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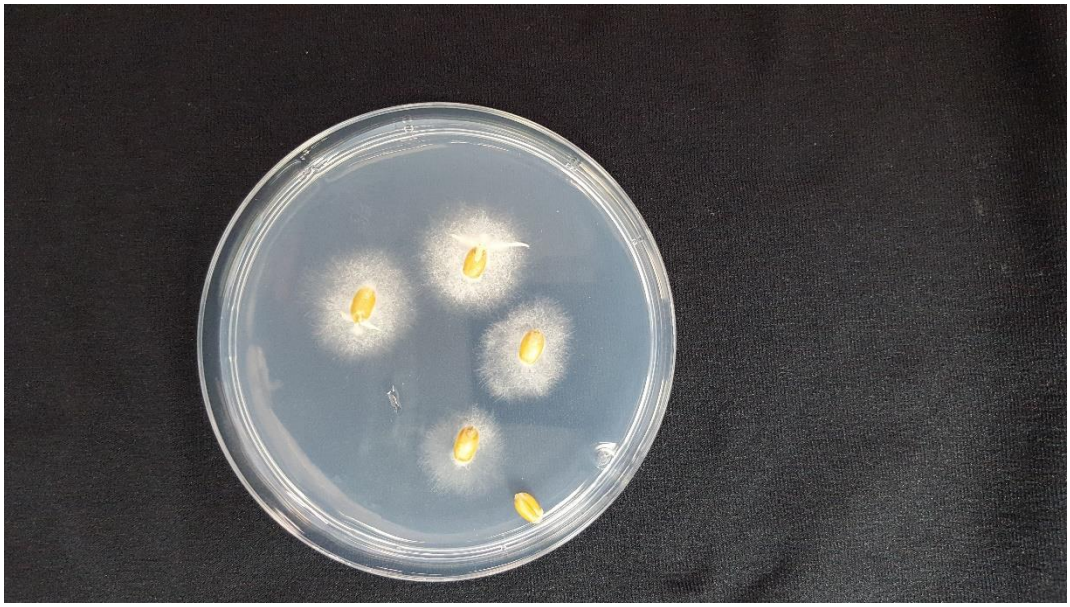

Master´s thesis • 30 credits
Agronomy program - plant & soil
https://stud.epsilon.slu.se 2020

Combining biocontrol fungus *Clonostachys rosea* with chemical fungicides – for integrated management of Fusarium foot/root rot

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Credits:	30 credits
Level:	Second cycle, A2E
Course title:	Independent project in biology
Course code:	EX0565
Programme/education:	Master in Biology / Agronomy program - plant & soil
Course coordinating department:	Department of Forest Mycology and Plant Pathology
Place of publication:	Uppsala
Year of publication:	2020
Cover picture:	Wheat seeds coated with germinating *Clonostachys rosea*, by Sebastian Bengtsson
Online publication:	https://stud.epsilon.slu.se
Keywords:	Biocontrol agent, *Clonostachys rosea, Fusarium culmorum*, *Fusarium graminearum*, prothioconazole, Proline, Fusarium foot/root rot, Integrated pest management

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Abstract

Fungal plant diseases have typically been controlled by the application of chemical pesticides. However, excessive use of chemical pesticides may produce undesirable side effects including fungicides tolerance in pathogens and environmental problems, if not handled correct. Application of biocontrol agents (BCA) alone or in combination with low dose of fungicides is one of the alternatives to the use of higher dose of chemical pesticides and lower the general use. The aim of this study was to investigate the potential of combining the fungal BCA *Clonostachys rosea* with a low dose of fungicides to control fusarium foot/root rot on wheat and barley, which is caused by the fungal plant pathogens *Fusarium graminearum* and *Fusarium culmorum*.

In order to find a fungicide and a dose that is compatible with *C. rosea*, an *in vitro* test was performed to analyse the fungicide tolerance of *C. rosea*. For this experiment, different concentration of six chemical fungicides, with different mode of actions, were selected. Fungicide tolerance/sensitivity of *F. culmorum* and *F. graminearum* to these fungicides was also analysed. The assay showed that *C. rosea* has different level of tolerance/sensitivity to different fungicides. In addition, our results showed that *C. rosea* has a relatively better ability to tolerate prothioconazole (commercial name Proline) at 1/30 and 1/60 concentration of recommended full dose compared to *F. graminearum* and *F. culmorum*. Based on *in vitro* result, Proline was selected for seed coating and *in planta* bioassay experiment against *Fusarium* foot rot on wheat and barley. A growth chamber sand seedling test showed significant reduction in disease severity in barley seedling when seeds were treated with *C. rosea* spores compared to control treatments. Similarly, barley seeds treated with full dose of Proline alone or a low dose of Proline in combination with *C. rosea* completely inhibited the *Fusarium* foot rot on barley. Our results showed no significant difference in disease severity on barley between Proline treated and *C. rosea* + Proline treated barley seeds. Analysis of plant health parameters showed that barley plants treated with *C. rosea* alone or with a combination of *C. rosea* and Proline had significantly higher shoot length, shoot fresh weight and dry weight compared to barley seedling from seed coated with only Proline. The bioassay experiment on wheat plants failed since no disease development was observed in any treatment, including the *Fusarium* control. In summary, the result from this study showed that the biocontrol fungus *C. rosea* can be combined with a low dose of Proline. The treatment with a combination of *C. rosea* and low dose of Proline showed similar effect to that of full dose of Proline in controlling fusarium foot rot on barley. The result from this study will help to formulate integrated pest management strategy by mixing the fungus *C. rosea* with Proline and apply it on the specific crop accordingly.

Keywords: Biocontrol agent, *Clonostachys rosea*, *Fusarium culmorum*, *Fusarium graminearum*, prothioconazole, Proline, Fusarium foot/root rot, Integrated pest management (IPM).

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1 Introduction

Biocontrol agents (BCA) in addition to good agronomic practices involving the use of resistant varieties, crop rotation and timely fungicide application have the potential to play a vital role in plant protection against the plant pathogens and thus in future integrated pest management (IPM) strategies. This is due to the biological services the BCAs contribute, such as a complex mode of action and lower or no side effects, leading to more sustainable cropping systems (Parolin *et al*, 2014). In addition, an application of BCA can help to develop both organic and conventional cropping systems. BCA contributes to more natural derived alternative of chemical pesticides, however only few products are marketed due to their inconsistent efficacy under field conditions. To improve the efficacy of the BCAs, mixing of two or more BCAs with complementary mode of action is an increasing practice among scientific community (Xu *et al*., 2011). Combined application with low dose of fungicides is an alternative for an efficient use of the BCAs. The mixture of fungal BCA *Trichoderma pseudokoningii* and bacterial BCA *Bacillus subtilis* was found to be more effective in controlling *F. oxysporum* f. sp. *fabae* and of *F. oxysporum* f. sp. *lupini*, (causing agents of wilt on broad bean and lupine, respectively) compared with either BCA used alone (Wahid, 2006). Similarly, *in planta* experiments showed a significant reduction in foot and root rot on tomato plants inoculated with fungal BCA *Clonostachys rosea* and *Psudomonas chlororaphis* compared with single inoculation of either of BCA (Kamou *et al*., 2016). However, the data analyses from published literatures on combined application of BCAs showed that only 2% of the total treatments had synergistic effects in controlling plant diseases (Xu *et al*., 2011). This suggest that a careful selection of BCAs is necessary while applying microbial mixture for biocontrol of plant diseases.

Combined application of BCAs with compatible fungicides can be crucial for controlling complex plant diseases and integrated pest management. However, in order to combine applications of fungicides and BCA, fungicide tolerance of the BCA (at least up to certain level) is essential. Combined soil application (seed coating) of the fungal BCA *Trichoderma virens* and lower dose of the compatible fungicide thiophanate-methyl was more effective than using either of them individually against *F. solani* and *F. oxysporum* in dry bean (Abd-El-Khair *et al*., 2019). Similarly, combined application of the BCA *T. harzianum* SH 1303 and lower dose of difenoconazole-propiconazole showed synergistic effect in controlling the southern corn leaf blight disease caused by *Cochliobolus heterostrophus* (Wang *et al*., 2019). Moreover, application of the *Trichoderma* agent (Tri-1) formulated with a reduced dose of carbendazim showed efficacy similar to a higher dose of carbenzazim alone in controlling *Sclerotinia sclerotiorium* on oilseed rape

(Hu *et al*., 2016). These results showed that biocontrol agents can be effectively combined with lower doses of compatible fungicides for use in an integrated pest management program.

Fusarium spp is a destructive fungal plant pathogen of cereal plant species and are distributed all over the world. *Fusarium graminearum* is ranked number 4 in the list of top 10 fungal plant pathogens based on their importance in science and agriculture (Dean *et al*., 2012). Fusarium head blight (FHB) and Fusarium foot and root rot caused by *F. graminearum* and *F. culmorum* can cause serious yield and economic loss (Dean *et al*., 2012). In addition, infection of *F. graminearum* and *F. culmorum* to floral tissue reduces the grain quality by producing several mycotoxins (Dean *et al*., 2012). The diseases caused by *F. graminearum* and *F. culmorum* is mainly controlled by azole fungicides, which are moderately effective (Dean *et al*., 2012). Therefore, there is an urgent need of developing alternate strategies to control *Fusarum spp.*

1.1 Aims and objectives

The aim of this study was to identify the fungicides that is compatible with the biocontrol agent *C. rosea*, and investigate the potential in combining applications of the fungal BCA *C. rosea* with compatible fungicides to control fusarium foot-and root rot on wheat and barley,

Hypothesis: A combined application with *C. rosea* and low dose of fungicides can provide a better protection against Fusarium foot-and root rot and consequently healthier wheat and barley plant compared to single treatment with either *C. rosea* or fungicide.

2 Background

2.1 Fungal biological control agents-an alternative of chemical pesticides

The commercial interest for fungal BCAs has increased rapidly during the last decade, with ongoing research on how to improve plant productivity by using sustainable agriculture practices. The commercialisation has confronted obstacles because it is hard to communicate the advantages with BCAs when there are good working fungicides on the market (Butt *et al*, 2001).

The fungal BCAs are considered an attractive alternative to control plant diseases caused by fungal pathogens but there is a need for better application timing to get the BCA to colonize on the targeted crop and inhibit the targeted disease at an effective level (Lima *et al*, 2008). The application of fungal BCAs could be a good alternative in conventional agriculture when fungicide registrations do not get prolonged and get phased out from the market or when there are problems with fungicide resistance. Problems that could be avoided with the application of fungal BCAs are contamination of groundwater which might harm microorganisms, animals and humans (Butt *et al*, 2001). These problems are the reason why new IPM should be integrated in the agriculture and thus it gives more sustainable and less toxic handling of chemicals. Sweden's government decided in the mid 80's that they would reduce the use of pesticides with 50 % within a decade. This was one of several goals that was historically put up and it was not fulfilled because there were no substitutes for the pesticides (Butt *et al*, 2001). BCAs are used as seed treatments and are often effective, but the results vary more than seed treatment with fungicides. A combined integrated seed treatment with both biological- and chemical agent has the potential to control fungal diseases better and increase the yield compared to BCA and fungicide separately (Harman, 1991).

There are some advantages and disadvantages linked with fungal BCAs application compared to fungicides. By using BCAs the risk of getting resistant pests is lower, and biological control can be used in specific areas where chemicals are not allowed. Successful application of BCAs will reduce the use of chemical pesticides and their environmental impact and will promote the IPM practices (Whipps & Lumsden, 2001). In addition, a fungal biocontrol agent can grow and colonize plant root surface and induce the defence response in plants. BCA can be active in places where it initially have not been applied (Harman, 1991). Countries worldwide are legislating the use of chemical pesticides (herbicide, insecticide and fungicide) and goals of reduced application of pesticides are suggested in the EU (European Commission, 2020). The consumers are getting more conscious about what they put in their mouths. The consumers drive the demand of organically produced food and the change to less pesticide application and more BCAs has just begun. Although, it is a long way to make the BCAs as effective as chemical pesticides (Whipps & Lumsden, 2001). By year 2017 Marrone (2009) estimated that the BCAs globally will be sold for $10 billion. The BCAs have an annual growth of 12 % while the chemical pesticides have an annual growth of 3 % (Marrone,

2009). The disadvantages associated with BCAs application is that they are expensive and are target specific. The lack of knowledge of handling and using these products in an optimal way makes it difficult to implement BCAs on the farm level. BCAs with low persistence, that are hard to implement and too site-specific and with unreliable effects could result in expensive cropping systems (Whipps & Lumsden, 2001).

2.2 Plant diseases caused by *Fusarium graminearum* and *Fusarium culmorum* and their control

Fusarium graminaerum and *Fusarium culmorum* are plant pathogenic fungus that causes Fusarium head blight (FHB) (Müllenborn *et al*, 2008; Bai & Shaner, 2004) and Fusarium foot and root rot on wheat and barley (Cook, 1980). *Fusarium graminaerum* and *F. culmorum* produces mycotoxins such as deoxynivalenol (DON), nivalenol (NIV) and zearalenone (ZEA) which contaminates food and fodder (Müllenborn *et al.*, 2008). FHB is common worldwide and is a big problem in some parts of the world with considerable yield losses (Parry *et al*, 1995). *Fusarium* species are mainly controlled by the fungicides tebuconazole and prothioconazole (Müllenborn *et al*, 2008) but also metconazole are effective against *Fusarium* and reduces the toxins significantly (Matthies & Buchenauer, 2000). The need of fungicide treatments varies between different areas, in some areas there are a high pressure of FHB in the fields and in others FHB is not a problem. Forecasting FHB is not easy but can gift the producers a hint of what to use, fungicide or BCAs (De Wolf *et al*, 2003). If there is a high risk of infection, fungicides should be sprayed on the crops at anthesis to reduce the risk of lower yield and contamination of toxins (Chala *et al.*, 2003). Experiments show that the treatment with triazoles can reduce the mycotoxin contamination at the level between 5-90 % (Matthies & Buchenauer, 2000; Chala *et al*, 2003). The choice of fungicides is important. Strobilurines has been reported to increase especially DON levels on the kernels but decrease the symptoms of FHB (Simpson *et al.*, 2001).

Cook (1980) claims that more intense cropping systems increases the risk of soil borne diseases, especially *F. graminaerum* and *F. culmorum*. For farms adapting to the economically more profitable and intense crop rotations it is important to understand the factors which contributes to the risk of e.g. fusarium foot rot in wheat. Plant drought stress can increase the problems with Fusarium foot rot disease, leading to a bigger impact of the disease, indicating the importance of understanding crop stress factors when controlling this type of soil borne diseases (Cook, 1980)

Müllenborn *et al* (2008) show that there are alternatives of different BCAs controllin*g Fusarium spp*. Antagonistic fungi were tested on many *Fusarium spp* and the results showed that the antagonistic fungi could reduce mycelial growth (Müllenborn *et al*, 2008). Palazzini *et al.* (2007) applied BCAs together with *F. graminearum* to prevent FHB. The aim was to cover spray (inoculate) the heads of the crop to evaluate the antagonistic performance of different BCAs against FHB. 354 bacterial strains interacting with *F. graminearum* were tested and the conclusion was that two bacterial strains will be tested further for controlling FHB, *Brevibacillus sp*. and *Streptomyces sp*.

Important factors when testing antagonists on diseases caused by *F. graminearum* are temperature and water since they affect the outcome of the disease.

2.3 *Clonostachys rosea* - a fungal biocontrol agent

Clonostachys rosea (*C. rosea*) is a soil borne fungus found worldwide (Karlsson *et al*., 2015). *Clonostachys rosea* is classified as Phylum: Ascomycetes; Class: Sordariomycetes; Order: Hypocreales; Family: Bionectriaceae. *Clonostachys rose* strain IK726 (used in this study) was isolated from barley roots infected with *F. culmorum*. This strain has shown antagonistic properties against several plant pathogenic fungi including *Fusarium spp* (Dubey *et al*., 2014; Karlsson *et al*., 2015; Nygren *et al*., 2018; Dubey *et al*., 2020; Fatema *et al*., 2018). In addition, it can colonize plant root surface and induce defence response in host plants (Karlsson *et al.,* 2015; Dubey *et al*., 2020). Previous studies have shown that *C. rosea* is an effective biocontrol agent several plant pathogenic fungi including *Botrytis cinerea*, *Alternaria spp F. graminearum* and *F. culmorum* and oomycetes *Pythium tracheiphilum* (Lübeck *et al.*, 2002; Knudsen *et al*., 1995; Jensen *et al*., 2004; Jensen *et al*., 2016; Moller *et al*., 2003). Research has shown that *C. rosea* were able to control 56-76 % of *F. culmorum* infections, which causes rot on foot and root in wheat (Harman, 1991). In addition, *C. rosea* can antagonize plant parasitic nematodes and can be used for nematode biocontrol (Iqbal *et al*., 2018). The biocontrol ability of *C. rosea* against plant pathogenic fungi is attributed to its ability of producing protein/enzyme like hydrophobins, LysM proteins, proteases, chitinases (Dubey *et al*, 2014, 2020 Tzelepis *et al*., 2015) and secondary metabolites including the polyketides (Fatema *et al*., 2018; Iqbal *et al*., 2019; 2020). In addition. *C. rosea* can tolerate secondary metabolites from plant pathogenic fungi in the rhizosphere (Dubey *et al*., 2014, 2016).

2.4 Tolerance of *Clonostachys rosea* to the fungicides

Clonostachys rosea has the ability to tolerate certain fungicides at higher dose compared to those recommended for controlling several fungal plant pathogens (Roberti *et al*., 2006; Jensen *et al*., 2011; Tzelepis and Logopodi, 2011; Dubey *et al*., 2014, 2016). Experiment has shown that *C. rosea* mycelia grows uninhibited on carboxin, guazatine, thiram and triticonazole but is inhibited by prochloraz. The same has been reported for conidia germination (Roberti *et al.*, 2006). When exposed for prochloraz the hyphae start swelling and this affects the growth and formation of conidia negatively (Roberti *et al*, 2006). *Clonostachys rosea* tolerance to fungicides is related to the higher gene copy number coding for ATP-binding cassette (ABC) and major facilitator superfamily (MFS) transporter proteins compared to another biocontrol agent (*Trichoderma atroviride*) and plant pathogenic *Fusarium spp* (Karlsson *et al*., 2015; Nygren *et al*., 2018).

2.5 Fungicide groups characteristics

Fungicides are divided into different groups based on presence of active compound and its mode of action. One fungicide may contain one or more active compounds. The fungicides that were used in this study are presented in Table 1. Appendix.

Table 1. List of used FRAC - groups.

No & Name	Active substance(s)	Mode of actions (MOA)
3, DMI - Fungicide	Propiconazole, difenconazole, prothioconazole	Sterol biosynthesis in membrane
7, SDHI – Fungicide	Isopyrazam	Respiration
9, AP - Fungicide	Cyprodinil	Amino acids and protein synthesis
11, QoI – Fungicide	Azoxystrobin	Respiration

2.5.1 Demethylation inhibitors

Triazoles are also called DMI-fungicides as they are demethylation inhibitors (DMI; FRAC [Fungicide resistance action committee], 2016). In cereals azoles are effective against eyespot, septoria tritici blotch, powdery mildew, yellow rust, crown rust, brown rust, *Fusarium* head blight and rhynchosporium. In addition, in oilseed rape, azoles are effective against light leaf spot, phoma, stem canker and sclerotinia stem rot according to the commercial description of group of active substances (Bayer crop science, 2016).

2.5.2 Succinate dehydrogenase inhibitor

Succinate dehydrogenase inhibitor (SDHI) fungicides (FRAC, 2016) has inhibiting efficacy on rust, septoria tritici blotch, net blotch, ramularia leaf spot and rhynchosporium regarding wheat and barley according to the commercial description of group of active substances (Syngenta, 2020).

2.5.3 Anilino pyrimidines

According to the FRAC, cyprodinil is put in the group anilino pyrimidines (AP – fungicides) and their target site on the fungus is the methionine biosynthesis (FRAC, 2016). Stereo (312, 5 EC) was registered in cereals from year 1999 until 2019 in Sweden. The active substances are especially effective against powdery mildew, net blotch, rhynchosporium leaf blotch, rust and other leaf blotches according to the commercial description of group of active substances (ADAMA, 2016).

2.5.4 Quinone outside inhibitors

Strobilurines are so called QoI-fungicides (Quinone utside Inhibitor) (FRAC, 2016). Azoxystrobin belongs to this group and has a preventive MOA, which means that treatment must be performed before attack of e.g. *Zymoseptoria*, *Fusarium* sp and rust fungi in cereals according to the commercial description of group of active substances (Syngenta, 2016).

2.6 Integrated pesticide management

Integrated pest management (IPM) is a system formed to achieve a more sustainable agriculture. This thesis is only aiming at the agricultural crops, not greenhouse crops where the use of BCAs is more common. The arable cropping systems should be adapted to the prevailing conditions and pathogens. With the IPM regulation the use of pesticides should be adapted to the need of pest or disease control (Whipps & Lumsden, 2001). The EU has issued the "sustainable use directive on pesticides" which stipulates that different practices should be combined to get the most sustainable agriculture, both ecologically and economically (Chandler *et al.*, 2011). One of the reasons for the minor use of BCAs is because the lack of sufficient efficacy compared with existing chemical fungicide market standards. A good way to implement fungal BCAs on the market is to make them compatible with fungicides. The big problem is to make sure that the fungal BCAs are tolerant to the actual fungicide. Seed coating has been tried with both fungicide and fungal BCAs, and the results showed that the fungicide gives a direct protection of the seed and the fungal BCA becomes active a while later in the seedling germination and can also give protection of the root system (Whipps & Lumsden, 2001).

If the governments continue to ban active substances in pesticides faster than new products can be introduced to the market, this means that there will be fewer products in the future. Chandler *et al,* (2011) states that BCAs will/can be a substitute for the phased-out pesticides which is a great opportunity to expand the biocontrol product use. They also said that the use of BCAs on field grown crops is far more complex than using it in greenhouses. Farmers need to be convinced than using BCAs works and is possible, the politicians need to make frameworks for future use in cropping systems and last but not least, the consumers must see a value in these new integrated pest management systems (Chandler *et al*, 2011).

3 Material and methods

3.1 Fungal strains and culture conditions

Fresh cultures of *C. rosea* strain IK726, *F. graminearum* and *F. culmorum* were obtained from department of Forest Mycology and Plant pathology, SLU. The cultures were grown and maintained on potato dextrose broth (PDA, Sigma-Aldrich, St. Louis, MO) or Czapek-dox media (Sigma-Aldrich, St. Louis, MO). Czapek dox medium was used for *in vitro* fungicide tolerance assays. During the whole experimental period the fungus was continuously re-cultured on agar plates to assure fresh mycelia and spores for the coming experiments.

3.2 *In vitro* assay for fungicides tolerance

3.2.1 In vitro assay for fungicides tolerance of *C. rosea* mycelia

The first part of the experiments was to investigate if and at what concentration *C. rosea* is tolerant to different fungicides. An *in-vitro* assay was performed to test *C. rosea* tolerance to four different FRAC (Fungicide Resistance Action Committee) groups: QoI – fungicides, also called strobilurines (Amistar and Stereo), DMI – fungicides, also called triazoles (Proline and Armure), and AP – fungicides (Kayak, Stereo and Bontima) (frac.info), see Appendix.

Table 1. List of used FRAC - groups.

No & Name	Active substance(s)	Mode of actions (MOA)
3, DMI - Fungicide	Propiconazole, difenconazole, prothioconazole	Sterol biosynthesis in membrane
7, SDHI – Fungicide	Isopyrazam	Respiration
9, AP - Fungicide	Cyprodinil	Amino acids and protein synthesis
11, QoI – Fungicide	Azoxystrobin	Respiration

The *in-vitro* tests were performed using three different concentration of fungicides i) recommended field doses ii) 1/ 30 of full dose and iii) 1/60 of full dose for each fungicide. The field doses were taken from the producing companies' recommendation. To practise this as close to reality as possible the field concentration per hectare are usually mixed with 200 litres of water. The field spray tank has normally liquid dose per hectare is 200 litres but that can vary depending on brands and equipment. To get a concentration of the fungicide the field dose (litres/ha) was divided by the amount of liquid in the sprayer (litres/ha).

$$\frac{Fungicide\ field\ concentration\ (\frac{l}{ha})}{Water\ amount\ (\frac{l}{ha})} = Fungicide\ (l)/water\ (l)$$

The fungicide (l)/water (l) was used to calculate how many microliters (µl) fungicide there should be added to 1 ml agar.

Table 2. Concentrations of fungicides in *in vitro* test

Product	Recommended field dose (l/ha)	Concentration on agar (µl fungicide/ml agar)
Kayak	1-1,25	6,25
Stereo	1-1,5	7,5
Bontima	2	10
Armure	0,4-0,8	4
Proline	0,6	3
Bontima	0,3-0,5	2,5

The experiment was set up with four replicates from each fungicide plus four replicates of the control. After autoclaving the Czapek-dox media 20 ml was poured into a falcon tube. The 20 ml mix was then divided on the four replicates, 5 ml on each plate. The corresponding field dose of each fungicide were added and mixed with the media on the plates. The plate was 5.2 cm in diameter. The concentration of fungicides used for *in vitro* assay are presented in Table 2. After the agar has solidified one agar-plug (5 mm in diameter) of active growing mycelia from *C. rosea* was added in the middle of each plate. The plates were stored in a 25°C room for ten days and the growth diameter of *C. rosea* was measured continuously with a ruler. Photographs were taken during the whole experiment.

3.3 *In vitro* assay to test fungicides tolerance of *F. graminearum* and *F. culmorum*

In the beginning of experiments, *F. graminearum* and *F. culmorum* were grown on Czapek – Dox agar. The Fusarium species were tested under similar conditions as *C. rosea* with 5 ml of Czapek – Dox media on each agar plate. The growth and inhibition on each of the six different fungicides were tested at full field dose and full field dose divided by 30 and 60 in four replicates.

3.3.1 In vitro assay to test fungicide tolerance of *C. rosea* spore

The next step in the experiments was to see if the germination of the spores (conidia) of *C. rosea* was affected by the different fungicide concentrations. By this time the oldest

inoculated *C. rosea* plates had started to sporulate and these spores were used to inoculate the Czapek – Dox plates with all six fungicides and a control. To harvest *C. rosea* spores, autoclaved distilled water was added to the culture. The spores were collected with a pipette and filtered through glass wool to get rid of mycelia in the solution. A hemacytometer (Bright-Line; Sigma-Aldrich) was used to count the spores following manufacturer's instructions. The spores were counted twice and mean value was used as a final spore concentration. The equation $Concentration_1 * Volume_1 = Concentration_2 * Volume_2$ was used and gave 100 spores per 1 µl of spore suspension and a 10 µl drop of spore suspension (1000 spores) was inoculated in one petri dish with 8.6 cm in diameter. The germination and growth rate of the spore colonies were measured after 4, 5, 7 and 11 days. The experiment was performed in five biological replicates.

3.4 Seed coating with *C. rosea* + prothioconazole

The most effective fungicide group against *F. graminearum* and *F. culmorum* is azoles (Becher *et al.*, 2010). Based on results from the *in vitro* tests and previously publish report of Becher *et al.*, (2010), prothioconazole was selected for further experiment. Bayer crop science has a product called Raxil Pro for seed coating, containing tebuconazole, metalaxyl and prothioconazole. The concentration of prothioconazole is 250 g/l and the recommended dose is 325 ml/100 kg seed (cropscience.bayer.ca). The prothioconazole concentration in Raxil Pro was used as guidance for the seed coating. The method used for checking *C. rosea* spore's compatibility with prothioconazole was done by using five different prothioconazole concentrations. The tested concentrations were recommended field dose of prothioconazole applied as Proline, Proline field dose divided by 120 and the other four treatments were adapted to the amount of prothioconazole used in Raxil Pro for seed coating. It was decided to use the protioconazole concentration that one seed get when coated with Raxil Pro which equals 0,0108 µl prothioconazole/ 1 ml agar. The other three prothioconazole coating concentrations equals to a concentration according to 5 coated seeds (0,054 µl prothioconazole/1 ml agar), 1/2 coated seed concentration (0,0054 µl prothioconazole/1 ml agar) and 1/4 coated seed concentration (0,0027 µl prothioconazole/1 ml agar). A control without prothioconazole was included in the experiment. 15 ml of Czapek – Dox agar was used for each plate and that is why the concentrations above are multiplied by 15. A 10 µl *C. rosea* spore suspension ($1x10^3$ spores) was added on the Czapek – Dox agar plates supplemented with different concentration of prothioconazole. The experiment was performed in five biological replicates. The germination and growth of the spores was continuously monitored.

3.5 *In planta* bioassay

The *in planta* bioassay experiment was performed on wheat and barley in the same way. For bioassay experiment, the seeds were first coated with *C. rosea* spores ($1x10^8$ spore/ ml) following procedure described before (Dubey *et al*., 2014, 2016). The seeds were immersed in spore suspension for 30 minutes with gentle shaking, and then air dried for 15 minutes before coating with the fungicide Proline. There were four different treatments, five seeds coated with *C. rosea*, five seeds with *C. rosea* + full seed

concentration Proline, five seeds with *C. rosea* + 1/2 seed concentration proline and five seeds with *C. rosea* + 1/4 seed concentration proline (Table 3). To get all seeds coated properly and make sure that all prothioconazole was absorbed the coating solution was diluted. The full seed coating concentration was diluted a hundred-fold, the half seed coating concentration was diluted two hundred-fold and one fourth of full concentration was diluted four hundred-fold. By using this method for coating both wheat and barley, the amount of liquid solution was the same for all the different prothioconazole treatments. Following calculations was used to find out prothioconazole dose per seed:

$$\frac{Wheat\ seed\ weight\ (g)}{Recommended\ seed\ weight\ per\ treatment(g)} = \frac{0{,}045}{100000} = 4{,}5 * 10^{-7}$$

$$4{,}5 * 10^{-7} * 375\ ml = 1{,}46 * 10^{-4}\ ml/seed\ = 0{,}146\ \mu l/seed$$

Thousand seed weight was used in the calculations and for wheat it is 45 g (Andersson, 1992).

Table 3. Wheat seed coating concentrations

Treatment	Prothioconazole concentration per seed (µl)	*C. rosea* coating concentration/ml
C. rosea	-	$1*10^8$
C. rosea + full concentration prothioconazole	0,146	$1*10^8$
C. rosea + ½ concentration prothioconazole	0,073	$1*10^8$
C. rosea + ¼ concentration prothioconazole	0,0365	$1*10^8$

Barley seeds were coated following the procedure described for wheat seed except *C. rosea* spore concentration (Table 4). To coat barley seeds, a *C. rosea* spore concentration of $1x10^6$ spore/ml was used. Following calculations was done to determine the prothioconazole dose per seed:

$$\frac{Wheat\ seed\ weight\ (g)}{Treated\ weight} = \frac{0{,}05}{100000} = 5 * 10^{-7}$$

$$5 * 10^{-7} * 375\ ml = 1{,}625 * 10^{-4}\ ml/seed\ = 0{,}163\ \mu l/seed$$

Thousand seed weight was used in the calculations and for barley it is 50 g (Andersson, 1992).

Table 4. Barley seed coating concentrations

Treatment	Prothioconazole concentration per seed (µl)	*C. rosea* coating concentration
C. rosea	-	$1*10^6$
C. rosea + full concentration prothioconazole	0,163	$1*10^6$
C. rosea + ½ concentration prothioconazole	0,081	$1*10^6$
C. rosea + ¼ concentration prothioconazole	0,041	$1*10^6$

Seeds that were used in the bioassay were controlled to verify the presence of *C. rosea*. They were controlled by taking five seeds from the treated wheat and ten seeds from the treated barley which was added and taken in for account before dilution already in the coating part of experiment. The seeds for control were rinsed and the spores were counted using two methods. First method was using the hemacytometer, five treated wheat seeds were diluted with 5 ml of distilled water (1 seed = 1 ml distilled water). From the new stock solution 10 µl was put on the hemacytometer two times and the spores was counted both times and divided by two to get a mean value. The second method was plating, 100 µl spore solution was spread on Czapek – Dox agar plate. The germination and colonies emerging were counted after approximately 48h. Series of dilutions were made, stock, 10^{-1}, 10^{-2} and 10^{-3}. The stock solution and the dilution series were used to count spores on the hemacytometer under the microscope.

The dilution series were also used on the spore germination on plates. Five replicates from each dilution series were used and the germinating spores were counted to get a mean value of all five replicates. The mean value was multiplied by 10 because one 10^{th} of the spore concentration from one coated seed was taken to plating. If the spore dilution is not from the stock the spore count is further multiplied e.g. $10^{-1} = 10$, $10^{-2} = 100$ and $10^{-3} = 1000$.

A sand seedling test was performed on wheat and barley seeds following a procedure described before (Dubey *et al*., 2014, 2016, 2020). The experiment was performed in a phytotron with 80 % air humidity, 120 – 150 lumens light and at temperature of 15°C. The wheat plants were watered approximately every fifth day to keep the moisture in the special sand in which they were sown. Each bioassay contained 5 trays (replicates) with

40 pots in each tray. All trays contained 8 different treatments (Table 5), 5 pots per treatment with 2 seeds in each pot. The 8 different treatments required 50 seed plus 10 seeds extra to count coated spores on each seed treatment.

Table 5. Bioassay treatment - wheat. All seeds were inoculated with *F. graminearum* except No. 1 Control.

No.	Treatments
1. Control	Clean seed
2. Control	*F. graminearum* control
3. *C. rosea*	$1*10^8$
4. Full fungicide	0,164 µl/seed
5. ½ fungicide	0,073 µl/seed
6. *C. rosea* + full fungicide	$1*10^8$ + 0,146 µl/seed
7. *C. rosea* + ½ fungicide	$1*10^8$ + 0,073 µl/seed
8. *C. rosea* + ¼ fungicide	$1*10^8$ + 0,037 µl/seed

The plants were grown for approximately four weeks. As soon as the sand started to dry out, it was watered. The height of the plants was measured after 14 days and before harvest (day 27). All germinated plants were measured in each treatment in one tray (replicate). The total height of plants in one treatment was divided by the number of germinated plants in that treatment, not the sown number of seeds (10), to get a comparative mean value. On day 27 the wheat was harvested. The plants were picked up gently to make sure all the roots were collected. The plants were sorted after replicates and treatments. The dry weight of the plants in each treatment from every replicate was determined separately. The plants were dried for 24 hours in a 60°C oven before measuring the dry weight. Photographs were taken during the whole experiment.

The treatments used for *in planta* bioassay on barley are presented in table 6. Before being coated with *C. rosea* and prothioconazole the barley seeds were surface sterilized in seven steps, 1. Wash with tap water for 5 minutes, 2. Cover seeds with water and add one drop detergent and wash for 15 minutes at 200 rpm in a baker, 3. Rinse with tap water to remove all detergent, 4. Put the seeds in an autoclaved flask or bottle, 5. Add 2 % sodium hypochlorite (NaOCl) and put at 200 rpm for 15 minutes, 6. Rinse the seeds from NaOCl with sterile water for several times and 7. Blot dry.

In total 560 seeds were used, 50 for each treatment plus 20 extra to investigate the effect of coating the seeds. The seeds that were not sown were rinsed with 1 ml distilled water/seed in a falcon tube. The seeds were mixed and vortexed for several minutes and the spore counting from the *C. rosea* treatments were made in a hemacytometer and by pipetting 100 µl stock solution and 10^{-1} dilution on Czapek – Dox agar plates, 5 replicates each.

Table 6. Bioassay treatment - barley. All seeds were inoculated with *F. graminearum* except No. 1 Control.

No.	Treatments
1. Control	Clean seed
2. Control	*F. graminearum* control
3. *C. rosea*	$1*10^6$
4. Full fungicide	0,163 µl/seed
5. ½ fungicide	0,082 µl/seed
6. *C. rosea* + full fungicide	$1*10^6$ + 0,163 µl/seed
7. *C. rosea* + ½ fungicide	$1*10^6$ + 0,082 µl/seed
8. *C. rosea* + ¼ fungicide	$1*10^6$ + 0,041 µl/seed

The watering of the barley plants was also executed differently because they were only watered when sown and after 8 days. The water amounts were limited to stress the plants and make them more susceptible to infection (Schoeneweiss, 1975) in this case by *F. graminaerum*. The growth rate was measured after 12 days in the same way as the wheat.

Plants that had symptoms due to the *Fusarium* were rated according to a scaling method called infection rating (IR). The plants were scored from 1-5, 1 (vigorous plant), 2 (minor disease), 3 (more visible disease), 4 (whole plant affected by disease) and 5 (dead plant) (Dubey et. al 2014)).

For the statistics in this thesis Fischer's method was used.

4 Results

4.1 *In vitro* tests of dose – response to different fungicides

4.1.1 Tolerance of *C. rosea, F. graminearum* and *F. culmorum* to fungicides

Growth rate of *C. rosea* (figure 1), *F. culmorum* and *F. graminearum* (figure 2) was measured on Czapek-dox media supplemented with Kayak, Stereo, Bontima, Armure, Proline or Amistar fungicides. At the concentration corresponding to full dose, *C. rosea* could grow only on Czapek-dox supplemented with Kayak or Amistar, see Figure 1. No measurable growth was recorded in the presence of Stereo, Bontima, Armure or Proline. Similar result was recorded for *F. graminearum* and *F. culmorum* (Figure 2). At 1/30 and 1/60 of full dose concentration, mycelial growth of *C. rosea* was recorded in the presence of all tested fungicides except Armure (Figure 4, 6). While at 1/30 and 1/60 of full dose concentration mycelial growth of *F. graminearum* and *F. culmorum* was recorded in medium supplemented with Kayak, Stereo, Bontima and Armure (figure 3, 5). In contrast to the growth of *C. rosea*, no growth of *F. graminearum* and *F. culmorum* was found on 1/30 and 1/60 of full dose concentration. In addition, *in vitro* fungicide assay showed that *C. rosea* cannot tolerate Armure fungicides at the tested concentration.

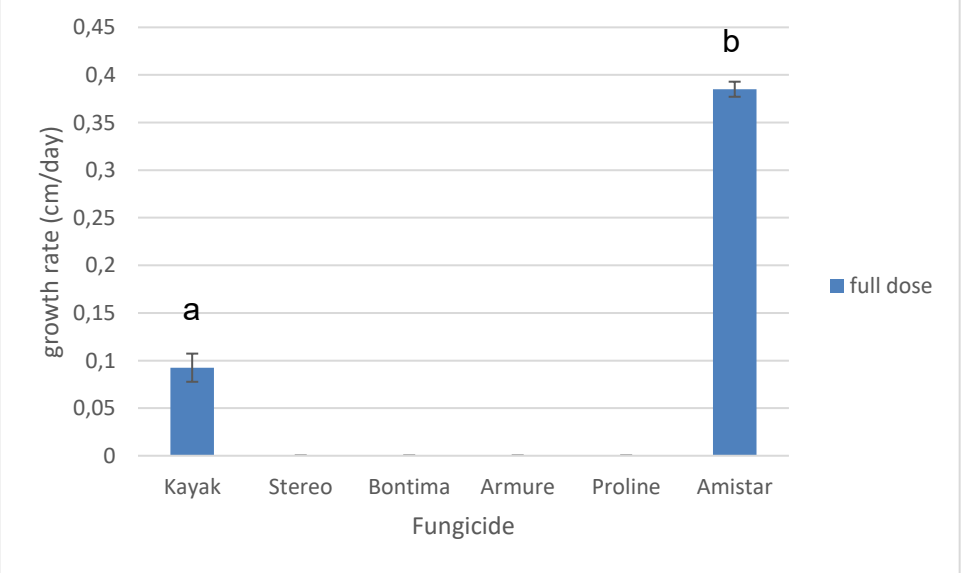

Figure 1. Growth rate of *C. rosea* on agar plates supplemented with full field dose fungicides. Error bar represents standard deviation based on five biological replicates. Different letter shows significant difference between the treatments.

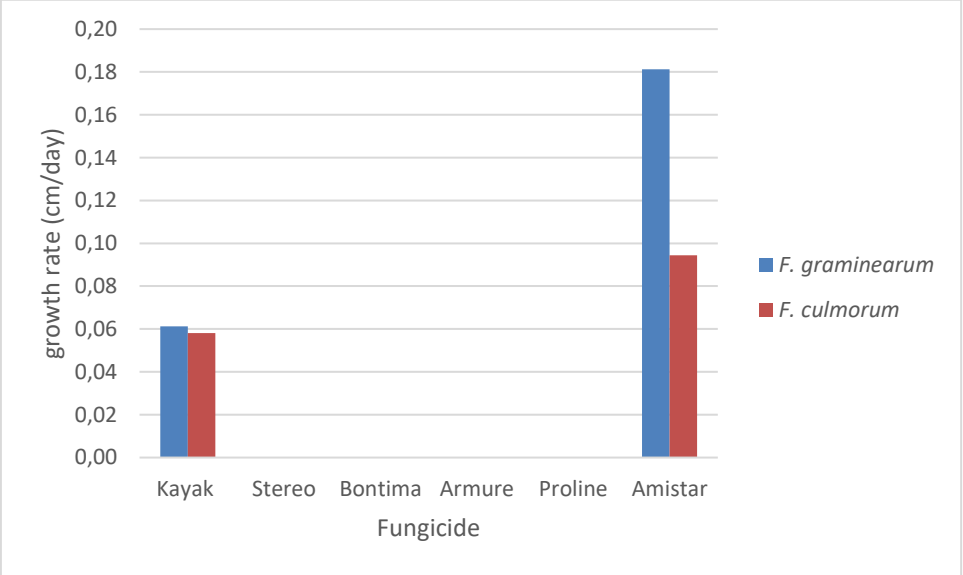

Figure 2. Mean growth rate of *F. graminearum* and *F. culmorum* on Czapek-dox medium supplemented with full field dose fungicides. The data is average of five biological replicates.

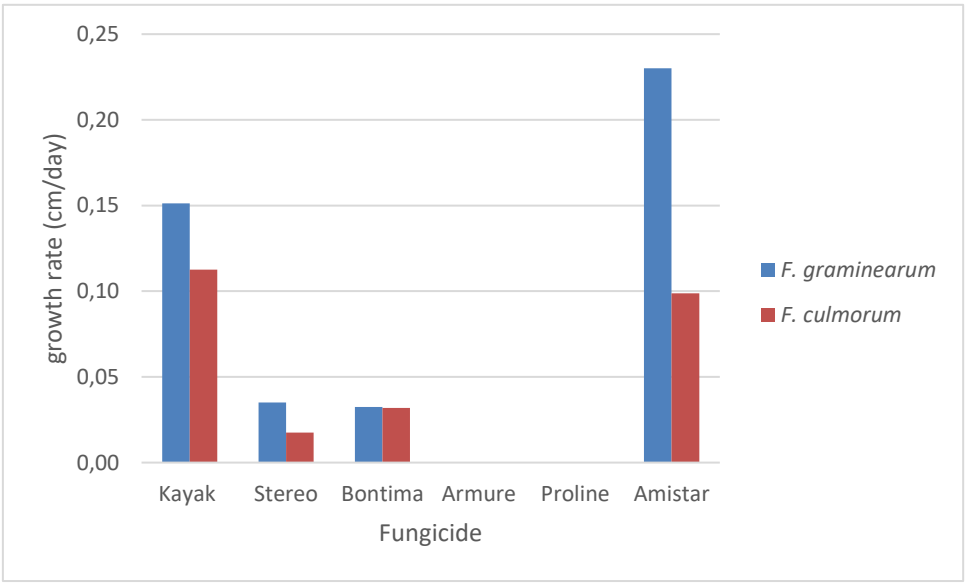

Figure 3. Growth rate of *F. graminearum* and *F. culmorum* on the agar plates with 1/30 (full dose divided by 30) of full dose fungicide concentration. The data is average of five biological replicates.

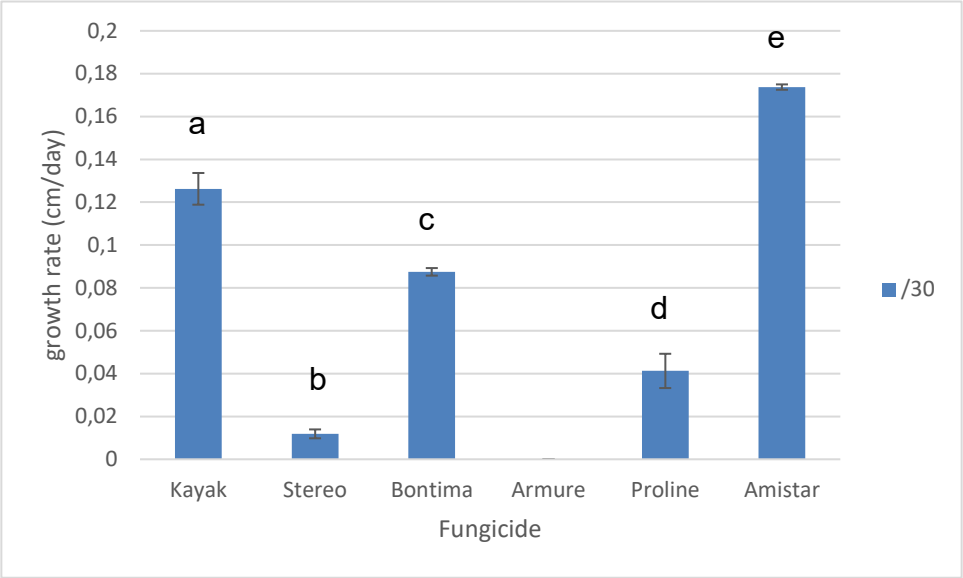

Figure 4. Growth rate of *C. rosea* on the agar plates with 1/30 (full dose divided by 30) of full dose fungicide concentration. Error bar represent standard deviation of five biological replicates. Different letter shows significant difference between the treatments.

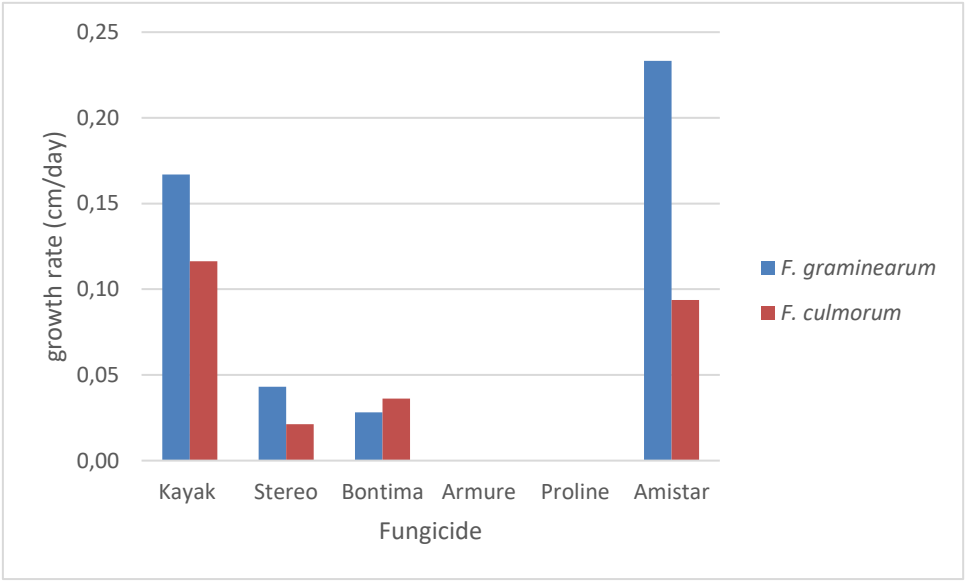

Figure 5. Growth rate of *F. graminearum* and *F. culmorum* on the agar plates with 1/60 (full dose divided by 60) of full dose fungicide concentration. The data is average of five biological replicates.

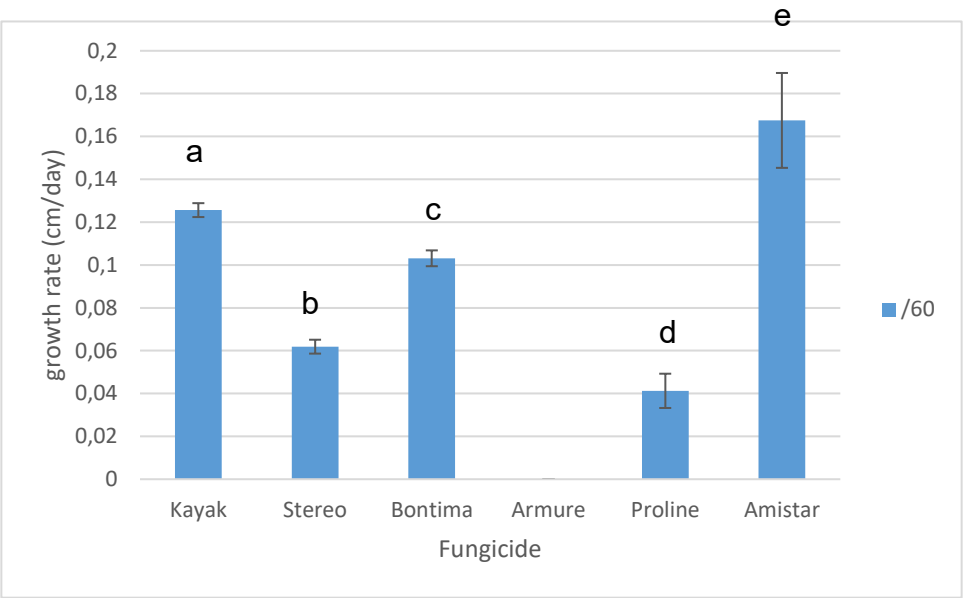

Figure 6. Growth rate of *C. rosea* on the agar plates with 1/60 times (full dose divided by 60) of full dose fungicide concentration. Error bar represents standard deviation of five biological replicates. Different letter shows significant difference between the treatments.

4.1.2 Tolerance of *C. rosea* spores to fungicide

An *in vitro* spore germination test was set up to study the tolerance of *C. rosea* spores to six selected fungicides at concentrations corresponding to 1/30 and 1/60 of recommended full dose. At the 1/30 dose *C. rosea* only grew on Kayak and Amistar, while at 1/60 dose it grew on Kayak, Stereo, Bontima and Amistar. No measurable growth of *C. rosea* was found in the presence of Armure or Proline (Figure 7). Similar to the mycelial growth experiments, *C. rosea* spores showed higher tolerance to Kayak and Amistar. Microscopic observation of spore germination showed that *C. rosea* spores could germinate in the presence of Armure and Proline 7 days post inoculation.

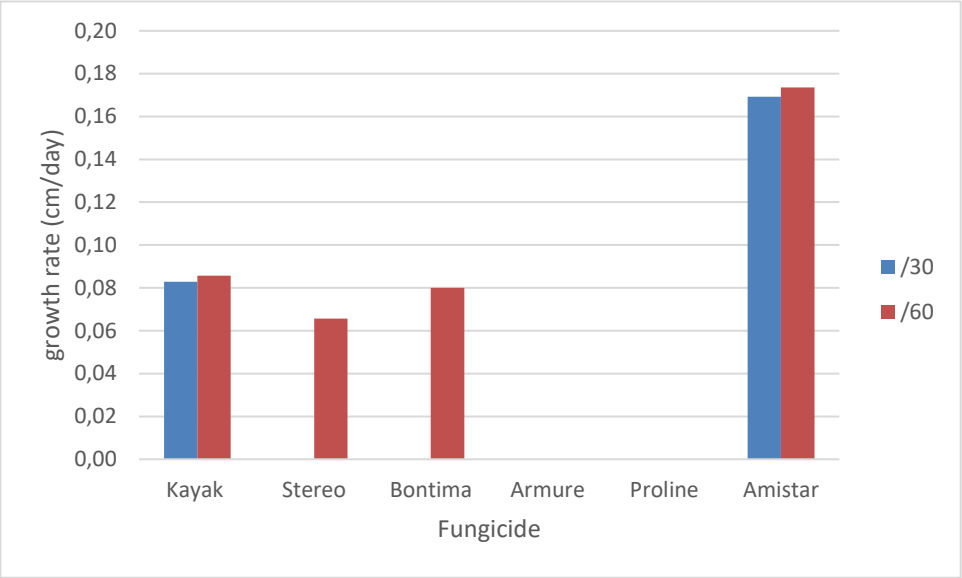

Figure 7. Colony diameter of *C.rosea* spores on Czapek-dox media supplemented with 1/30 of recommended fungicide concentration 7 days post inoculation. The data is average of five biological replicates.

Eleven days after inoculation, the colony diameter of *C. rosea* on Czapek-dox supplemented with Kayak was 2.08 cm, Stereo 1.23 cm, Bontima 1.15 cm, The corresponding colony diameter for *C. rosea* on Czapek-dox with Amistar was not measured as the mycelia had grown to the edges of the agar plate.

At 1/60 concentration, *C. rosea* spores could germinate and grow in presence of all tested fungicides except Armure (Figure 7). Eleven days after inoculation, spore colonies on Kayak had grown 2.3 cm, Stereo 1.13 cm, Bontima 1.69 cm. The fungus had just begun to grow on Proline day 11 while the colonies on Amistar had grown all over the plate. This experiment shows that DMI - fungicides are inhibiting spore germination and growth effectively, AP – fungicides are less effective and QoI – fungicides inhibits the grow of *C. rosea* very little.

4.2 *C. rosea* germination at different prothioconazole seed coating concentrations

Based on result from the *in vitro* test where *C. rosea* could grow in the presence of Proline, while *F. graminearum* and *F. culmorum* could not, *in vitro* experiment was performed to test the tolerance of *C. rosea* spores to the different concentration (based on seed treatment) of Proline (prothioconazole). In figure 8, 1 seed treatment is equal to the same prothioconazole concentration per seed which Raxil Pro gives when treated according to standard recommendations (cropscience.bayer.ca).

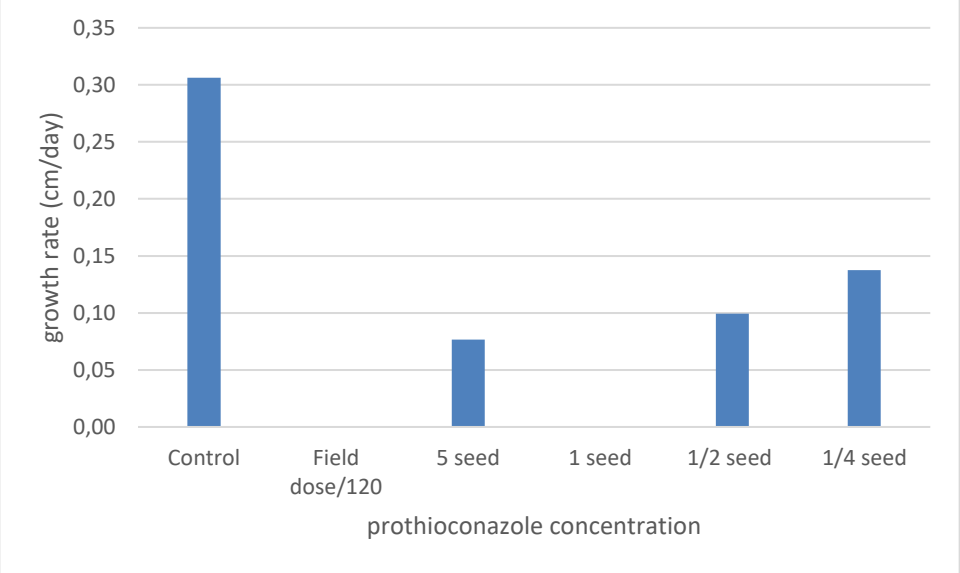

Figure 8. Chlonostachys rosea's spore germination and mycelia mean growth rate per day on Czapek – Dox agar with different prothioconazole concentrations. The data is an average of five biological replicates. 5 seed treatment is five times higher dose than 1 seed etc.

The concentration of prothioconazole per seed was calculated as described in materials and methods section. *Clonostachys rosea* spores germinated and grew 0.08 cm/day at 5 seed concentration (five time more than recommended for per seed), 0.1 cm/day at 1/2 seed concentration, 0.14 cm/day at 1/4 seed concentration. At the concentration of /120 of the recommended full dose and 1 seed concentration, no measurable growth rate was observed, however, microscopic analysis showed that *C. rosea* spores were germinating on the agar plates.

4.3 *In planta* biocontrol assay against *F. graminearum* on wheat

A winter wheat variety called Stava was used for the first bioassay experiment. The result of seed coating is presented in table 7. *Fusarium graminearum* was inoculated at all five biological replicates except the control. Each replicate contained five pots with two seeds in each pot i.e. 10 plants per replicate. The seed emergence was 89 % and 94 % after 14 days and 27 days of experiment, and no difference in seed germination was observed between the treatments.

Table 7. Table show the spore count from *in vitro* and from the hemacytometer. Spores counted from seeds treated with *C. rosea* and *C. rosea* together with different prothiconazole dosages. Prothioconazole is called fungicide in tables and figures.

Treatment	Hemacytometer (spores/seed)
C. rosea	$2*10^5$
C. rosea + ¼ fungicide	$4{,}25*10^5$
C. rosea + ½ fungicide	$3*10^5$
C. rosea + full fungicide	$7{,}75*10^5$

Among the five sets (five trays), one was not watered enough so there was uneven germination and dried out plants. That is why the results from the wheat bioassay only have four sets with five replicates in each tray in the result.

Figure 9 shows the shoot length (height in cm) of wheat plants after 14 days of incubation. The combination treatments (*C. rosea* + fungicide) were not significant different in shoot length compared to the control. However, shoot length of wheat treated with full or half dose of Proline was significantly shorter ($P \leq 0.001$) compared with the control treatment (Figure 9). In addition, shoot length of *C. rosea* treated plant was significantly higher ($P \leq 0.005$) compared to the Proline treated plant (Figure 9). *Chlonostachys rosea* treated plants were significantly shorter than the control ($P = 0.02$). No significant difference was found between control (only wheat plant) and fusarium control treatment. The results show that the control treatment was significantly different ($P \geq 0.005$) from all other treatments except control and *C. rosea* + ¼ fungicide. The wheat seeds coated with full dose Proline showed lowest ($P \geq 0.039$) shoot length among all treatments (Figure 9), while the combined treatment of *C. rosea* with ¼ dose of Proline showed the highest shoot length.

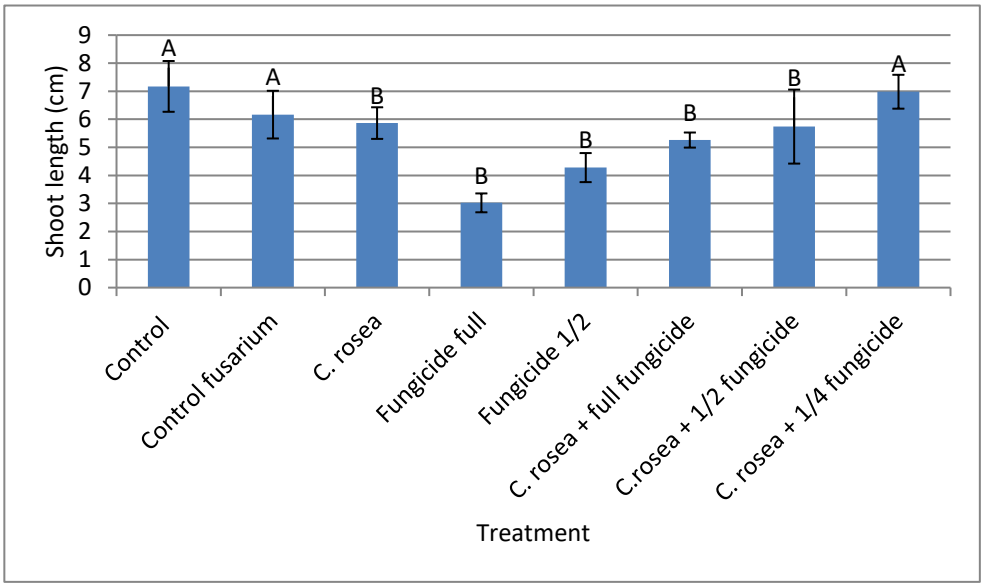

Figure 9. Shoot length of wheat plants at 14 days of experiment. The experiment was performed in four biological replicates. Error bar represent standard deviation. Significant difference ($P \leq 0.05$) is treatment compared to Control and is based on Fisher's exact test. Bars indicated by a common letter are not significantly different.

After 27 days in the phytotron the height of the wheat plants was recorded again. The *C. rosea* + fungicide treatments had tendencies to a higher mean height compared to single treatments with either *C. rosea* or fungicide, but not at a $P \geq 0.005$ significance level (Figure 10). All three *C. rosea* + fungicide treatments had significantly higher shoot length ($P \geq 0.005$) than the full fungicide treatment. The plants in the full fungicide treatment was significant shorter than all treatments except the 1/2 fungicide treatment ($P = 0.11$). The mean shoot length of *C. rosea* was 7.88 cm and the mean length of the full fungicide treatment was 6.69 cm which made *C. rosea* treatment significant higher ($P = 0.039$) than the full fungicide seed treatment

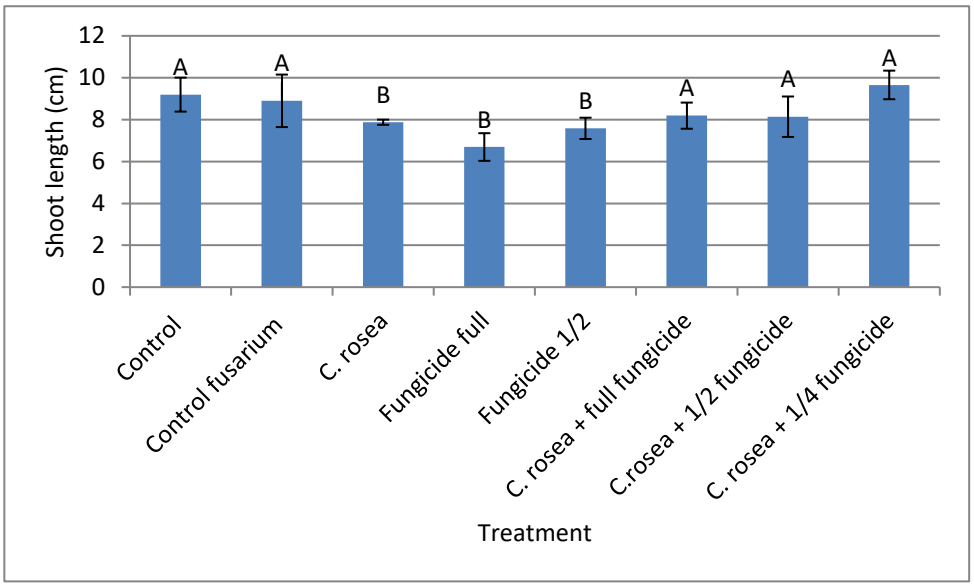

Figure 10. Shoot length of wheat plants at 27 days of experiment. The experiment was performed in four biological replicates. Error bar represent standard deviation. Significant difference ($P \leq 0.05$) is treatment compared to Control and is based on Fisher's exact test. Bars indicated by a common letter are not significantly different.

After 27 days of experiment the wheat plants fresh weight and dry weight was measured. Figure 11 show the biomass fresh weight from the different treatments (root + leaves). The combined treatments with *C. rosea* and 1/4 of full fungicide (Proline) had higher biomass ($P \leq 0.045$) compared with the biomass of plants treatment, except ½ fungicide treatment ($P = 0.095$).

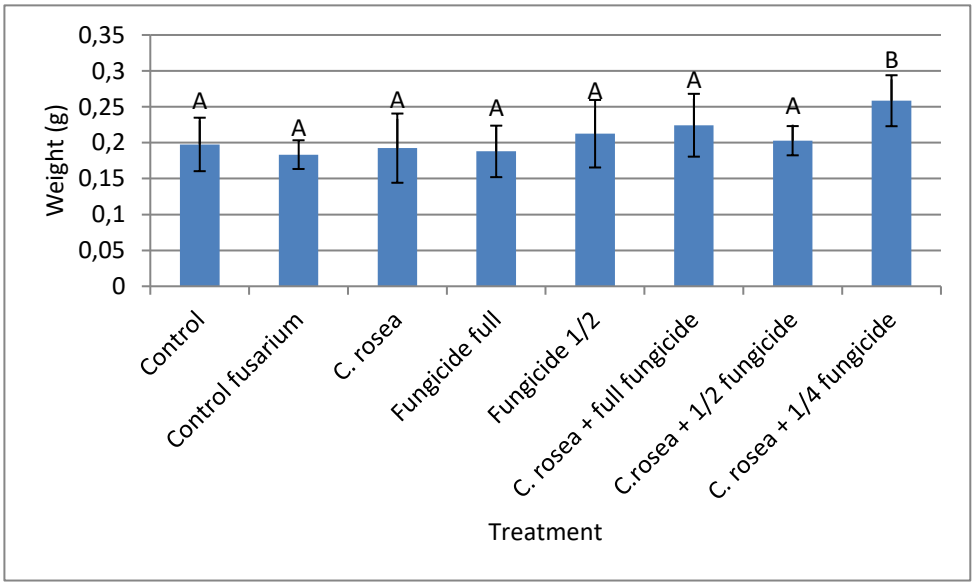

Figure 11. Fresh shoot weight of wheat plant at 27 days of experiment. The experiment was performed in four biological replicates. Error bar represent standard deviation. Significant difference ($P \leq 0.05$) is treatment compared to Control and is based on Fisher's exact test. Bars indicated by a common letter are not significantly different.

After drying wheat plants approximately 24 hours in a 60° oven the plants were weighed to obtain the dry weight. The mean value from all replicates show that, again, the combination of *C. rosea* + fungicide treatments had a higher dry weight (Figure 12). The dry weight biomass show more significance between different treatments compared to fresh weight. *C. rosea* + ¼ fungicide had a significant higher biomass than Control *Fusarium* (P = 0.046), *C. rosea* (P = 0.019), full fungicide (P = 0.046) and ½ fungicide (P = 0.02). Otherwise *C. rosea* + full fungicide treatment had significant higher biomass than *C. rosea* (P = 0.03) and ½ fungicide (P = 0.03) treatments.

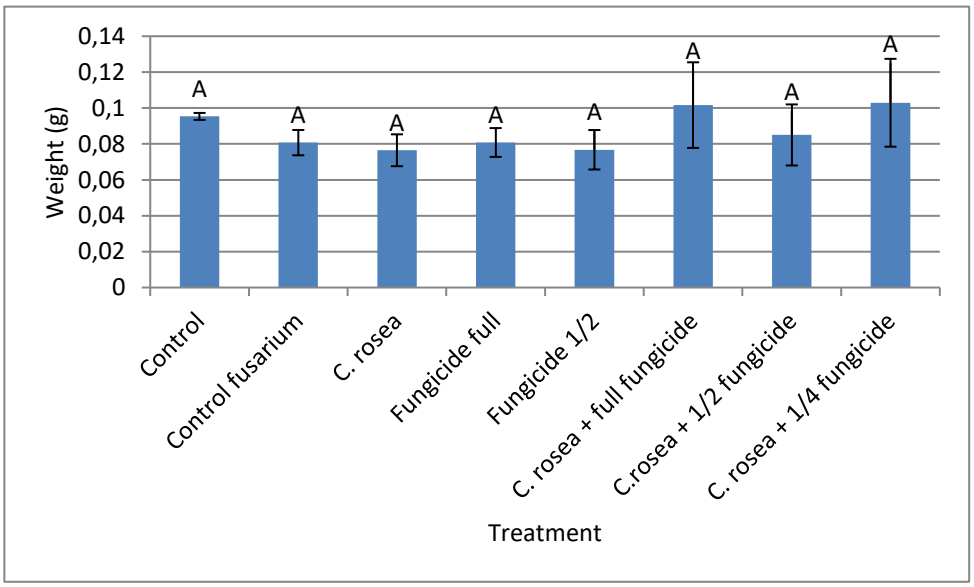

Figure 12. Dry shoot weight of wheat plant at 27 days of experiment. The experiment was performed in four biological replicates. Error bar represent standard deviation. Significant difference ($P \leq 0.05$) is treatment compared to Control and is based on Fisher's exact test. Bars indicated by a common letter are not significantly different.

4.4 *In planta* biocontrol assay against *F. graminearum* on barley

The second bioassay experiment against *F. graminearum* was performed on barley. The experiment was performed in five biological replicates with 10 plants per replicates. The seed emergence was 67% and 72% after 12 days and 21 days of experiment respectively, and no difference in seed germination was observed between the treatments. Extra seeds from the treatments coated with *C. rosea* and *C. rosea* + fungicide (prothioconazole) were washed and diluted to see how many spores were in fact attached to one seed (see table 8.). The *in vitro* inoculation of the barley seed coated with *C. rosea* were not growing but spores were found in all treatments except *C. rosea* + ½ fungicide treatment. The spore count per seed made with the hemacytometer gave measureable results in three of four treatments.

Table 8. The calculated mean presence of *C. rosea* spores from the four different treatments with *C. rosea*. (nd = none detected)

Treatment	Hemacytometer (spores/seed)
C. rosea	$2{,}75*10^5$
C. rosea + ¼ fungicide	$1{,}25*10^5$
C. rosea + ½ fungicide	nd
C. rosea + full fungicide	$2{,}5*10^5$

The height of barley was measured at day 12 and at 21 day of experiment (harvest day). The barley plants from seed treated with *F. gramineaum* showed a reduced growth rate compared with the control (P = 0.003). After 12 days, the plants from seed treated with *C. rosea* spores, *C. rosea* + ½ fungicide and *C. rosea* + ¼ fungicide were significantly ($P \geq 0.005$) taller compared to fusarium control and to seeds treated with full dose Proline (one seed treatment). However, no significant difference in plant height between fusarium control and fungicides treated plants was recorded (Figure 13). The control grew significant taller than the *Fusarium* control and the full fungicide treated replicates.

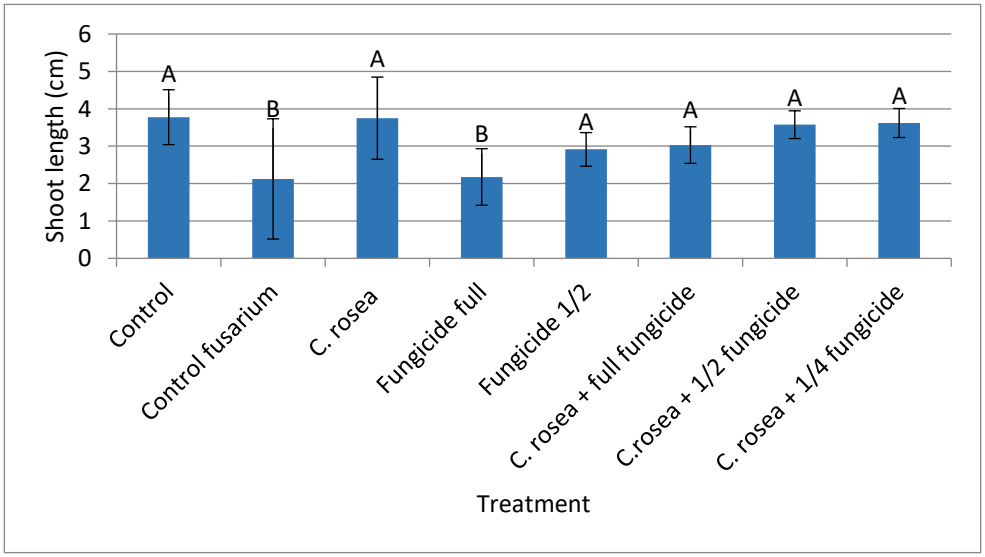

Figure 13. Shoot length of Barley plant at 12 days of experiment. The experiment was performed in five biological replicates. Error bar represent standard deviation. Significant difference ($P \leq 0.05$) is treatment compared to Control and is based on Fisher's exact test. Bars indicated by a common letter are not significantly different.

The plant height was also recorded after 21 days. The result showed more differences between the treatments compared to the 12 days recordings. In comparison to *Fusarium* control, all other treatment had significant ($P \leq 0.05$) higher shoot length (Figure 14). In addition, barley seed treated with *C. rosea*, *C. rosea* + ½ fungicide and *C. rosea* + ¼ fungicide showed significantly ($P \leq 0.05$) higher shoot length compared with barley plant treated with full dose Proline. The mean values of all replicates show that *C. rosea* combined with fungicide were higher than seed treatments made only with fungicide.

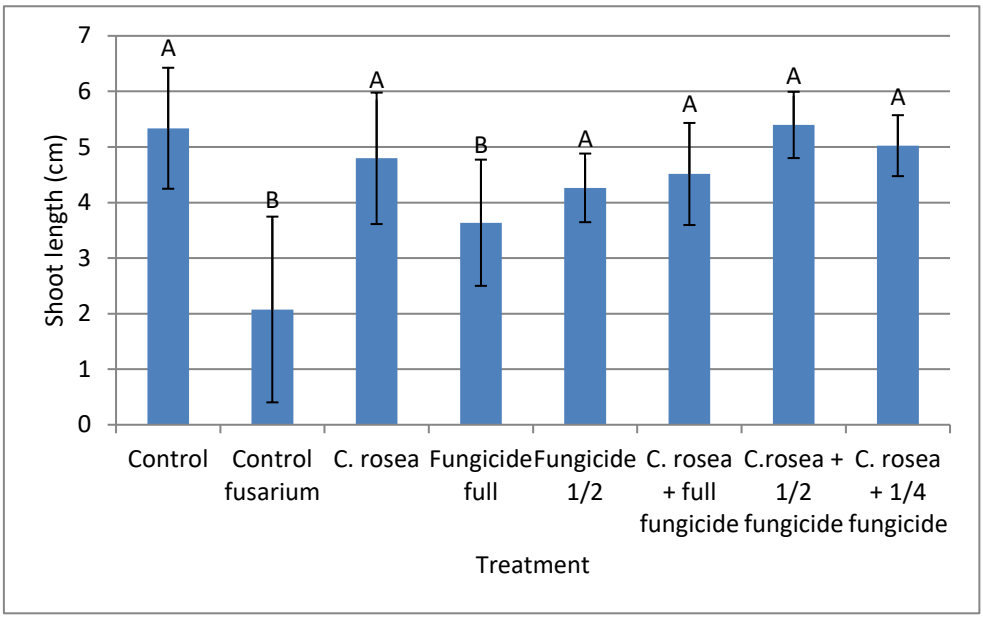

Figure 14. Shoot length of Barley plant at 21 days of experiment. The experiment was performed in five biological replicates. Error bar represent standard deviation. Significant difference ($P \leq 0.05$) is treatment compared to Control and is based on Fisher's exact test. Bars indicated by a common letter are not significantly different.

The fresh and dry shoot weight of barley plant was measured at 21 day of experiment. The *Fusarium* control i.e. the plants infected only with *Fusarium* showed significantly reduced fresh shoot weight compared with barley control and all treatments except plant treated with *C. rosea* and full dose of fungicide (Figure 15). In addition, the barley plant treated with *C. rosea* + ¼ fungicide was significant ($P \leq 0.05$) higher than all other treatments except *C. rosea* + fungicide and *C. rosea* + ½ fungicide.

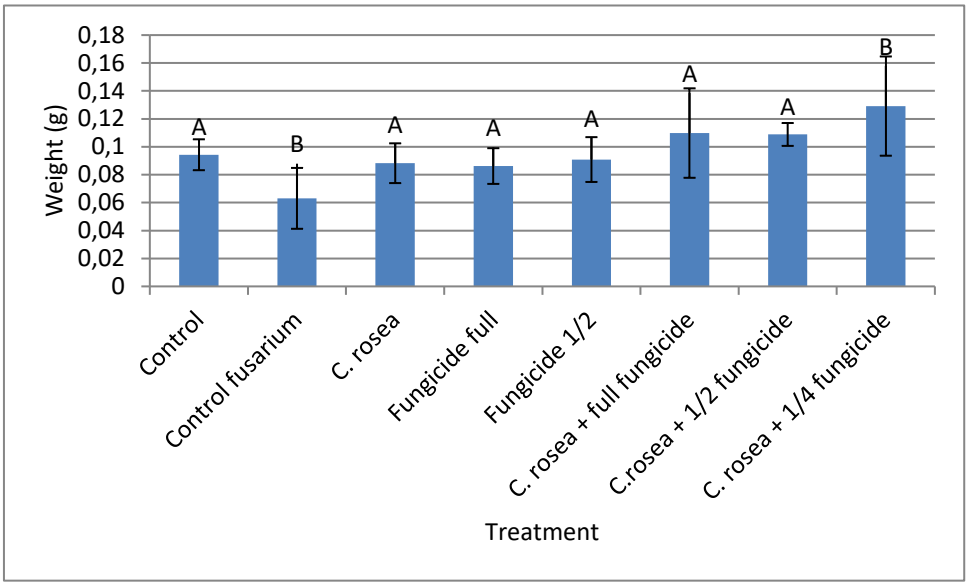

Figure 15. Fresh shoot weight of Barley plant at 21 days of experiment. The experiment was performed in five biological replicates. Error bar represent standard deviation. Significant difference ($P \leq 0.05$) is treatment compared to Control and is based on Fisher's exact test.

The plant shots were dried and the dry weight was measured. The biomass measurement showed that the dry weight of the barley plant with various treatment had significant differences similar to the fresh shoot weight (Figure 16). The combination treatments showed higher dry weight compared with all other treatments. The combination treatment *C. rosea* + fungicide had significant higher dry weight than the straight *C. rosea* treatment ($P \leq 0.05$).

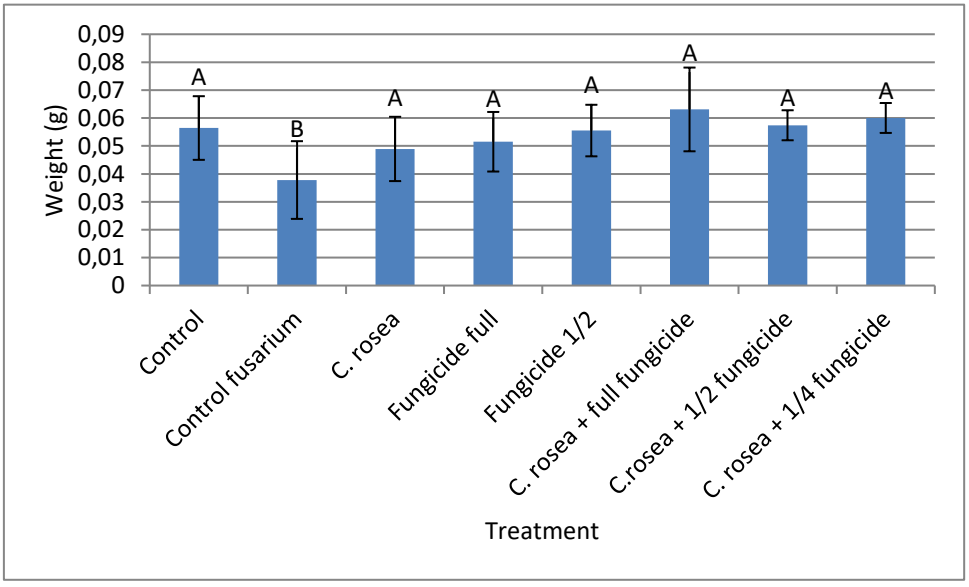

Figure 16. Dry shoot weight of Barley plant at 21 days of experiment. The experiment was performed in five biological replicates. Error bar represent standard deviation. Significant difference ($P \leq 0.05$) is treatment compared to Control and is based on Fisher's exact test.

The biocontrol performance various treatments against *F. graminearum* foot rot disease on barley was determined using a 0 – 3 disease scoring scale as described before (Dubey *et al*., 2020). The results from the disease scoring on barley showed a significant difference ($P \leq 0.001$) between Control *Fusarium* and all other treatments. The mean score; control *Fusarium* 3.55 and *C. rosea* 1.79. Treatment *C. rosea* + fungicide had a mean score of 0.04 and the other treatments had 0 as mean score due to no visible diseases.

5 Discussion

5.1 *C. rosea* and *Fusarium spp*. *In vitro* dose – response to fungicides

The *in vitro* experiments were conducted using fungicide concentrations chosen in order to correspond to normal fungicide field doses used in Sweden. The concentrations were calculated based on spraying 200 litres of water per hectare, but modern spray tanks can vary their spraying volume from 30 litres to 600 litres per hectare depending on crop needs, target pest and type of pesticide. In the beginning of my experiments, the calculations on fungicide concentration per ml agar were made according to the recommended field doses for each fungicide product. The concentration of fungicide product should be the same in all replications in the experiments but since there were considerably smaller amounts of some fungicides e.g. 3 µl Proline per ml agar, it was difficult to make sure that the fungicide was evenly distributed in the agar medium. This may have led to variations between experiments.

In the beginning it was hard to find a suitable concentration for *C. rosea* to grow on because it did not grow well or at all on the full field dose in the in vitro experiments. To determine concentrations where *C. rosea* was able to grow, the EC_{50} value for cyprodinil on *Botrytis cinerea* (0,006 – 0,054 µg/ml) was used (Petsikos-Panayotarou *et al.*, 2003).

A common factor for *C. rosea* and the *Fusarium* species tested was that they all were sensitive to triazoles but not strobilurines or AP-fungicides. This was in accordance to findings by Roberti *et al* (2006), showing that triazoles, in that case prochloraz, were the only ones that could inhibit *C. rosea*. In my studies, Stereo did not have the same effect against *C. rosea* or the *Fusarium* species as Armure and Proline. This was probably due to the composition of Stereo, with both a triazole and an AP-fungicide, but with a lower concentration of triazoles compared to the other two products. Stereo contains 62.5 g/l propiconazole compared to Armure which contains 150 g/l and in the experiments on the agar plates the concentration of propiconazole was approximately 28 % higher at the Armure plates compared to Stereo.

5.2 Compatibility – *C. rosea* and prothioconazole made a couple

From the introduction in this thesis Abd-El-Khair *et al*., 2019 and Wang *et al*., 2019 mentioned that combination of a BCA and a fungicide was more effective than using them separately. The results from the inoculation of *C. rosea* and *Fusarium* species on fungicide agar are not convincing for further testing because *C. rosea* was inhibited. In what extent was the inhibition affecting *C. rosea*'s vitality and characteristics as a BCA? It is, however, interesting that *C. rosea* was inhibited and *Fusarium* did not grow at all on prothioconazole. In my results, *C. rosea* had a lower growth rate on agar plates containing Proline compared with fungicide free control plates and plates with other fungicides. When *C. rosea* spore germination and growth were tested on Proline agar plates there was no growth detected, during measurement period, whereas the fungi grew fast on the fungicide free control and gradually covered the whole agar plate. When the control plate was covered in mycelia from *C. rosea*, the germinated spores started on plates with Proline. Thus, Proline did not inhibit the development of *C. rosea* completely,

but delayed it. Proline was the fungicide that separated the BCA (*C. rosea*) and *Fusarium* the most, and it was therefore the most interesting fungicide to bring for further research.

In the seed coating tests with spores (paragraph 4.2), it was often hard to see any fungal growth, but the spores of *C. rosea* were germinating to a large extent and surviving. The intention in the experiment was that the spores would survive seed coating with retained concentrations, and grow together with the seedling as a protection. When the seed coating with *C. rosea* + prothioconazole was done, seed samples were taken out and inoculated on Czapek-Dox agar. Through this, I could confirm that C. *rosea* grew nicely together with the germinating seeds. When coating the seeds with prothioconazole and *C. rosea*, it was also found that a higher concentration prothioconazole resulted in inhibited growth of *C. rosea*. An interesting observation was that more spores seemed to be attached to the seeds when they were coated with prothioconazole compared with seeds that were not treated with prothioconazole.

5.3 Bioassay with two different plant species: wheat and barley

In the bioassay, there was an interesting difference between wheat and barley. Unlike barley, wheat did not express any disease symptoms from *F. graminearum* infection. This was most likely due to a combination of different factors. In contrast to the barley plants, the wheat plants always had good turgor pressure because of frequent watering. This probably contributed to healthier and less stressed plants. Stressed plants are more susceptible to diseases and the wheat were probably too vigorous to be susceptible to the disease. As another reason for less disease in wheat could be that *F. graminaerum* grown on Czapek-Dox agar did not produce enough metabolites to cause severe disease. *Fusarium graminaerum* was therefore grown on PDA before inoculation in the barley bioassay. The idea was that Czapek-Dox contains sucrose molecules as a carbon energy source whereas PDA contains dextrose which may contribute to more metabolites. More metabolites increase the chance of the crop getting infected. One additional explanation for the difference in disease expression could be the choice of varieties of wheat and barley - that the barley cultivar was more susceptible than the wheat cultivar.

The differences in disease symptoms caused by *F. graminearum*, was that also the reason for different length and weight of plants from the different treatments? No, most likely the different lengths and weights depended on the different treatments. The result showed that the seed germination was prolonged significantly when coated with only fungicides. Coating with *C. rosea* did not affect the germination at all. Especially in barley, single *C. rosea* treatment resulted in significantly higher plants and plants with higher biomass compared to prothioconazole treatments. In both wheat and barley bioassays, the combination of *C. rosea* and prothioconazole resulted in a higher biomass than the non-treated control, *F. graminaerum* infected control, *C. rosea* and full dose prothioconazole treatments. These tests tell us that a combination of BCA and fungicide increase the plant biomass and at the same time give the plants effective protection against diseases caused by *fusarium spp*. This is a good start for integration to a future IPM system.

The wheat bioassay was harvested after four weeks and the barley was harvested after three weeks. This resulted in that wheat had more significant differences in length when measured after 12 days than at harvest day (27). One possible explanation to this pattern

is that the plants in treatments resulting in lower growth rates were “catching up” on the plants in other treatments.

5.4 Integrated pest management with integration of more BCA

Before starting my experiments, my idea of controlling FHB was by spraying BCAs and fungicides in the crop. Since the crops needs a few months of growth before treatments can be done, the experiments would have taken too much time and I had been forced to work outside the time frame. When the result showed that full field doses of the fungicides are not possible in mixtures with *C. rosea*, seed coating became more relevant and opened up new opportunities. Seed coating with prothioconazole is now (year 2020) registered in Sweden. Hopefully, we will see more registrations of seed coating treatments in the future, both BCAs and chemical products. The fungicide seed coating is an effective way to give the crops a good protection in the start from soil and seed borne diseases and at the same time, the concentrations of fungicide are low compared to regular field doses, keeping the negative environment impact to a minimum.

If this new IPM system would be implemented only as simultaneous spraying with chemical products and BCAs, there will be a problem with persistence of the BCAs when mixed with high concentrations of fungicides or maybe other pesticides. One strategy could therefore be to separate the spraying occasions so that the BCAs are sprayed first, since it gives more long-term protection (given that it germinates and survives), and when it is established in the crop, the fungicides could be sprayed separately to enhance and give a faster protection against diseases.

Although the most important tool in a IPM strategy to control FHB is still by choosing less susceptible cultivars (Gilbert & Fernando, 2004), the second most effective way could be to integrate more BCAs in the system. BCAs can be used as separate treatments or together with chemical pesticides. Based on the results in this thesis, combinations with *C. rosea* and prothioconazole make the plants more vigorous and give them a better start in the growing season, which is important to make them reach their full potential in terms of yields. To get the best out of IPM, other actions like crop rotation, tillage and good decisions systems, plays an important role.

6 Conclusions

Concentrations of fungicides comparable to full field doses recommended by fungicide producing companies are too high for germination and growth of the BCA *C. rosea*. The concentrations used in seed coating were more suitable for *C. rosea*. It germinated and grew on the lower seed coating concentrations.

Clonostachys rosea and fungicides (prothioconazole) can be used together in a mixture when for example coating seeds. They are, according to this thesis, compatible although high concentration of prothioconazole may inhibit both mycelial growth and spore germination of *C. rosea*.

Clonostachys rosea and fungicides (prothioconazole) can provide a good protection against diseases caused by *Fusarium*, in this case foot and root rot, together and separately. The efficacy is clear and the results from the bioassay, especially with barley, showed that combination treatments with chemical fungicide and BCA gave healthier plants. This suggests that this type of combination can contribute to a more sustainable IPM in the future.

6.1 Prospects for further research

The outcome of testing whether *C. rosea* was compatible with different fungicides or not gave rise to some thoughts. *Clonostachys rosea* was tolerant to prothioconazole at lower concentrations. During the growth inhibition test, however, the growth mode of *C. rosea* was different. The fungus mycelium was not growing at the same speed compared to the control and the growth mode showed that the mycelia did prefer to grow on the *C. rosea* inoculated agar plug instead of the fungicide agar. Could this affect the viability of mycelium growth or spores? This raises the interest to conduct tests with maybe another fungicide that does not inhibit *C. rosea* as much as prothioconazole did, especially the spore germination and growth. Could there be any more synergy effects?

Regarding *in vitro* testing, *C. rosea, F. culmorum* and *F. graminearum* were put on agar plates with fungicides and sealed. How much of the inhibition from the fungus growth came from the actual fungicide active substance in the Czapek-dox media? Is it precluded that the fungicide has zero inhibition via vaporized fungicide? If vaporized fungicides are as potent as they are when deposited in the agar, this could possibly be a reason of another source of inhibition of the active growing fungus. Though it was clear that even if the agar plugs with e.g. active growing *C. rosea* did not germinate on the Czapek-dox agar with chemical fungicide, *C. rosea* still grew on the inoculated plug where it did not have contact with the fungicide agar. If it was vaporized chemical fungicide present, there was not enough concentration to inhibit the growth of fungus on the agar plug which sides were not in contact with chemical fungicide agar. This mean that there is a difference between *in vitro* testing and the seed coating in terms of fungus being affected by vaporized fungicide, since the agar plates are sealed, and the coated seeds are dried and not sealed together with fungicide.

The next step in testing chemical fungicides and *C. rosea* would be in large scale trials under field conditions in for example winter wheat or spring barley. That would provide possibilities to test application methods against Fusarium head blight. In that case the sprayings would be done at the correct timing probably during heading of the crop.

Hopefully now and in the future, we will see more research about these important topics brought up in this thesis. Research leading to commercial production which enables use for farmers, to grow more sustainable and healthier food. Cropping systems containing further developed IPM systems containing biological control agents together with natural and synthetic derived pesticides.

7 Acknowledgement

I would like to send my biggest thanks to my supervisor Mukesh Dubey who has guided me through the work with my thesis. From the first day to the last day of work you have been patient and supportive. Also, thank you for discussions and sharing of experience leading to better experiments and laboratory work.

Hanna Friberg, co-supervisor. Thank you for your support and encouraging e-mails.

Dan Funck Jensen, co-supervisor. Thank you for contributing with your experience which has made the road forward in my work clearer.

Last I would like to thank the Department of Forest Mycology and Plant Pathology at SLU which made it possible for me to do laboratory work whenever I wanted, weekdays, weekends, day time and night time.

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9 Appendix

9.1 Commercial fungicides used in experiments

Name	Active substance(s)	FRAC – group, No.
Kayak	Cyprodinil 300 g/l	AP – fungicide, 9
Stereo	Propiconazole 62,5 g/l + Cyprodinil 250 g/l	DMI, 3 + AP – fungicide, 9
Bontima	Cyprodinil 187,5 g/l + Isopyrazam 62,5 g/l	AP – fungicide, 9 + SDHI, 7
Armure	Propiconazole 150 g/l + Difenconazole 150 g/l	DMI, 3
Proline	Prothioconazole 250 g/l	DMI, 3
Amistar	Azoxystrobin 250 g/l	QoI, 11

9.1.1 Kayak

Kayak is the commercial name for a fungicide with the active substance cyprodinil. According to the Fungicide resistance action committee (FRAC) cyprodinil is put in the group anilino pyrimidines (AP – fungicides) and their target site on the fungus is the methionine biosynthesis (FRAC, 2016).

The company Syngenta is the provider of this product. Kayak is mainly used in barley against eyespot, net blotch, powdery mildew and rhynchosporium. The company recommends that Kayak should not be sprayed by itself but be combined with another fungicide with another mode of action (MOA). Maximum individual dose is 1,5 litres per acre and maximum 3 litres per acre during the growth season (Syngenta, 2016).

9.1.2 Stereo

Stereo is a mixture of two active ingredients against fungus, propiconazole and cyprodinil. Stereo has characteristics from both triazoles and AP-fungicides. Triazoles are also called DMI-fungicides because they are demethylation inhibitors (FRAC, 2016).

Stereo (312,5 EC) are registered in cereals. The active substances are especially effective against powdery mildew, net blotch, rhynchosporium leaf blotch, rust and other leaf blotches. The product is also recommended by ADMA to be mixed with another MOA fungicide. Maximum dose is 1,5 litres per acre (ADAMA, 2016).

9.1.3 Bontima

The two active substances in Bontima are isopyrazam and cyprodinil. Again, an AP-fungicide and a SDHI-fungicide (succinate dehydrogenase inhibitor) (FRAC, 2016).

Isopyrazam and cyprodinil together are effective against net blotch, rhynchosporium secalis, ramularia collo-cyni, rusts and powdery mildew. The recommended dose is 2 litres per acre at the time (Syngenta, 2016).

9.1.4 Armure

This fungicide contains the active substances propiconazole and difenconazole which means that there are two DMI-fungicides (FRAC, 2016).

In Sweden Armure has a very thin spectrum for treatment, just at anthesis. It is effective against brown rust, yellow rust and leaf blotches. A normal dose is 0,4 – 0,8 litres per acre together with another fungicide (Syngenta, 2016).

9.1.5 Proline

Proline is also a DMI-fungicide with the active substance prothioconazole (FRAC, 2016).

This triazole is produced by Bayer crop science and is also recommended to mix with another fungicide e.g. strobilurine. Cereals and oilseed rape are common crops that are treated with Proline, about 0,6 litres per acre and maximum 1,2 litres per acre and year. The MOA in cereals are effective against eyespot, *Zymoseptoria*, powdery mildew, yellow rust, crown rust, brown rust, fusarium ear blight, *Rhynchosporium* and in oilseed rape it is effective against light leaf spot, phoma, stem canker and sclerotinia stem rot (Bayer crop science, 2016).

9.1.6 Amistar

Amistar is the only strobilurine used in this work and the active substance is azoxystrobin. Strobilurines is called QoI-fungicides because it is a quinone outside inhibitor (FRAC, 2016).

Azoxystrobin has a preventive MOA, which means that treatment can be performed before attack of e.g. *Zymoseptoria*, *Fusarium* and rust in cereals. Amistar is also widely used in special crops such as potatoes and onions for example against different leaf spots, powdery mildew and foliar diseases. Since the product is widely used the doses per acre are between approximately 0,3 – 1,0 litres (Syngenta, 2016).

Combine application of fungal biocontrol agent with low dose of chemical pesticide for integrated pest management

Plant disease causing severe yield losses to agricultural and horticultural systems are effectively controlled by chemical pesticides. However, their excessive use has led to certain problems including pesticides contamination in food and feed and development of pesticides resistance in targeted and non-targeted organisms. Biological control of plant diseases using naturally occurring microorganisms is a promising alternative to the chemical pesticides. Although, biological control of plant diseases is attractive, their use in crop production system is limited due to big variations often seen in field performance of biocontrol agents (BCAs). Therefore, for efficient use of BCAs and to reduce the use of chemical pesticides, one aspect is to evaluate the prospects of combining BCA with low dose of chemical pesticides.

The aim of this study was to investigate the potential of combining a fungal BCA with low dose of chemical fungicides to control fusarium foot/root rot on wheat and barley caused by fungal plant pathogens *Fusarium graminearum* and *Fusarium culmorum*. In addition to fusarium foot/root rot, *F. graminearum* and *F. culmorum* are responsible for Fusarium head blight (FHB) that can cause serious yield and economic loss to the agriculture production system. In addition, infection of *F. graminearum* and *F. culmorum* to floral tissue reduces the grain quality by producing several mycotoxins. In this study, a biocontrol fungus *C. rosea* strain IK726, which was isolated from the barley roots infected with *F. culmorum* in Denmark was used as a biological control agent. *C. rosea* IK726 and has been shown to be an effective biocontrol agent against several plant pathogens including *F. graminearum* and *F. culmorum*. Furthermore, *C. rosea* can tolerate relatively high concentrations of chemical pesticides, in relation to commercial doses recommended for controlling fungal diseases of plants.

Our result from an *in vitro* experiment showed that *C. rosea* has a relatively better ability to tolerate prothioconazole (commercial name Proline) at 1/30 and 1/60 concentration of recommended full dose compared to *F. graminearum* and *F. culmorum*. A combination of low dose of proline and *C. rosea* IK726 showed complete inhibition of the fusarium foot/root rot on barley seedling in a growth chamber sand seedling test. Analysis of plant health parameters showed that barley plants treated with *C. rosea* alone or with a combination of *C. rosea* and low dose of Proline had significantly higher shoot length, shoot fresh weight and dry weight compared to barley seedling from seed coated with only Proline. The result from this study will help to formulate integrated pest management strategy by mixing the fungus *C. rosea* with Proline and apply it on the specific crop accordingly. However, more experiment is needed to evaluate the both long-term and short-term effects of this combination in arable fields. In addition, *C. rosea* and Proline combination can also be tested to control fusarium head blight. The thesis gives thoughts in theory how to develop and use a different integrated pest management system to control specific plant pathogens.