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Swedish University of Agricultural Sciences

**Department of Forest Mycology and Plant Pathology**

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## Abstract

Fungicide use is the dominant direct method to control fungal pathogens on crops in agriculture. While effective, excessive use of them can be harmful to biodiversity and lead to fungicide resistance in pathogen populations. Biocontrol is an alternative where natural antagonistic organisms, called biocontrol agents (BCA), are used to protect crops. One such BCA is the mycoparasitic fungus *Clonostachys rosea* which has a wide host range, including *Fusarium graminearum,* an important fungal pathogen causing the disease fusarium head blight in cereals. One important challenge facing the use of biocontrol is finding cost-effective ways of identifying strains of BCAs that incorporate the desired attributes. The purpose of this project is to evaluate three different *in vitro* assays for evaluation of *C. rosea's* antagonism against *F. graminearum* and to find if they correlate with previous climate chamber experiments that more accurately represent field conditions. Six different strains of *C. rosea* with differing levels of expected biocontrol efficacy were used. The results indicate that the assays used do no correlate well with the biocontrol potential of the tested strains. There was also some indication of variation in phenotypic expression when different growth media were used. These results have support in the literature as *in vitro* screening for BCAs have been shown to miss strains of organisms that proved to be effective under field conditions. Further optimization of these assays is suggested to determine whether they can be useful for screening purposes. Improved protocols of these assays could also serve as a cost-effective means to study variations in phenotypes, relevant to biocontrol, between strains as well as other attributes important for BCAs to be implemented in commercial uses.

*Keywords: Biocontrol, Biocontrol agent, Clonostachys rosea, Fusarium graminearum,*

## Sammanfattning

Användning av fungicider är idag den främsta metoden för att direkt bekämpa växtpatogena svampar inom jordbruket. Trots deras effektivitet kan en överanvändning av dem vara skadligt för biodiversitet, samt leda till fungicidresistens i populationer av patogena svampar. Biologisk bekämpning är ett alternativ där naturliga antagonister används för att skydda grödor. En sådan antagonist är mykoparasiten *Clonostachys rosea* som har en bred värdpreferens, inklusive *Fusarium graminearum*, en viktigt patogen som orsakar axfusarios i spannmål. En viktig utmaning för biologisk bekämpning är att på ett kostnadseffektivt sätt hitta isolat av antagonister som är effektiva under fältförhållanden. Syftet med detta projekt är att utvärdera tre olika *in vitro* metoder för att bestämma *C. rosea's* antagonism mot *F. graminearum* och undersöka huruvida dessa metoder korrelerar med tidigare utförda experiment i klimatkammare, där förhållandena till en större grad efterliknar de i fält. Sex olika isolat av *C. rosea* användes med olika nivåer av förväntad biologisk bekämpning. Resultaten indikerade att metoderna inte korrelerar väl med isolatens förväntade förmåga. Det förekom även indikationer på fenotypisk variation när olika tillväxtmedia användes. Dessa resultat har stöd i litteraturen där det konstaterats att *in vitro* metoder för screening av organismer för biologisk bekämpning inte har kunnat identifiera isolat som visats vara effektiva i fält. Ytterligare optimering av dessa metoder är nödvändiga för att kunna avgöra om de är användbara i screeningsyfte. Förbättrade protokoll av dessa skulle även kunna fungera som kostnadseffektiva medel för att undersöka fenotypiska variationer, med relevans for biologisk bekämpning, mellan isolat samt andra attribut som är viktiga för kommersiell implementering av biologisk bekämpning.



*Nyckelord:* Biologisk bekämpning, *Clonostachys rosea*, *Fusarium graminearum*

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# 1 Introduction

## 1.1 Background

Practices for more sustainable agricultural production systems are required in order to support a growing human population, while at the same time minimizing negative environmental impacts. The Swedish Parliament has adopted 16 environmental quality objectives to be achieved by 2020 (Proposition. 2004/05:150). Among these goals is ”A rich diversity of plant and animal life” where one of the challenges described is an agricultural system that does not deteriorate, but contributes to biodiversity. According to a study from 2010 (Geiger *et al.*, 2010), a declining diversity in both bird and plant populations can be attributed to intensive use of fungicides. One way to reduce fungicide use is to employ alternative methods, such as biocontrol, to control plant pathogens in agricultural production.

The purpose of this work was to develop and evaluate an effective *in vitro* assay method to quantitatively measure antagonism of the fungal biological control agent *Clonostachys rosea* against the cereal pathogen *Fusarium graminearum*. Such assays can be used to screen large numbers of *C. rosea* strains in a cost-effective manner with applications in biological control and research, including selection of strains for future biocontrol solutions.

## 1.2 Fusarium head blight

Fusarium head blight (FHB) is a global plant disease on cereals, such as wheat and maize. It can cause both yield reduction, like the epidemics in northern United States and Canada during the early 1990s with losses of up to 50% (Windels, 2000), as well as reduction in grain quality (Spanic *et al.*, 2018). The cause of FHB includes several species of pathogenic fungi from the *Fusarium* genus. In Europe, the dominant species is *F. graminearum*, which has increased in importance since the 1950s in northern Europe (Nielsen *et al.*, 2011), followed by *F. culmorum* and *F.*

*avenaceum*. One reason for the disease's significance is the pathogens ability to produce trichothecene mycotoxins. In the case of *F. graminearum*, two important toxins produced are deoxynivalenol and zearalenone (Kosawang *et al.*, 2014), which contaminate the grain and poses a safety hazard to both humans and animals. The toxicity of deoxynivalenol is particularly problematic which, when ingested, causes vomiting and diarrhoea along with other symptoms, especially in pigs (Pestka and Smolinski, 2005). As a result, the yield loss can be total in severe cases when the grain cannot be used for either human consumption or animal feed.

A range of agronomical and environmental factors have been proposed as explanations for the increased occurrence of FHB, and *F. graminearum* in particular. Shifts towards practices such as no-tillage or reduced tillage with crop residues left in the field from the year before and short crop rotations including wheat and maize has been put forth as possible reasons (Nielsen *et al.*, 2011). These practices have also been suggested as the reason for the shift toward *F. graminearum* among the complex of species causing FHB (Weber *et al.*, 2001). Since *F. graminearum* is favoured by a warm and humid environment, the change in climate and precipitation patterns have been suggested to contribute to the increase, both directly and indirectly. With a warmer climate, cultivation of maize becomes feasible further north, thus, as mentioned above, increasing the risk of FHB. In addition, changes in precipitation can lead to more humid weather during anthesis, when crops are most vulnerable to infection (Gilbert and Haber, 2013). *Fusarium graminearum* has been ranked as the 4th most important plant pathogenic fungus in the world from an economic and scientific perspective (Dean *et al.*, 2012).

## 1.3 Fungicides

Since the 1940s, chemical fungicides have been the predominant way of combating and controlling fungal pathogens in cereals. According to the Swedish Board of Agriculture, 70% of the wheat grown in Sweden was treated with fungicides in 2010 (not primarily for control of FHB), and the amount of fungicides sold in 2016 compared with 2010 increased by 12% (Jordbruksverket, 2018). The importance of fungicides in maintaining high yields is hard to dispute, however, there are several reasons why a decrease in the use of fungicides would be favourable.

### 1.3.1 Environmental effects

Fungicides applied to fields can also affect non-target organisms in the surrounding environment (Karlsson *et al.*, 2017). Although different fungicides differ in their specificity towards the target organisms, the use of fungicides as a whole has been

linked to decreased biodiversity in both plants and birds (Geiger *et al.*, 2010) as well as fungal communities in the phyllosphere (Karlsson *et al.*, 2014).

### 1.3.2 Resistance

The use of chemical fungicides also brings the risk of pathogens developing fungicide resistance. Most new fungicides introduced after the 1970s have been based on modes of action that target a specific site in a pathogen's metabolism (Russell, 2005), thus providing a certain level of specificity in action. These fungicides are typically systemic, meaning they are absorbed and transported with the xylem to other parts of the plant. These attributes contribute to the high efficacy of modern fungicides in combating plant pathogens resulting in higher yields. However, another consequence of this is that these compounds exert a high selection pressure on the pathogens they target. This leads to a situation where most pathogen individuals die, but individuals carrying mutations that confer insensitivity towards the fungicide can be quickly propagated. Continued fungicide applications can result in pathogen populations with very high proportion of insensitive individuals. This means that the agricultural systems that rely on fungicides run the greatest risk of causing them to lose their effectiveness (Lucas, Hawkins and Fraaije, 2015).

An additional reason for concern regarding development of resistance is that the selection pressure isn't only applied to the phytopathogens for which they are intended. Other fungi in the environment, some of which might be human pathogens, are also exposed to the fungicide. This can lead to strains with cross-resistance to medical fungicides making treatment of humans more difficult (Chowdhary *et al.*, 2013; Lucas, Hawkins and Fraaije, 2015).

In the case of FHB, it has been suggested that *F. graminearum* exhibits intrinsic insensitivity towards several classes of fungicides (Dubos *et al.*, 2011, 2013; Fan *et al.*, 2013). This means that the effectiveness of fungicides to control FHB is challenged from the start (Lucas, Hawkins and Fraaije, 2015). Triazoles are the most used fungicide group against FHB, and it is especially important to avoid resistance to this group in pathogens causing FHB to ensure future control of the disease.

## 1.4 Biocontrol

To reduce the dependency on fungicides, and thus maintaining their effectiveness, other methods to control pathogens are required. One method for achieving this is with the use of cultural practices such as removing residues from preceding crops, using resistant crop varieties, sowing at appropriate times and crop rotations, all of which have been shown to be important for controlling fungal diseases in cereals,

particularly FHB (Shah et al., 2018). However, these methods are often lacking when compared to chemical controls. As a supplement to these practices, the use of biological control has a potential to further the efficiency of non-chemical agricultural systems. The concept of biocontrol can be defined as the use of living organisms to control pathogens (Jensen et al., 2016). Using the terminology suggested by Eilenberg et al. (2001), implementing microorganisms in biocontrol can be termed microbial control and when released into an environment where they are not expected to persist beyond fulfilling their intended use, it can be classified as a form of inundation biological control.

### 1.4.1 Biological control agents

The first obstacle to implementing microbial control is identifying the actual organisms that can control the pathogens, called biological control agents (BCA).

The mechanism in which direct control is exerted by BCAs on pathogens is primarily through three modes of action (MoA). The first is by competing for nutrients, thus, starving the pathogen. The second is antibiosis, where the BCA produces metabolites and enzymes with antibiotic properties that inhibit growth or kill the pathogen. The final MoA is parasitism, called hyperparasitism if the host is also a parasite, where the BCA directly attacks its host to use it as a nutrient source (Jensen *et al.*, 2016). The parasitism of a fungal hyperparasite typically involves coiling of hyphae around the conidia and hyphae of the pathogen, and penetration of the pathogen cell walls with lytic enzymes (Barnett and Lilly, 1962).

Certain BCAs can also reduce plant infection indirectly by activating defence responses in the plant. Plants have evolved a complex system of immune responses that are activated during infection by pathogens, sometimes described through the zig-zag model of plant immunity (Jones and Dangl, 2006). Studies have shown that BCAs can in some cases induce these defensive reactions in the plants, referred to as induced systemic resistance, thus providing additional control of the pathogen beyond the direct effects of the BCA (Shoresh, Harman and Mastouri, 2010).

Several other challenges exist when it comes to finding and selecting candidates that might be suitable and this is part of the reason why still so few products have made it to market. These challenges include the rate at which screening and selection can be done, using assays that properly reflect the field conditions where the BCAs would be applied, selecting candidates that are compatible with commercial scale production (Schisler and Slininger, 1997) and also maintains their viability through storage (Jensen, Knudsen and Funck Jensen, 2002).

### 1.4.2 *Clonostachys rosea*

One organism currently used as a BCA is the fungus *Clonostachys rosea* (previously *Gliocladium roseum*). *C. rosea* is a saprophytic ascomycete with a worldwide distribution. It can be found in a wide range of environments, mainly in soils but also on plants, both as a decomposer of senescent material and as a non-pathogenic colonizer of living parts (Sutton *et al.*, 1997). It is also known as a mycoparasite capable of infecting a large number of other fungi (Barnett and Lilly, 1962). Whether it acts as a saprophyte or mycoparasite depends to a large degree on the circumstances of the environment. Studies have shown that when *C. rosea* is exposed to low nutrient levels, it can switch from being saprotrophic to mycoparasitic (Rodriguez *et al.*, 2011).

Besides its mycoparasitic abilities, the antagonism of *C. rosea* is also based on its nutrient competition with pathogens (Sutton *et al.*, 1997), as well as production of secondary metabolites with anti-fungal properties and cell wall degrading enzymes (Rodriguez *et al.*, 2011; Fatema *et al.*, 2018). *C. rosea* can also activate the plants own defence by inducing the expression of pathogenesis-related proteins, including chitinases and peroxidases (Roberti *et al.*, 2008). Its effectiveness as an antagonist and BCA is, however, to a large degree dependent on the strain used, as different isolates show considerable variation in their antagonistic potential in biocontrol studies (Teperi *et al.*, 1998).

## 1.5 Hypothesis

The hypothesis of this study is that strains of *C.rosea* will show correlation between their biocontrol efficacy, as established in climate chamber experiments, and their performance *in vitro* with regards to antagonism against *F. graminearum.*

Three different *in vitro* methods will be used to test this and subsequently be evaluated for further use with the purpose of screening organisms and strains for biocontrol use.

# 2 Methods

## 2.1 Initial cultures and strain selection

*C. rosea* strains were revived from glycerol stocks stored at -80°C in the culture collection at the Department of Forest Mycology and Plant Pathology at the Swedish University of Agricultural Sciences. The selected strains were previously evaluated for their ability to control fusarium foot rot disease on wheat (Karlsson et al., unpublished) in climate chamber experiments (Kosawang *et al.*, 2014). Six strains where chosen, three with high biocontrol ability, IK726, SDT-5-1 and 178.28, one mediocre, 1882, and two with poor potential for biocontrol, GG-1-2 and SYP-4-2. All strains where maintained on potato dextrose agar (PDA) at 20°C.

## 2.2 Dual-plating assay

Mycelial growth inhibition of *F. graminearum* by *C. rosea* was measured in a plate confrontation assay. *C. rosea* was inoculated on a 90 mm diameter PDA petri dish using a 4 mm diameter agar plug with actively growing mycelium. After 13 days, *F. graminearum* was inoculated, also with a 4 mm agar plug, 40 mm from the front of *C. rosea's* mycelia (Figure 1). 5 repetitions were made for each *C. rosea* strain. A control with 5 repetitions was made for *F. graminearum* (without *C. rosea*) and controls for each *C. rosea* strain (without *F. graminearum*) was made with 3 repetitions. Measurements were taken of *F. graminearum's* growth after 3, 5, 8, 10 and 14 days using a ruler. If the mycelia coalesced before the end of the experiment, no further measurements were taken beyond the previous measurement.

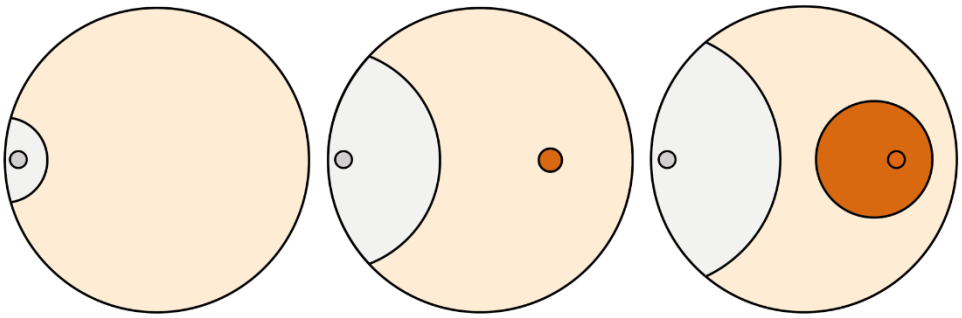

*Figure 1 Schematic of dual-plating assay on PDA petri dishes at three different timepoints. Initial inoculation (left) with agar plug of active C. rosea mycelia. After 13 days of incubation (center), an agar plug with active F. graminearum mycelia is inoculated 40 mm from the front of C. rosea's mycelia. The progressive growth of mycelia (right) is observed and measured following F. graminerum inoculation*

## 2.3 Liquid medium interaction assay

In this assay, the antagonistic effect was measured by mixing spore solutions of the two fungi together in a liquid nutrient medium and counting viable spores after a specific time of interaction. In this assay, only the IK726 and SYP-4-2 strains were used due to work constraints. A schematic representation of this assay can be seen in Figure 2.

### 2.3.1 Stock solutions

To produce stock solutions, *F. graminearum* was grown in 90 mm petri dishes on synthetic nutrient agar (SNA) (Leslie and Summerell, 2007) media at 20ºC in darkness. After 12 days, when the mycelia covered the entire plates, the culture was exposed to 30 minutes of sunlight to stress the fungi and promote conidia production. SNA was used because of its low nutrient content, also in order to promote conidia production. After 14 days, 4 ml of water was pipetted onto the dish and the mycelium was scraped with a spatula to release the conidia. The water-mycelia mixture was then filtered through a funnel lined with cheesecloth to remove mycelial debris. The number of spores in the solution was determined by counting with a haemocytometer. The solution was then diluted to $7.5\times10^5$ spores/ml.

Of the *C. rosea* strains, SYP-4-2 was cultured for 16 days on PDA, also in 90 mm petri dishes at 20ºC in darkness. IK726 was cultured for 20 days. Conidia were harvested in the same manner as above and then diluted to $1\times10^7$ spores/ml.

### 2.3.2 Exposure

100 µl of stock solution of *C. rosea* was inoculated in 800 µl of 1/10 strength potato dextrose broth (PDB) in a 2 ml screw cap tube and incubated at 25ºC on a shaker to prevent anaerobe conditions at the bottom of tube. After 4 hours, 300 µl of *F. graminearum* stock solution was added. The solution thus contained $8.33\times10^6$ spores of *C. rosea* and $1.875\times10^5$ spores of *F. graminearum*. The mixture was then further incubated at 25ºC on a shaker for either 4 or 16 hours. Each treatment had 5 repetitions. A control with *F. graminearum* (without *C. rosea*) was also made, and one for both *C. rosea* strains (without *F. graminearum*) with one repetition each. At the end of each treatment, the tubes were stored at 4ºC, to minimize further activity, for no more than 20 hours.

### 2.3.3 Dilution series

In order to determine the number of viable spores after incubation, a 10-fold dilution series was made for each repetition down to $10^{-4}$. For each repetition, 100 µl from the dilutions of $10^{-2}$, $10^{-3}$ and $10^{-4}$, were inoculated on PDA in 90 mm petri dishes and incubated at 20ºC. Each dilution had 3 technical replicates. One drop of 80% tween was added to each dilution before inoculation to break the surface tension and separate individual spores. After 3 days, colonies for both species were distinguished based on morphology and the number of colony forming units (cfu) was determined.

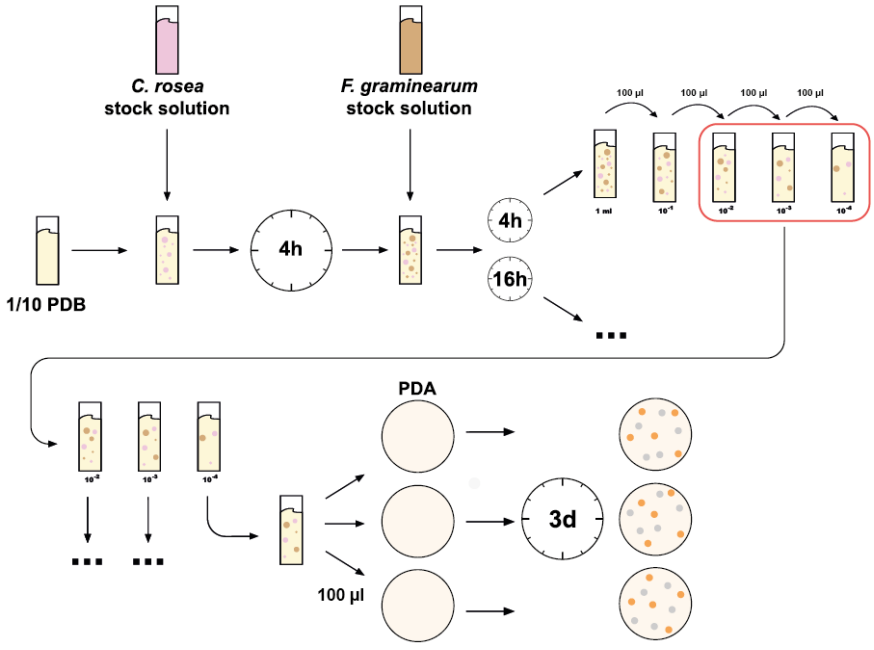


*Figure 2 Schematic of Liquid medium interaction assay.*

## 2.4 Solid medium interaction assay

In this assay, conidia production was measured after a mixed spore solution was inoculated on nutrient media. A schematic representation of the assay can be seen in Figure 3 *C. rosea* strains IK726 and SYP-4-2 was used for this experiment. The same stock solutions were used as in the assay described in the previous section. 100 µl of *C. rosea* solution was added to 800 µl of 1/10 strength PDB and incubated at 25ºC for 4 hours in darkness on a shaker. After 4 hours, 450 µl of *F. graminearum* solution was added. Thus, the solution contained $2.5\times10^5$ conidia of *F. graminearum* and $7.41\times10^5$ conidia of *C. rosea*. 100 µl of mixed conidial solution was immediately pipetted to either SNA or 1/10 strength PDA in 90 mm petri dishes and spread around the dish with a spatula. The plates were then incubated at 20ºC in darkness for 33 days. After incubation, the conidia were harvested by adding 3 ml of sterile water to the petri dish and scraping the mycelia with a spatula. The mixture was then filtered through a cheese cloth and funnel and counted using a hemocytometer. *F. graminearum* and *C. rosea* conidia were distinguished based on morphology.

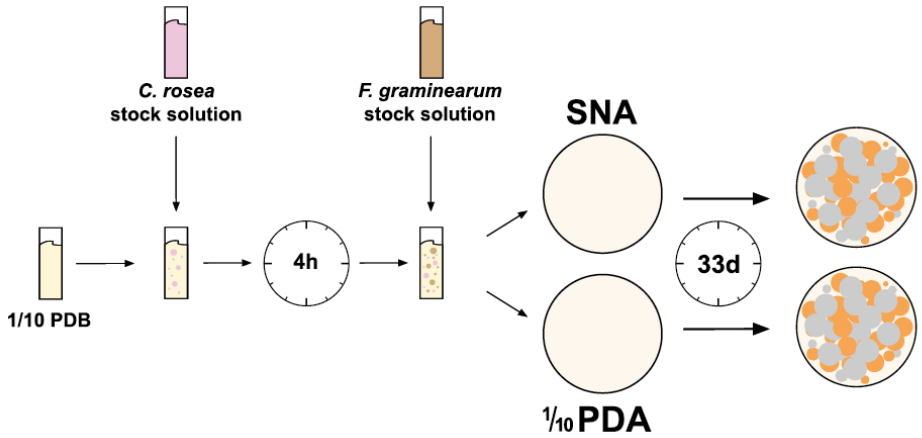


*Figure 3 Schematic of Solid medium interaction assay.*

## 2.5 Statistical analysis

Significant difference between treatments in the dual-plating assay was tested for using one-way analysis of variance (ANOVA) in Minitab 16 statistical software. Pairwise comparisons were performed using Fisher's least significant difference (LSD) method at the 95 % significance level. All other analyses were done using Student's t-test in Excel 16.

# 3 Results

## 3.1 Dual-plating assay

The growth of the mycelia was measured on five occasions, after 3, 5 ,8, 10 and 14 days. An example of the assay's progression can be seen in Figure 4. By the time of the third observation, the mycelial fronts had coalesced in three of the treatments. Therefore, only the first two measurement, 3 and 5 days, were included in the results. Since growth rate of the mycelia before the two fungi meet is the quantifiable measurement in this assay, once they meet in any of the repetitions, no measurements for any of the treatments can be used. This is because including any repetition where the fronts have collided in the data of subsequent measurements is not possible since they can no longer be measured. Conversely, excluding them means skewing the data towards the treatments with repetitions that haven't collided yet.

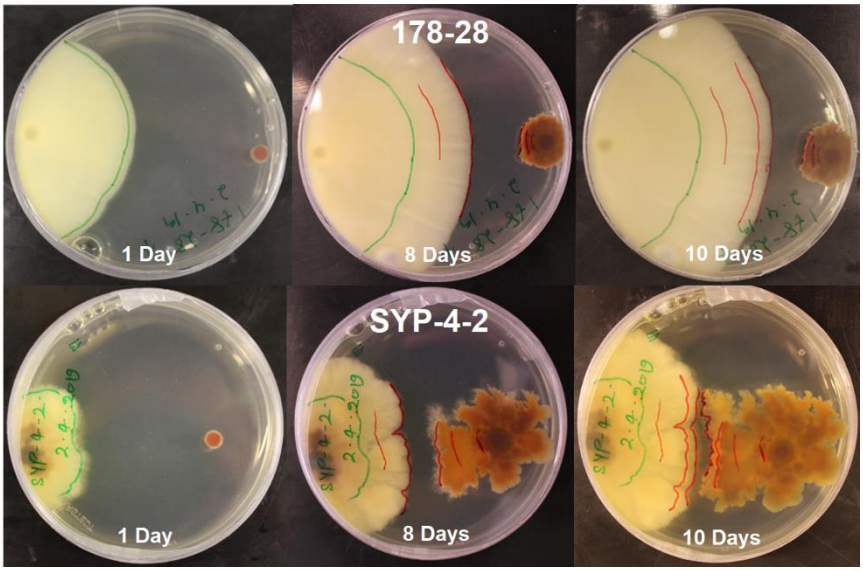


*Figure 4 Examples of dual plating assay. Two different treatments at three different time points, day 1, day 8 and day 10.*

Figure 5 shows the mycelial growth (radius) of *F. graminearum* after five days of interaction with *C. rosea* strains. Only GG-1-2 and 178.28 inhibited growth significantly ($P \leq 0.05$) more than the control.

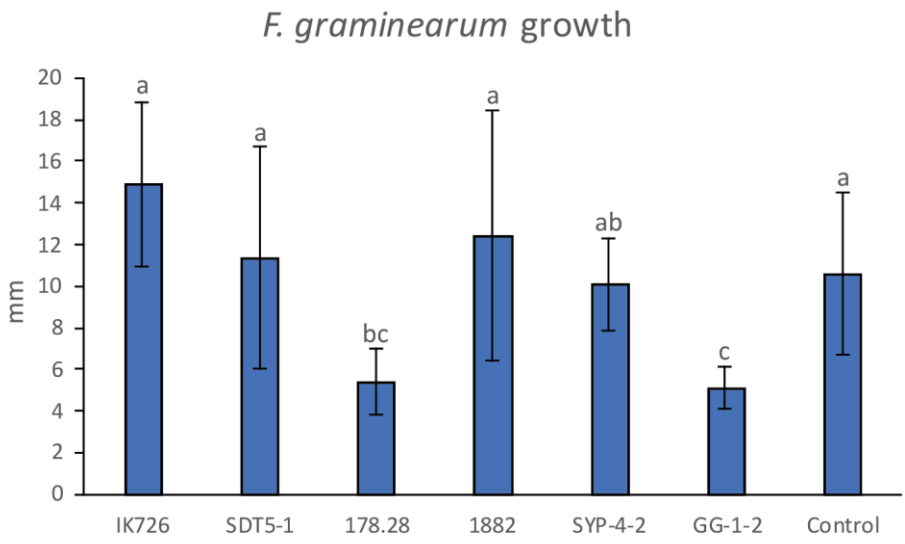


*Figure 5 Mycelial growth (radius) of* F. graminearum *after four days in dual-plating assay with different strains of* C. rosea*. Staples with letters in common indicate no significant difference in growth inhibition. Error bars indicate standard deviation.*

## 3.2 Liquid medium interaction assay

After 3 days, germinated conidia were distinguishable (Figure 6). Colonies from germinated *C. rosea* conidia were small, about 5 mm in diameter, with dense mycelial growth at the centre. The colonies of *F. graminearum* had grown larger, to about 2 cm in size. Some *F. graminearum* cultures showed yellow colouration, especially when forming near *C. rosea* cultures. Plates inoculated with the least diluted solution, i.e. $10^{-2}$, provided the optimal colony density for quantification. Microscopy of the solution after incubation but before inoculation on the agar plates confirmed gemination of both types of spores. There was no identifiable instance of *C. rosea* parasitizing on *F. graminearum*.

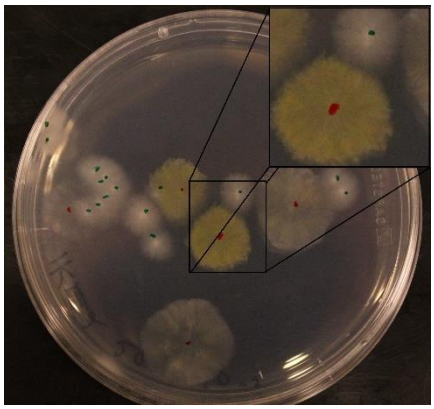

*Figure 6 Germinated conidia were distinguishable after 3 days of incubation. Colonies of C. rosea were small and white, F. graminearum were more than twice as large and some showed yellow coloration. Photo: Victor Olsson*

### 3.2.1 Viability of *F. graminearum* spores

After 8 hours of incubation, (4 hours of confrontation with *C. rosea*) in liquid media, the number of viable *F. graminearum* conidia per plate was 65 and 45 for respective treatment (SYP-4-2 and IK726). This is a difference of 44% more *F. graminearum* cfu when confronted with SYP-4-2 compared to IK726, which was statistically significant difference (P = 0.004) (Figure 7).

After 20 hours of incubation (16 hours of confrontation), the number of cfu in the SYP-4-2 treatment had dropped to 47. This change from the previous timepoint for this treatment was significant (P = 0.009). However, in the IK726 treatment, the number after 16 hours was unchanged at 45, meaning no further reduction of viable *F. graminearum* conidia had taken place between 8 and 20 hours. The difference between the strains after 20 hours, 47 and 45, was not significant.

A lack of *F. graminearum* conidia when preparing stock solutions precluded proper replication of the *F. graminearum* control treatment.

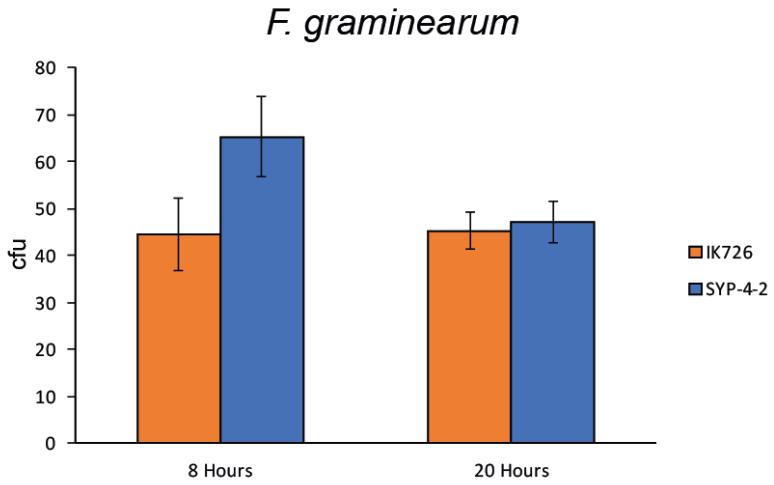


*Figure 7 Average number of* F. graminearum *colonies per plate inoculated with spore solution diluted to 10^-2. The difference between* C. rosea *strains SYP-4-2 and IK726 was significant (P = 0.004) at 8 hours, but not at 20 hours Bars indicate standard deviation.*

### 3.2.2 Viability of *C. rosea* spores

The number of viable conidia from SYP-4-2 proved to be greater than IK726's at both timepoints. After 8 hours, the number of viable IK726 conidia was 41% lower than SYP-4-2. After 20 hours, that number was 43% (Figure 8). These differences were significant (8 hours; P = 0.032, 20 hours; P = 0.019). Differences between timepoints within treatments were however not statistically significant.

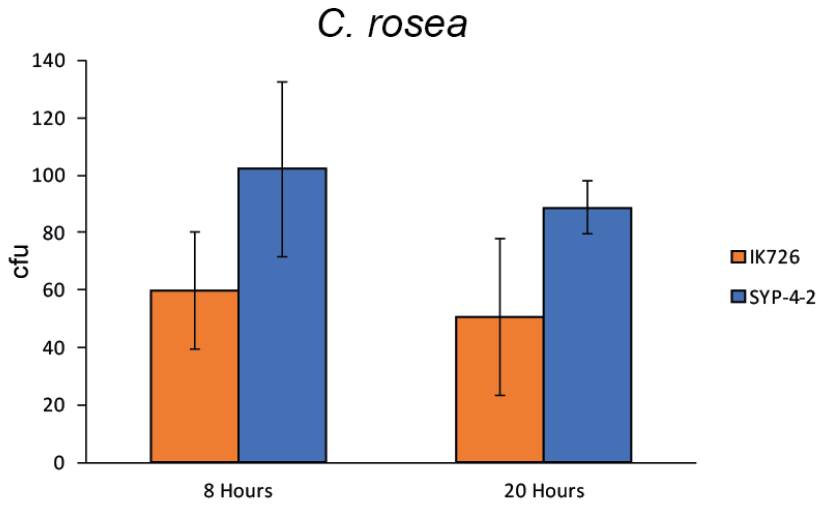


*Figure 8 Average number of C. rosea cultures per plate inoculated with 10^-2 dilution. The difference between strains at both 8 hours (P = 0.032) and 20 hours (P = 0.019) was statistically significant. Bars indicate standard deviation.*

## 3.3 Solid medium interaction assay

### 3.3.1 Inhibition of *F. graminearum* conidia production

The number of *F. graminearum* conidia from the solid medium interaction assay was significantly higher (P = 0.006) after interaction with IK726, compared to SYP-4-2 on 1/10 PDA. Comparing the treatments, the number of *F. graminearum* conidia was 6.9 times greater for the IK726 treatment (Figure 9). While there was some conidia production of *F. graminearum* when grown on SNA, the number was close to zero for most repetitions. Due to this, no significant results were obtained in regard to *F. graminearum* conidia on SNA.

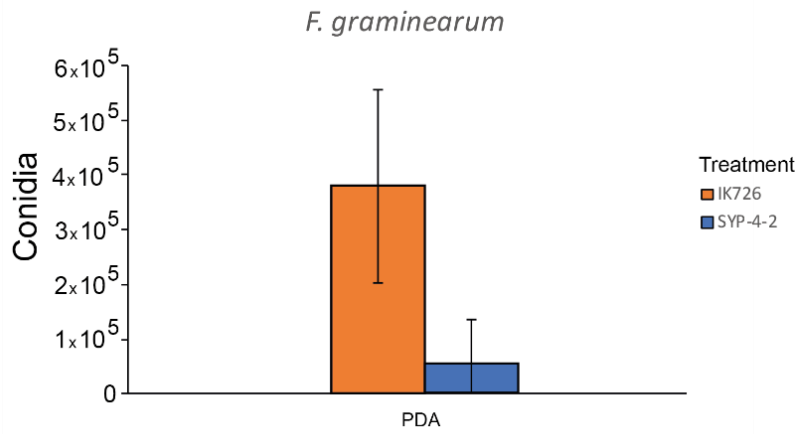


*Figure 9 Average number of F. graminearum conidia produced on 1/10 PDA petri dish, when incubated together with C. rosea, strain IK726 or SYP-4-2. Incubation time was 33 days. The difference between treatments was significant (P = 0.006) Bars indicate standard deviation.*

### 3.3.2 Inverse conidia production of *C. rosea*

For the production of *C. rosea* conidia, the results show an inverted relationship between the strains on the two media (Figure 10). When inoculated on PDA, IK726 produced 65% fewer conidia than SYP-4-2 (P = 0.026). In contrast, when inoculated on SNA, SYP-4-2 produced 63% fewer conidia compared with IK726 (P = 0.008).

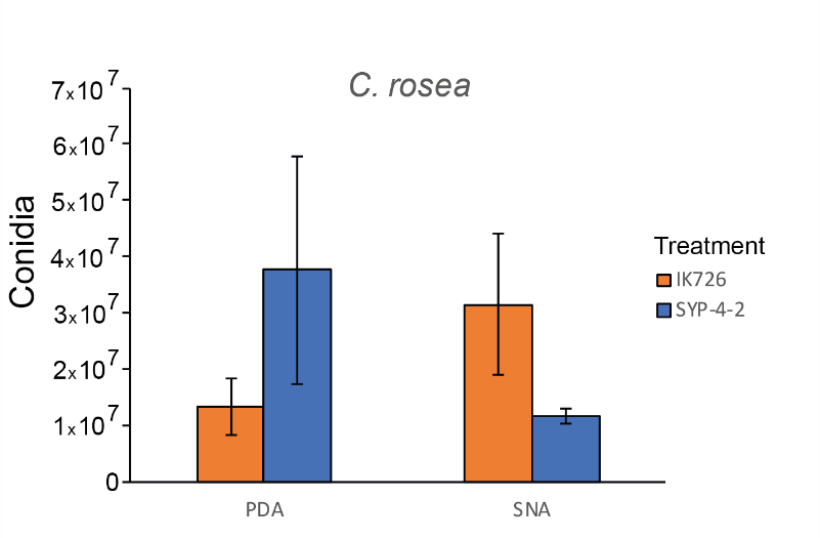


*Figure 10 Average number of C. rosea conidia produced per petri dish, 33 days after inoculation with spore solutions containing both F. graminearum and C. rosea, on two different media. Differences on both PDA (P = 0.026) and SNA (P = 0.008) were statistically significant. Bars indicate standard deviation.*

# 4 Discussion

## 4.1 Dual-plating assay

In this assay, IK726, the strain with the highest potential for use in biocontrol according to climate chamber experiments, displayed no greater inhibition than the negative control (Figure 5). On the other hand, GG-1-2, with a relatively low estimated biocontrol ability, displayed the greatest inhibition of *F. graminearum*. The relationship is however not inverse, since the strain 178.28, which had good biocontrol efficacy, also showed effective inhibition.

Since the results in this assay is measured in growth before the mycelial fronts meet, i.e. there is no direct contact between the fungi, the antagonistic effect is assumed to be due to secreted secondary metabolites or enzymes. These molecules interact with *F. graminearum* by diffusing throughout the media, as well as potentially through the air, in the case of any volatile metabolites (Rodriguez *et al.*, 2011). This work indicates a considerable variation between strains in the production of these molecules.

Studies have shown that antagonistic effects of BCAs, including *C. rosea* strain IK726, can vary depending on which media is used (Fatema *et al.*, 2018; Iqbal *et al.*, 2018). Including different growth media in an assay such as this would be interesting in order to see whether strains react uniformly to different growth conditions or if there is variability amongst them with regards to antagonistic potential. These results suggest that the latter is the case, since growth on PDA doesn't correlate with the climate chamber experiments. However, a more dedicated experiment with that in mind would have to be used to determine this.

Also influencing the results of this assay, apart from the antibiotic activity evaluated, is the effect of *F. graminearum* on *C. rosea*. Since only three positive controls were made for each strain of *C. rosea*, there were not enough samples to gain statistical significance on its growth rate. This was unfortunate since those

measurements might indicate *C. rosea's* ability to detoxify *F. graminearum's* mycotoxins such as zearaleone. *C. rosea* has previously been shown to be highly tolerant towards zearalenone, through physical detoxification by the enzyme zearalenone lactonohydrolase ZHD101 (Takahashi-Ando *et al.*, 2002), and efflux by ABC transporter ABCG5 (Dubey, Jensen and Karlsson, 2014). Both ZHD101 and ABCG5 contribute to the biocontrol effect of *C. rosea* against *F. graminearum* (Dubey, Jensen and Karlsson, 2014; Kosawang *et al.*, 2014). However, since *C. rosea* had been incubated for 13 days prior to the start of the assay, the causality of such measurements might have proven harder to define anyway. Considering that *C. rosea's* metabolites could already have been produced and have an effect on *F. graminearum* regardless of that strains ability to tolerate mycotoxins. Therefore, using this assay with regards to that parameter should perhaps be avoided. Other methods that might be more useful in determining the detoxifying ability of different strains would be either using transcriptomic analysis (Nygren *et al.*, 2018). However, such a method is quite expensive and might not be practical. A cheaper alternative would be to use the method of incubation on culture filtrates of *F. graminearum*, similar to that used by Dubey *et al.* (2014).

## 4.2 Liquid medium interaction assay

The MoA's being evaluated in this assay is antibiosis as well as physical parasitism, e.g. when hyphae of *C. rosea* penetrates the cell walls of *F. graminearum* with the use of chitinases (Jensen *et al.*, 2016). Spores of *C. rosea* can have an inhibitory effect on the germination of *F. graminearum's* spores, as was shown by Hue et. al. (2009). In that paper, the researchers used a similar method, combining macroconidial solutions and observing the germination rate after some time.

The results in Figure 7 indicate intrinsic differences in the speed at which the two strains activate biosynthesis of antibiotic metabolites. Of the two strains, IK726 appears to initially be more effective at inhibiting germination when compared to SYP-4-2. It is curious however that the number of viable *F. graminearum* conidia did not decrease further at the second observation for IK726.

As with the previous assay, *C. rosea's* ability to detoxify *F. graminearum* mycotoxins is also part of the interaction (Dubey, Jensen and Karlsson, 2014). In this assay however, the head start of *C. rosea* was only 4 hours, compared to 13 days, and the results may be a stronger indicator of the strains differing levels of mycotoxin tolerance. There were more viable conidia of SYP-4-2 left at both timepoints (Figure 8). One hypothesis is that SYP-4-2 is able to more effectively neutralize *F. graminearum's* toxins under these specific conditions.

Though this assay was also meant to measure parasitic antagonism of *C. rosea* on *F. graminearum* (Barnett and Lilly, 1962), this was not observed. A possible reason for this was the use of a shaker during inoculation, which might have hampered interaction between spores and prevented physical parasitism. Future experiments might benefit from an alternative method of preventing anaerobic conditions.

Furthermore, as the focus of this assay was to measure the inhibition of viable *F. graminearum* spores, no control was done with only *C. rosea*. However, in light of the results (Figure 8), which indicate a stronger viability of the SYP-4-2's spores, a control would've provided information whether this was due to the interaction with *F. graminearum* or general differences in viability between the strains.

Since circumstances prevented a control with only *F. graminearum*, the performance of the strains is only relative to each other. To further develop this assay, I would suggest using more timepoints to provide a clearer picture of the development over time. This would increase the workload substantially though, so this would preferably be done with only one strain in order to optimize the assay for further use. Using other media would also be interesting, however, simpler methods might be more suitable for investigation of those relationships, such as the dual-plating assay.

## 4.3 Solid medium interaction assay

The antagonism measured in this assay can be considered to be more holistic than that of the previous two. The strain's ability to parasitize, produce antibiotic molecules and compete for nutrients is assumed to be factors contributing to the results. However, low nutrient media was used to limit the fungi's resources and promote antagonism, thus likely lowering the effect of nutrient competition on the results.

Perhaps the most interesting result of the assay is *C. rosea's* conidia production. As presented in Figure 10, the strain's ability to produce conidia seem to be inverted on the two different media. On PDA, SYP-4-2 produce more conidia than IK726, while on SNA, the relationship is inverted. This measurement could indicate differences in phenotypic expression, either in terms of being able to utilize the media or detoxifying *F. graminearum's* mycotoxins, when grown on different media. This therefore further supports the theory of media playing a part in phenotypic expression between strains (Iqbal *et al.*, 2018). However, it is important to note that at the end of the assay, the conidia production *F. graminearum* was close to zero on SNA. Therefore, extrapolating estimates for *C. rosea's* ability to tolerate mycotoxins might not be prudent since any meaningful presence of *F. graminearum* is not established.

On PDA, the outcome of the assay did provide a measurable antagonistic effect, indicating that *F. graminearum* had been inhibited to a larger extent when exposed to SYP-4-2 than IK726 (Figure 9). As in the two other assays, and in accordance with the literature (Knudsen *et al.*, 1997), this *in vitro* assay therefore does not correlate very well with the climate chamber experiment either, as IK726 is expected to be a more effective antagonist.

As there were no results for *F. graminearum* conidia production on SNA, the antagonism could not me measured on that media. This would suggest that optimization is needed to make the assay useful, either by shortening the incubation time, further inducing conidial production in *F. graminearum* or reducing the incubation time of *C. rosea* before combining the conidial solutions and inoculating the agar plates. The germination rate of the conidia produced at the end of the assay might also be of interest since under this method, the conidia of *F. graminearum* is exposed to the fully developed mycelia of *C. rosea*, unlike in the previous assay where interaction only took place between conidia, and potentially germ tubes.

## 4.4 Conclusion

Although the results from none of these assays correlated with the previous climate chamber experiment, it is possible that further optimization would provide better correlation. More careful execution of the assays is also important to allow inclusion of proper controls in all of them. Beyond this, these methods also serve the purpose of illuminating which attributes in a strain are important for biocontrol efficacy. While the production of secondary metabolites and enzymes would seem crucial for biocontrol, the results of the dual-plating assay clearly show that there is more to it, as was supported in the literature (Teperi *et al.*, 1998). However, assays such as the dual-plating and liquid medium interaction assay, where the MoA is isolated could be useful to determine which attributes are important. Beyond this, similar assays to these could also be used for evaluation of other characteristics. How different strains react to different environments could be useful knowledge when considering what strains to use in commercial applications and whether agronomical conditions need to be taken into account when choosing it.

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