the observed symptoms (Fig. 24), counted as yellow leaves per plant, is showed in figure 25 and gave the highest amount of yellow leaves for the treatment with both *C. rosea* and *V. longisporum*, followed by the *V. longisporum* treatment, the *C. rosea*, and at last the control treatment. There was a statistically significant difference ($P < 0.05$) between all treatments except the control and the *C. rosea* treatment (Fig. 25).

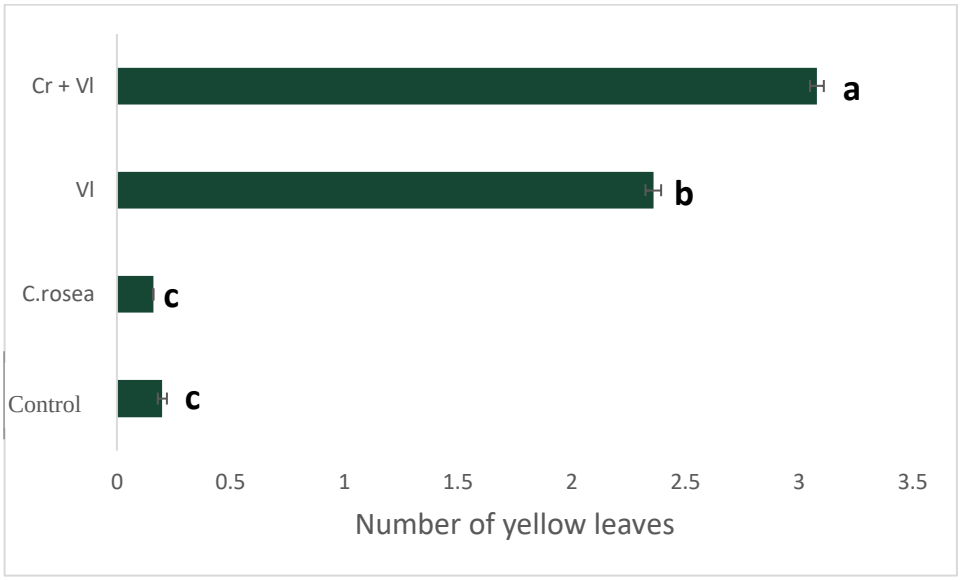

Figure 24. Bar chart of the ratio of yellow leaves per plants and treatment. From above: both C. rosea and V. longisporum, V. longisporum, C. rosea and control. The letters to the right of the bars display a statistical significant difference between bars without the same letter. Mean ± S.D., n= 27 (control), n=22 (C. rosea), n=25 (Vl and Cr+Vl). T-tests, p-value < 0.05.

Figure 25. Yellow leaves of infected plant (left) and green leaves of a healthy plant (right).

5. Discussion

The aim of this project was to test *C. rosea* as a biological control agent against verticillium stem striping disease, caused by the pathogen *V. longisporum*, in oilseed rape. The main *in planta* experiment showed no indication of *C. rosea* being able to control *V. longisporum* infections on oilseed rape. This was shown as two of the symptoms of verticillium stem striping disease are stunted plants and yellow leaves, and the treatment with both pathogen and biocontrol agent produced the most stunted plants and the highest number of yellow leaves. Nevertheless, the experimental set-up was appropriate, since the two treatments with the pathogen produced significantly less plant biomass than the two treatments without pathogen, which implies a successful pathogen infection.

Altough not being able to prevent infection, *C. rosea* had no negative effect on the growth of oilseed rape when inoculated alone. Plants in this treatment produced almost the same fresh weight biomass as plants in control treatment, and they produced the lowest number of yellow leaves, although there was no statistical significant difference between the two treatments. Seemingly, *C. rosea* somehow caused a possible negative synergistic effect on the growth of oilseed rape when inoculated together with *V. longisporum*, as the treatment with both fungi produced the lowest fresh weight biomass and the highest number of yellow leaves.

Due to a technical error, all plants did not dry successfully and a few replicates even started to grow mold. The results for the dry weight biomass might therefore not be fully reliable. Additionally, the soil of the pathogen treatments was notably more wet than the others. There was also visible mycelium covering much of the top soil. Perhaps this affected the growth of oilseed rape and the data attained.

All of the three *in vitro* experiments did on the other hand, to a certain degree, point to *C. rosea* being a promising agent to combat *V. longisporum*. The dual culture *in vitro* experiment showed that *C. rosea* can inhibit the growth of *V. longisporum* when in direct contact. On CDA medium *V. longisporum* was completely overgrown in half of the replicates, while on the other half it was partly overgrown. On PDA medium, it first seemed to be overgrown, but then managed to fight back. The lytic zone that appeared on PDA media prevented the growth of *C. rosea* compared to its solo growth, which could mean that the cell wall degrading chitinase of *C. rosea* either broke down its own cell walls, or that *V. longisporum*

also produce some similar substance. This also shows that the mycoparasitic enzyme production of *C. rosea*, its antagonistic effect, differs with media.

The growth in liquid culture filtrate *in vitro* experiment showed that the secondary metabolites produced by *C. rosea* can inhibit the growth of *V. longisporum*. There was a statistical significant difference between the control treatment, without *C. rosea*, and the treatments with the secondary metabolites produced by *C. rosea*. There was no difference depending on the time *C. rosea* was allowed to produce these metabolites, meaning that *C. rosea* produced some kind of secondary metabolites very quickly that later inhibited the growth of *V. longisporum.* Nonetheless, it does evoke the question whether *C. rosea* simply might have depleted the amount of nutrients in the media and consequently limited *V. longisporum's* potential growth.

The quantification of pathogen biomass *in vitro* experiment indicated that the presence of *C. rosea* in the plant, oilseed rape, inhibit the presence of *V. longisporum*. Unfortunately, this experiment did not end up with enough biological replicates for a conclusive result, so there was no statistical significant difference between the treatments. This might be due to the protocol followed in DNA extraction not being fully suitable for oilseed rape, since it was designed for *Arabidopsis thaliana.*

Other studies where *C. rosea* has been used to suppress clubroot on canola have shown that the antibiotic traits of *C. rosea* are effective in inhibiting the growth of fungal pathogens, due to cell wall-degrading enzymes and antibiotics produced by *C. rosea* (Lahlali & Peng 2013). This is in accordance with the results of this projects *in vitro* experiments. However, Lahlali & Peng (2013) also showed that the whole of the biofungicide product Prestop (*Clonostachys rosea*) was more effective than any of its components seperately (*C. rosea* conidial suspension or the culture filtrate). Perhaps by using *C. rosea* as a biofungicide in our project the effect *in planta* would confirm the effects observed *in vitro*.

By conducting several types of experiments we get a more complete picture of the two fungi's interactions, and if several experiments point towards the same conclusion this provides greater support for it. The more crude and controlled environment in the *in vitro* experiments gives a good setting to study specific mechanisms, with more control over the variables and more precise measurements where the variables can be measured with high accuracy, and it is easier to reproduce the studies to replicate and validate the findings. However, the simplicity of the *in vitro* setting can make the findings difficult to translate into a more complex setting such as an *in planta* or field setting, where many more factors are involved that may not have been considered in the controlled environment.

The limitations of this project are numerous. To achieve a credible result in the *in planta* experiment many replicates of each treatment, as well as several runs of the whole experiment, are necessary. Unfortunately due to this projects time

limitation (10 weeks) only one execution of a rather small-scale experiment was possible.

The method of both pathogen and biocontrol agent application are not indisputable. The inoculation of plants by root dipping in a conidial suspension differ from natural infections which originate from microsclerotia (Depotter et al. 2016). This may affect the project's credibility as a screening process, and other methods of application such as seed coating, spray application, and application to soil or growth substrate, could prove to be more appropriate.

One more control treatment could have been included in the growth in liquid culture filtrate experiment, where *V. longisporum* was allowed to grow in the media before removed and incolutated with new *V. longisporum*, to make sure that it was not a lack of nutrients that inhibited the growth.

In the quantification of pathogen biomass experiment a new protocol for DNA extraction need to be tested.

For further research, a longer growing period and several runs of the *in planta* experiment is a good start, since the growth condititions of oilseed rape and the impact of the fungi in the *in planta* experiment may be questionable. They should be sufficient for the screening process, but in the case of verticillium stem striping disease the symptom development is most prevalent during the ripening of the crop, at the end of the growing season. Conducting the same experiment with a longer time frame, where oilseed rape is allowed to grow until ripening, might give a more correct assessment of the biocontrol ability of this biocontrol candidate.

The effects are also different in an *in planta* setting compared to an *in vitro* setting, and would likely also differ to a field setting. Field trials would therefore be desirable, but should due to practical and economic reasons be done once a promising biocontrol agent is found.

One possibility for further screening of a biocontrol agent is the use of cross-protection, where in the case of vascular pathogens non-pathogenic or non-aggressive strains of the pathogen are used as biocontrol agents. In this case it would mean using a less aggressive strain of *V. longisporum* to combat a more aggressive one (Vega-Marin & Tiedemann 2022).

To summarize, our findings in the *in vitro* experiments indicate that *C. rosea* could be able inhibit the growth and presence of *V. longisporum*, but our findings in the *in planta* experiment point towards the opposite direction - no beneficial effect on oilseed rape by inoculating *C. rosea* was observed. Therefore, further research to study the interactions between these two fungi on the plant oilseed rape is necessary.

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