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Microbial biocontrol agents in IPM strategies

- reducing pesticide use in wheat and lowering the risk of fungicide resistance

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1. Preface

The project was carried out by University of Copenhagen, Department of Plant and Environmental Sciences (KU) in collaboration with Aarhus University, Department of Agroecology (AU) and The Swedish University of Agricultural Sciences, Department of Forest Mycology and Plant Pathology (SLU). The study was funded by the Danish Ministry of Environment's Research Program for Pesticides (J. no. MST-667-0025).

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2. Summary

The motivation of this project was the urgent need to generate knowledge for the development of reliable IPM strategies for implementation of biological disease control in field crops to reduce the input of chemical fungicides. Wheat is the main agricultural crop in Denmark. However, grain yield and quality is highly reduced by fungal diseases, if not efficiently controlled. Septoria tritici blotch (STB) is the most important wheat disease and the total loss per season may be as high as 10-30 hkg/ha if not treated with fungicide 2-3 times per season. Fungicide resistance in the *Zymoseptoria tritici* populations is an increasing problem due to frequent fungicide applications and access to only few active ingredients. Fusarium head blight (FHB) also reduces yield and more importantly lead to accumulation of mycotoxins, which are harmful to the health of humans and livestock.

The main questions of the project were (i) Can STB and FHB be efficiently controlled by microbial biological control agents (BCAs) alone or combined with traditional chemical fungicides applied in low dosages? (ii) Can the development of fungicide resistance in the *Z. tritici* pathogen population be reduced by the combined use of BCAs and fungicides as compared to repeated chemical fungicide applications? And (iii) can accumulation of mycotoxins in harvested grain be reduced by the use of (BCAs)?
The project included three BCAs. The versatile fungal BCA *Clonostachys rosea* IK726 isolated from barley roots in Denmark and the two registered bacteria-based products Cedomon (*Pseudomonas chlororaphis*, strain MA341) and Serenade ASO (*Bacillus velezensis*, syn. *B. amyloliquefaciens*, strain QST713). The azole fungicide Proline EC 250 (a.i. prothioconazole) belonging to the group of demethylase inhibitors (DMIs) was chosen as a model fungicide in order to look for possible changes in fungicide sensitivity and mutations in the CYP51 gene of *Z. tritici* as fungicide resistance to azoles in the *Z. tritici* population has developed in Denmark.and the Nordic countries in recent years.

To address the research questions, a series of field, greenhouse and growth chamber experiments were conducted. Field trials were conducted in 2018, 2019 and 2020 to test efficacy of the BCAs as single applications, as mixtures of two BCAs and as combined application of BCA and half dosage of Proline EC 250 (0.4 L/ha). Plots were treated either ones at growth stage GS 37-39 or GS 61-65 or twice at both growth stages. The effects on disease severity, grain yield and mycotoxin content were tested as well as effects on fungicide resistance development, fungal community structure and persistence of the fungal BCA (only *C. rosea*) in wheat. Furthermore, greenhouse and growth chamber experiments were conducted mainly to test efficacy of *C. rosea* and mechanisms involved in STB control as well as in FHB control in relation to accumulation of the mycotoxin deoxynivalenol (DON).

Across the 3 seasons, several BCA treatments reduced the severity of STB significantly when applied either once or twice, providing moderate control depending on the timing of application. Indeed, at several assessments BCA treatments gave disease control at similar levels as the fungicide Proline EC 250 when assessed during the growth period from early heading (GS 51) until early to medium grain development (GS 73-75). However, at later assessments (GS 79), the effect from BCAs was often reduced as compared to some fungicide treatments especially at high STB attacks. This indicated less persistence of BCAs when applied alone. In 2018, the efficacy of the best BCA treatments applied alone was in the range of 50-75% STB reduction where relatively high STB attacks were seen at the early growth stages while infection of the flag leaf at later growth stages was insignificant. In 2019, STB was more severe throughout the season. Whereas the best treatments with applications of BCAs reduced STB by 40-60% until the end of June, their effect failed at the last assessment in July where the flag

leaf of the untreated control had reached approximately 80% STB. In comparison, Proline EC 250 treatments (0.4 L/ha) and (0.8 L/ha) applied both at GS 37-39 and GS 61-65 reduced STB by 50-70% at the first assessments and by 50-60% at the final assessment in 2019. For the 2020 trial, where the STB attack did not exceed 6% at any time point, the best single BCA treatments reduced STB in the range of 60-90%. Particularly, *C. rosea* consistently gave moderate control of STB when applied only at the early timing (GS 37-39) irrespective of the differences in STB disease pressure between seasons.

Unexpectedly, the single treatment with *C. rosea* at GS 37-39 reduced STB on the uppermost leaves, which had not developed when the *C. rosea* spores were sprayed onto the canopy approximately 4-5 weeks earlier. This points towards a systemic effect of the BCA treatment. Microscopy of the *Z. tritici* infection processes further supported this as penetration efficiency of *Z. tritici* spores was lower and the pycnidia-formation was hampered in leaves pre-treated with *C. rosea* 24h earlier indicating activation of plant's own defence mechanisms by the BCA.

The control of FHB by BCAs were similarly variable in 2019 and 2020 where FHB attack was observed. The best treatments with BCAs alone reduced FHB by approximately 50% at the first disease scoring while the effect diminished at the last assessment. However, treatments including Proline application at GS 61-65 were superior and reduced FHB attack in the range 50-90%. Interestingly, the single application of *C. rosea* at GS 37-39 also reduce FHB by approximate 50% even though *C. rosea* was applied more than three weeks before the *Fusarium* infection took place and the DON content was reduced by approximately 30% in 2019. This again could indicate a systemic effect of the BCA as also suggested in relation to STB. Results from the greenhouse experiments confirmed the strong potential of a *C. rosea* pre-treatment for reduction of FHB and reduced DON accumulation in spikes.

Detailed analyses of treatments only including *C. rosea*, Proline EC 250 (0.4 L/ha) and the combined treatment, indicate that inclusion of BCAs in disease control schemes can play a role in an IPM strategy. It could be relevant to substitute the early Proline EC 250 application with *C. rosea* as both these treatments controlled STB and FHB to the same level. Therefore, one could consider combining early *C. rosea* with late Proline application in order to maintain efficient disease control during the growth season. Such alternation of BCA and fungicide treatment will potentially reduce the fungicide input while maintaining high disease control capacity. However, further studies are needed to determine whether this will also be the result in case of using more effective fungicides like Propulse SE 250 or newer active ingredients, now available. Basically, field trials testing different timing of BCA application should conducted in order to identity the optimal application strategy which may not be similar to the one used for fungicide application as BCAs and fungicides rely on very different modes of action. This would be important for designing strategies for alternation between BCAs and fungicides to stabilise efficacy and reduce selection pressure on plant pathogens.

Application of BCAs in a crop protection scheme may also have another beneficial effect since by using different modes of action, resistance in the pathogens against traditional fungicides may be reduced. However, there were no apparent differences in the sensitivity to Proline EC 250 in isolates recovered from the different treatments. Furthermore, successfully full-length sequencing of the *Z. tritici* CYP51 gene using PacBio revealed no clear picture of BCA mediated changes despite some smaller differences in specific mutation frequencies between *C. rosea* treatments and Proline EC 250. What these changes mean in relation to increased fungicide resistance is under further investigation.
A relatively unexplored effect of the use of BCAs for crop protection in the phyllosphere is related to how the natural microbial communities present in the ecosystem may be affected by such applications. We identified the wheat pathogens *Zymosepetoria*, *Blumeria graminis*, *Puccinia striiformis* and *P. recondita* as well *Sporobolomyces* and *Alternaria* among the most

abundant taxa using PacBio amplicon sequencing. However, no major effects of BCA treatments on the mycobiome composition was revealed suggesting that the indigenous mycobiota was largely undisturbed by the BCAs applied. Hence, the community analyses confirmed that *Clonostachys* amplicons were extremely rare and not more frequent in the *C. rosea* treated samples

The current study has not revealed any clear economic benefit, as only one of 3 trials gave a significant and positive yield increase with fungicide and BCA treatments. In order for BCA's to provide attractive solutions, trial data should verify an economic benefit for the farmers in a range of 3-4 Dt/ha in order to pay for the cost of treatments. If traditional fungicides should be substituted by e.g. one fungicide treatment, BCA products should have an advantage due to expected lower taxation following a better toxicity profile. Most BCAs are permitted for use in organic production. As this production segment is increasing, BCAs could become an important tool for future disease control in this branch as well.

3. Introduction

3.1 Aim of project and main research questions

The motivation of this project was to generate knowledge urgently needed in order to develop reliable IPM strategies for implementation of biological disease control in field crops to reduce the input of chemical fungicides.
Wheat is the main agricultural crop in Denmark. However, grain yield and quality is highly reduced by fungal diseases, if not efficiently controlled. Septoria tritici blotch (STB) is the most important wheat disease and the total loss per season may be as high as 10-30 hkg/ha if not treated with fungicide 2-3 times per season. Fungicide resistance in the *Zymoseptoria tritici* populations is an increasing problem due to frequent fungicide applications and availability of only few active ingredients. Fusarium head blight (FHB) also reduces yield and more importantly lead to accumulation of mycotoxins, which are harmful to the health of humans and livestock.

The overall aim of the project was to investigate the potential of integrating microbial biocontrol agents (BCAs) with low dosages of fungicides for control of the important wheat diseases STB and FHB.
Main question of the project were:

- Can STB and FHB be efficiently controlled by (BCAs) alone or combined with traditional chemical fungicides applied in low dosages?
- Can the development of fungicide resistance in the *Z. tritici* pathogen population be reduced by the combined use of BCAs and fungicides as compared to repeated chemical fungicide applications?
- Can accumulation of mycotoxins in harvested grains be decreased by the use of (BCAs)?

The project included three BCAs. The versatile fungal BCA *Clonostachys rosea* IK726 isolated from barley roots in Denmark and the two registered bacterial based products Cedomon (*Pseudomonas chlororaphis* strain MA341) and Serenade ASO (*Bacillus velezensis*, syn. *B. amyloliquefaciens,* strain QST713). The azole fungicide Proline EC 250 (a.i. prothioconazole) was used as a model fungicide in the project as it belong to the demethylase inhibitors (DMIs) where fungicide resistance in the *Z. tritici* population has developed in Denmark

To address the research questions above, a series of field, greenhouse and growth chamber experiments were conducted:
(i) Field trials were conducted in 2018, 2019 and 2020 to test efficacy of the BCAs in a single applications, as combination of two BCAs or BCA(s) were combined with half dosages of Proline EC 250. Here, effects on disease severity, grain yield and mycotoxin content were tested as well as effects on fungicide resistance developments, fungal community structure and persistence of the fungal BCAs on wheat.
(ii) Greenhouse and growth chamber experiments were conducted. Firstly, to test compatibility between *C. rosea* and prothioconazole. Secondly, to test efficacy of the fungal BCA and mechanisms involved in STB control as well as FHB control in relation to accumulation of the mycotoxin DON.

3.2 Background and state of the art

Healthy plant production resulting in high yield of quality products relies on the right crop management regime including diverse disease control measures. Crop rotations, use of disease resistant cultivars, ploughing or other ways of soil management, forecasting for predicting dis-

ease outbreak and biological control are examples. Fungicides have historically been very efficient in plant disease control and fungicide use is at present a major disease control measure in many different plant crops. However, pesticide use has resulted in various side effects that must be prevented. In particular, repeated use of specific pesticides over the last decades has led to development of fungicide resistance in organisms causing disease (pathogens) and therefore such fungicides become inefficient in the control of a particular disease when the pathogen population is frequently exposed to a specific group of actives with the same mode of action.

The interest and demands for biological solutions are increasing due to a need for increased and sustainable food production (Chakraborty & Newton, 2011). This process is partly driven by increasing consumer demand for plant products without pesticides, thus prioritizing IPM and organic production principles (Pedersen and Sewohl 2013).

For environmental reasons, and for the production of healthy food and feed with no or low content of chemical pesticide residues, EU legislation has now put strict regulations on pesticide use. The goal of the EU legislation on pesticides is sustainable plant production with reduced or no input of chemical pesticides and with more emphasis on other non-chemical control measures such as those mentioned above. Biological control measures are especially emphasised in the EU legislation (Directive 2009/128/EC of 21 October 2009). Included in the legislation is also an effort to prevent pesticide resistance when planning disease control strategies (Directive 2009/128/EC of 21 October 2009).
To meet this goal, it is now a requirement that all major plant crops in Europe must be grown organically or following Integrated Pest Management (IPM) strategies as defined in 8 IPM principles (Barzman et al., 2015). The EU goal is also in line with the 17 UN global sustainable development goals and the FAO initiative “International Year of Plant Health (IYPH2020)” dedicating year 2020-2021 to improve the global understanding of the importance of plant health for food security (http://www.fao.org/plant-health-2020/home/en/). Recently, the goal to reduce pesticide use has become even more urgent with the new framework of the Farm to Fork Strategy of the European Green Deal, where the European Commission will take additional action to reduce the overall use of chemical pesticides by 50% by 2030 (https://ec.europa.eu/food/horizontal-topics/farm-fork-strategy_da).

3.2.1 Biological disease control – pros and cons

Biological control is defined as the use of living organisms (biological control agents, BCAs) for controlling insect pests, weeds and plant diseases and is thus placed under a shared terminology umbrella “bioprotection” where other nature-based substances of other origin are not considered biocontrol, for example plant extracts used in plant protection (Stenberg et al., 2021). Here, we focus on microorganisms as they are used for biological disease control.

BCAs have the potential to play an important role in future IPM strategies. BCAs are often considered to be less harmful than pesticides due to fewer adverse health effects compared to pesticides and for that reason, there is a wish for revision of the registration process of beneficial microorganisms in the EU including microbial biocontrol agents as reviewed and recommended by (Sundh and Eilenberg (2021)) and Sundh et al. (2021). There is also a very low risk of pathogens developing resistance against BCAs based on experience over the past 40 years, chiefly relying on the complex mode of action of the BCAs. Furthermore, BCA application generally has no pre-harvest interval restrictions as opposed to chemical pesticides. In addition, organic crop production, where most biocontrol measures are permitted has increased in recent years.

It is a challenge to succeed in using biological control. It requires a profound understanding of the disease cycle of target pathogens to know the correct timing and placement for optimal BCA application. Understanding the environmental conditions where the plants are vulnerable

to pathogen attack is also important to consider when using both fungicides and biocontrol. Knowing the mode of actions of the BCA is additionally a prerequisite for developing efficient biocontrol strategies and for registration purposes. In most cases, the BCAs work through a concerted action of several mechanisms that can be direct actions on the pathogens or indirect disease control via BCA-induction of resistance (enhancing inherent defence responses) in the host plant (Jensen et al., 2021). New evidence is also emerging that the choice of plant cultivar/genotype can have an important impact on how efficient the biological disease control will be. Finally, the BCAs meet a harsh environment where they will have to survive and successfully cope with the soil- and/or plant microbiomes in addition to exerting its biocontrol activity (Collinge et al., 2022).

Prior to application to the plants, the BCA product must meet several quality parameters such as a high survival of the biocontrol organisms, also following storage of the product (a good shelf life). This also include a fast germination and high activity of the BCA when applied after storage (Jensen et al., 2021). The BCA must also be designed for application to the plant crop. Depending on the disease(s) and crop in focus, application can take place to the foliage in a way similar to spraying fungicides. Other methods of application of BCA products can be seed treatments, dipping root or cuttings in BCA suspensions or the BCA can be applied as a soil drench or by furrow application when sowing the plants.

In the project reported here, the focus was on biological control of plant diseases by spray application of fungi or bacteria to the crop for disease control (i.e. augmentative biocontrol) as part of an integrated pest management (IPM) strategy.
The aim was to verify that biological disease control can play an important role in IPM strategies for disease control, leading to reduced input of chemical fungicides and help to prevent development of fungicide resistance. Thus, the project served two IPM goals. Additionally, the role of biocontrol for preventing mycotoxin accumulation was addressed.

Wheat was used as the model crop in the project because wheat is one of the most important crops for food and feed, both in Denmark and worldwide. Two main diseases on wheat were addressed.

3.2.2 Two important diseases in Danish wheat production

Several fungal pathogens play an important role in decreasing wheat yields across the world. The yield losses due to fungal pathogens vary depending on climatic conditions, farming practices and cultivars grown (Savary et al., 2019). Septoria tritici blotch (STB) caused by *Zymoseptoria tritici* is the most important disease under north-western European growing conditions (Torriani et al., 2015). To control the disease, European farmers mainly rely on the use of fungicides and to a lesser extent on resistant cultivars. STB is also considered the most important wheat disease in Denmark and the total loss per season may be as high as 10-30 hkg/ha if not treated with fungicide 2-3 times per season (Jørgensen et al., 2021).

Fusarium spp. infects cereals such as wheat causing Fusarium head blight (FHB) (Bottalico & Perrone, 2002, Jørgensen et al 2008, Xu & Nicholson, 2009, Rojas et al, 2018). FHB is one of the most destructive diseases in cereals world-wide causing yield loss and reduced grain quality mainly due to the ability of the fungi to produce mycotoxins (Bai & Shaner, 2004). FHB is caused by a complex of up to 19 pathogens (Liddell, 2003), which vary in their mycotoxin profiles (Logrieco et al., 2002). In Danish wheat production, at least 8 different *Fusarium* species give FHB (Nielsen et al., 2011). The risk for FHB varies considerably between seasons, but sensible crop rotations, ploughing and resistant cultivars can help to reduce the risk of attack. However, commonly fields in Western Europe are treated with fungicides during flowering to ensure control of FHB, particularly in humid and warm seasons, where there is an increased risk of attack. *Fusarium culmorum* and *F. graminearum* are considered the most important

Fusarium species in wheat in Northern Europe and produce deoxynivalenol (DON), nivalenol (NIV) and zearalenone (ZEA) (Nielsen et al., 2011).

3.2.3 Fungicide use and resistance problems

The current control strategy in conventional farming relies heavily on the use of fungicides and approximately 60% of the total input of fungicides used in DK are applied to cereals, mainly winter wheat (Bekæmpelsesmiddel statistikken 2019). The main fungicides used are the demethylase inhibitors (DMIs) and the succinate dehydrogenase inhibitors (SDHIs). Frequent use of fungicides commonly leads to the development of resistance as particularly seen for the *Z. tritici* population, while fewer problems have been described in relation to control of Fusarium head blight.

DMI fungicides have been used for control of STB for more than 40 years (Russell, 2005, Jørgensen & Heick, 2021) and are regarded to be linked to a moderate risk for developing fungicide resistance (https://www.frac.info/home). Despite the lower risk, the DMI fungicides have experienced a significant decrease of sensitivity over the last decades (Cools & Fraaije, 2013, Mäe et al., 2020, Leroux et al., 2007, Heick et al., 2020).This shift can be explained by the accumulation of mutations in the *Cyp51* target gene of DMIs, along with overexpression of *Cyp51* and efflux transporters (Cools et al., 2012, Omrane et al., 2015, Omrane et al., 2017). Over 30 mutations have been found conferring DMI resistance at varying levels (Huf et al., 2018). As in most of Europe, a major shift in sensitivity has also occurred in the Danish *Z. tritici* population, where field performance has dropped from ca. 80% to 40-50% control (Heick et al., 2020). In the current project, we included prothioconazole as reference because this is the most widely used azole for STB control under Danish conditions.

SDHI fungicides have been on the market since 1960, but only been registered for STB control since 2003, where a new generation of SDHIs was introduced (Rehfus et al., 2018). Decreased sensitivity to fungicides of this group has been detected in several countries (Rehfus et al., 2018, Stammler et al., 2015). The main resistance mechanisms are the result of amino acid substitutions in the three subunits of the target gene *Sdh Succinate dehydrogenase*, i.e. *SdhB*, *SdhC*, and *SdhD* (Sierotzki & Scalliet, 2013). Several mutations have been identified (FRAC 2021) with low-to-moderate resistance impact. A specific mutation H152R has been shown to cause a loss of sensitivity to all SDHI fungicides (Dooley et al., 2016, Hellin et al., 2020). In the current project, we also included the product Propulse, which is a mixture of prothioconazole and fluapyram - a mixture of an azole and a SDHI fungicide. Mixtures are commonly used to stabilise or minimize development for resistance.

3.2.4 How to measure resistance development in the *Z. tritici* population

Monitoring fungicide resistance is carried out by independent national research institutions and companies that need to verify that their products still provide sufficient control. Collection of sensitivity data from yearly surveys to determine the status of resistance development and frequency of mutations will show the efficacy of different fungicides against the most important fungal pathogens in the major crops worldwide. Resistance might be observed in the laboratory, but it is essential to conduct field trials and evaluate the level of control attained in the field for a given fungicide, before concluding on the resistance levels of a given population in a given area (Brent & Hollomon, 2007).

EC_{50}

Fungicide resistance is an arbitrary expression and interpretation of the measured fungicide sensitivity can be based on either shifting in sensitivity of single isolates or obtained changes in field efficacy across several seasons. A method used to monitor resistance development is monitoring the half-maximal effective concentration (EC_{50}) for different compounds for individual isolates. An EC_{50} value is defined as the concentration at which 50% of fungal growth is

inhibited in *in vitro* studies (Oliver & Hewitt, 2014). Fungal pathogens that are non-obligate are grown and harvested from agar plates. Fungal spores produced on the plates are suspended in a liquid before being added to 96-well microtiter plates, in which increasing concentrations of the respective fungicide are present. The plates are then inoculated for a period before the growth of each isolate, at each concentration, is measured on a microplate reader, which measures the absorbance. The data produced can then be used to calculate the EC_{50} values for each isolate, to give an estimate of the degree of inhibition offered by the particular fungicide. Since the EC_{50} values only offer a concentration at which 50% of the fungal growth is inhibited, a resistance factor (RF) is often calculated, to provide a better interpretation of the resistance levels. The RF is calculated as the ratio of EC_{50} of a "resistant" isolate to that of a sensitive isolate, usually an isolate known to have high sensitivity to the fungicide (Oliver & Hewitt, 2014). The resistance factor is arbitrary, but can be divided into three groups: low (>5), moderate (5-20) and high (>20). Resistance is considered to be present when an isolate exceeds moderate-to-high RF values, while lower resistance factors can be interpreted as variation and different tolerance of the strain to the fungicide.
When an explanation of resistance is needed, molecular tools offer several methodologies that explain the mechanisms of resistance. This includes both search for target site mutations, efflux, over-expression, etc.

Sequencing to determine mutations in the CYP51 gene
Monitoring of fungicide resistance can play a vital role in understanding the distribution, evolution and management strategies of *Z. tritici* populations. The in vitro sensitivity assays such as measurement of EC_{50} is a traditional method to determine fungicide tolerance and its association with CYP51 mutations in *Z. tritici* known to lead to fungicide resistance (Siah et al., 2010). In previous years, PCR assays combined with RFLP (restriction fragment length polymorphisms) and Sanger sequencing have been used to identify and detect mutations in fungicide gene targets, including the *CYP51* and *cytochrome b* genes in *Z. tritici* isolates (Zhan et al., 2006, Leroux & Walker, 2011, Estep et al., 2015, Huf et al., 2018, Kildea et al., 2019, Stammler et al., 2008). The performance of these methods is limited due to lack of accuracy, required time scale and costs. PCR-RFLP can be used to determine only few mutations due to limited availability of restriction enzymes. In addition, Sanger sequencing or RFLP analysis provides limited opportunity to detect mutations in a heterogeneous population.

Next-generation sequencing (NGS) that generates millions of sequence reads per reaction can readily be used for a rapid and accurate detection and quantification of mutations in a particular gene. In a recent study, Illumina NGS was used to detect mutations in the *cytochrome b* gene leading to QoI (strobilurin) resistance in *Z. tritici* (Pieczul & Wąsowska, 2017). The result showed that NGS can be applied for large-scale detection and monitoring of gene mutations in mixed populations of strains with a varied level of sensitivity and resistance against fungicides. However, short reads of Illumina sequencing may hamper the ability to detect various haplotypes of *Z. tritici*, which are rapidly increasing in populations. Due to the rapid build-up of mutations in the CYP51 gene (Estep et al., 2015, Huf et al., 2018, Kildea et al., 2019), determination of *Z. tritici* haplotypes is necessary for monitoring of fungicide resistance in *Z. tritici* populations under field conditions. As increasing information becomes available on the CYP51 mutations and the development of azole fungicide resistance, a better strategy for detecting *Z. tritici* haplotypes is needed. The PacBio sequencing methods can achieve this, providing long reads with an average read-length of 10 kb or more. In addition, an error correction method (circular consensus sequencing; CCS) and repeated sequencing of a single-molecule DNA template can result in extremely high accuracy (Wenger et al., 2019). In a recent study, Samils et al. (2021) used PacBio sequencing for haplotype determination in a mixed *Z. tritici* population and at a large scale. In the current project, we used both methods to investigate the effect of BCA spray treatments on CYP51 mutations in the *Z. tritici* population.

3.2.5 What is known about biocontrol of STB and FHB in wheat?

Not much work has been done with biocontrol of STB and FHB at field level and, even under controlled conditions in greenhouses or growth chambers, few studies are published. Field trials are the ultimate way of testing the efficacy of BCAs – an evaluation method often seen as being more challenging due to variable and unpredictable weather conditions. Nevertheless, there are interesting examples of disease control using bacteria, yeast and filamentous fungi. In 2012-2017, field experiments for controlling FHB have been carried out as GEP experiments at Aarhus University, Flakkebjerg, Denmark, contracted by the Swedish University of Agricultural Science (Dan Funck Jensen, unpublished). In several years, significant control of FHB was observed and in some of the experiments on FHB control, there were significant disease control using bacteria-based BCAs (i.e. *Pseudomonas chlororaphis*) either alone or in combination with *Clonostacys rosea* (Dan Funck Jensen, unpublished). Interestingly, the results also showed significant biocontrol of STB with *C. rosea*. A PCT announcement for patent was published in 2019 (PCT announcement: WO2019/125294 A1). The Patent Cooperation Treaty (PCT) is an international treaty with more than 150 contracting states. The PCT makes it possible to seek patent protection for an invention simultaneously in a large number of countries by filing a single “international” patent application instead of filing several separate national or regional patent applications. The granting of patents remains under the control of the national or regional patent offices in what is called the “national phase”. Patent applications for the “national phase” have been applied for granting in the USA, Canada, Australia and the EU on biocontrol of STB with BCAs based on the fungal species *Clonostachys rosea*. Decision is still under way. There are commercial BCA products based on strains of *Clonostachys rosea* on the marked mainly for biological control in greenhouses but also in some cases in the open field. These include products for biocontrol of damping-off and soilborne diseases caused by *Fusarium* spp., *Pythium* spp. and *Rhizoctonia solani* in ornamentals, in several herbs and in vegetable crops. There are also products available for grey mold control caused by *Botrytis cinerea* in strawberries, raspberries and in orchard fruit production. In addition, a *C. rosea* product is marketed for use on golf courses against several soilborne pathogens (i.e. https://middeldatabasen.dk/). The fact that this BCA already is registered and commercialised for use in many different crops will facilitate the registration of *C. rosea* based BCAs for use against STB in wheat in the DK/EU.

A few published studies have explored the potential of plant-associated microorganisms for biological control of STB, with varying success (Cordo et al., 2007, Lynch et al., 2016, Samain et al., 2017, Samain et al., 2019). In many cases, the experiments were limited to *in vitro* studies and controlled environments. In the few cases, where the potential biocontrol agents were brought to the field, no or inconsistent STB control was found (Flaishman et al., 1996, Kildea et al., 2008). Recently, Latz et al. (2020) showed a minor reduction in STB in one field experiment using the two fungal strains *Penicillium olsonii* ML37 and *Acremonium alternatum* ML38. Serenade ASO, a BCA based on *Bacillus velezensis* (Serenade ASO) has recently been approved for use control of various wheat diseases, including STB (Middeldatabasen: https://middeldatabasen.dk/middelvalg.asp). The efficacy of Serenade ASO is, however, ranked as weak to moderate compared with traditional chemical solutions, which reflect that the efficacy in many cases not has been seen to be significant compared with untreated controls (Matzen et al., 2019, Reiss & Jørgensen, 2017).

Several organisms have been tested for control of FHB and DON accumulation in grains. In Canadian field trials, isolate ACM941 of *C. rosea* showed a significant reduction of FHB as well as a 22-33% reduction in the DON content following spray treatments at flowering (Hue et al., 2009, Xue et al., 2014). There are also promising results both from field and greenhouse trials showing significant reductions in DON accumulation in grains of plants treated with bacterial isolates (*Streptomyces* spp.), e.g. (Palazzini et al., 2017, Palazzini et al., 2018) and *Bacillus* spp. (Pan et al., 2015). Further details on the ability of these microorganisms to reduce DON are outlined in Table 3.1.

TABLE 3.1. Overview of studies testing the ability of spray treatments with bacterial or fungal BCAs to reduce the DON content in grains as compared to the untreated controls.

Species	Strain	Environment	DON content in untreated plots	DON reduction	Reference
Streptomyces spp.	RC 87B	Field trial	1.6-1.8 µg/g	69-85%	(Palazzini et al., 2017)
Streptomyces spp.	RC 87B	Field trial	4.4 µg/g	51%	(Palazzini et al., 2018)
Bacillus spp.	RC 218	Field trial	4.4 µg/g	50%	(Palazzini et al., 2018)
Bacillus spp.	SG 6	Field trial	17.5 µg/g	69%	(Zhao et al., 2014)
Bacillus spp.	BM 1 BS 43 BSM 0 BSM 2	Field trial	6.8 µg/kg	50-89%	(Pan et al., 2015)
Clonostachys rosea	ACM941	Field trial	1.7-12.0 µg/g	21%	(Hue et al., 2009)
Clonostachys rosea	ACM941	Field trial	9.6-21.6 µg/g	22-33%	(Xue et al., 2014a)

It has been observed that reduction in FHB symptoms, and in particularly reduced biomass of the pathogen in grains, is often positively correlated with a low mycotoxin accumulation (Yang et al., 2010). However, the use of BCAs could potentially affect this relationship in an additional way by degrading and/or transforming mycotoxins into a less toxic compound. For DON; detoxification, oxidation and de-epoxidation are important enzymatic transformations to reduce its toxic effect as discussed by Mielniczuk & Skwaryło-Bednarz (2020). *C. rosea* IK726 has ability to detoxify ZEA *in planta* (Kosawang et al., 2014), which suggests a potential for further mycotoxin reduction when using BCAs for FHB control.

3.2.6 What is known about combined BCA and fungicide control of STB and FHB in wheat?

There are only a few field studies testing the combination of BCAs and fungicide in wheat and none for STB control as recently reviewed by Ons et al. (2020). Some indication of the relevance of this IPM approach was shown in a field study of FHB control in wheat. Here, the BCA *Lysobacter enzymogenes* C3, in combination the fungicide tebuconazole, was consistently more effective in controlling FHB as compared with the bacterial strain or the fungicide alone (Jochum et al., 2006).

In a few projects, combinations of BCAs with traditional fungicides have been tested for control of yellow rust and powdery mildew in cereals. The aim has been to replace one of two chemical treatments with BCA products. No clear benefits from such combinations have so far been seen (Reiss & Jørgensen, 2017, Matzen et al., 2019).
Furthermore, in relation to preventing azole fungicide resistance in *Z. tritici* populations, only one study has been published. Thus, Heick et al. (2017) carried out field experiments for controlling STB, focusing on the fungicide prothioconazole that belongs to the DMI fungicide group. They made strategies for substituting prothioconazole with other fungicides from the DMI group or substituting it with fungicides with another mode of action. In one of the treatments, the BCA Serenade ASO was applied together with a chemical fungicide at all three time points where the plants were sprayed. There was a clear pattern in the pathogen population showing changes in the frequencies of mutations that are known to lead to fungicide resistance (i.e. mutations in the CYP51 gene). This was also the case where BCA applications were part of the pesticide treatments, although it was not possible to rule out if this played a role in such changes in mutation frequencies and the biocontrol effect on STB was too weak in these preliminary tests (Heick et al., 2017). However, to allow its large-scale implementation, further knowledge is needed, comprising timing, number and interval of repeated BCA applications and their compatibility with fungicides. The compatibility of BCAs with fungicides might differ when applied in a mixture or when used in alternation (Ons et al., 2020).

3.2.7 The indigenous fungal microbiome - effects of BCAs and fungicides on community structure in the phyllosphere

The composition of the fungal wheat leaf community is dynamic and may depend on environmental factors such as the climatic conditions, experimental site, physiological stage and tissue of the plant, cultivation practices, fungicide applications as well as the wheat cultivar used (Knorr et al., 2019, Sapkota et al., 2015, Sapkota et al., 2017, Karlsson et al., 2014, Karlsson et al., 2017, Latz et al., 2021) However, the underlying mechanisms behind the dynamics of leaf community composition are not fully known. For example a positive effect of organic farming as compared to conventional farming on species richness of wheat phyllosphere fungi has been reported (Karlsson et al., 2017). Knorr et al. (2019) showed that fungal communities from fungicide-treated plots could be separated from the communities in non-treated plots and that fungicide effects on the communities were related to dosis, timing and product used. Some fungi, including the target organism *P. striiformis* were effectively controlled by most of the fungicide applications whereas some yeasts and also *P. tritici-repentis* increased after treatments. So far it has not been studied whether treating wheat crops with BCAs has an effect on the microbial communities in the phyllosphere.

4. Materials and methods

4.1 BCAs and pathogens – inoculum production for field, greenhouse and growth chamber

Field trials: Three BCAs were applied in the field. The fungus *Clonostachys rosea* strain IK726, isolated from barley roots (Knudsen et al., 1997) was produced on a mixture of sphagnum peat and wheat bran, dried and milled as described by Jensen et al. (2000). The inoculum produced each year was stored at 4°C until application in 2018, 2019 and 2020, respectively. On the day of spraying, inoculum was mixed with water and the surfactant Tween 20 (0.01%) to obtain a concentration of 2 x 10^6 spores/ml. This resulted in application of 1x 10^7 spores per m^2. The bacterium *Pseudomonas chlororaphis* strain MA341 (the active ingredient of Cedomon) was applied in the 2018 trial. In the 2019 and 2020 trials, Serenade ASO (4 L/ha), containing the bacterial strain *Bacillus velezensis* QST713 was applied which resulted in application of 1x 10^9 spores per m^2.

Fusarium inoculum for inoculation consisted of a mixture of two *Fusarium* species (*F. graminearum* and *F. culmorum*). The inoculum was produced using different strains of *Fusarium* in the lab and mass produced following inoculation of sterilized grain incubated in long-wave (UV-A) ultraviolet light (black light) for 4 weeks. Following this period, spores were washed off, spore concentrations measured and suspensions frozen for later use.

Growth chamber and greenhouse: *Clonostachys rosea* strain IK726, *Penicillium olsonii* strain ML37 and *Acremonium alternatum* strain ML38 were cultured on Potato Dextrose Agar plates (PDA, Difco) for 14-21 days at room temperature under ambient light conditions. Spores were harvested by adding autoclaved MilliQ water to the plates and gently scraping the surface of the mycelium. The suspensions were filtered through Miracloth® or three layers of gauze. Spore concentrations was adjusted according to the requirements in the different experiments. Stocks of the BCAs, either spore suspensions or agar plugs, were maintained in 10% glycerol and stored at -80°C until use. Inoculum of *Zymoseptoria tritici* (isolates IPO323, OP15.1 and ZT07) for growth chamber and *in vitro* experiments was produced on PDA. The isolates were grown at room temperature under ambient light conditions for 4 days. Blastospores were harvested by adding autoclaved demineralised water to the plates and gently scraping the surface. The suspensions were filtered through a layer of gauze and the concentrations adjusted according to the requirements in the different experiments.
For production of *Fusarium graminearum* macroconidia (isolate WC-091-7, AU Flakkebjerg), three agar plugs from a 7-day-old culture were inoculated in 20 ml sporulating RA liquid medium (Imholte & Schramm, 1968) for 7 days at 180 rpm. Macroconidia were filtered through Miracloth® and centrifuged at 6500 rpm for 7 min. The spore pellet was washed twice in sterile MilliQ water to remove traces of the culture medium. Finally, the concentration of macroconidia was adjusted to 10^6 conidia/ml and aliquotes were stored in 10% glycerol at -80°C until use.

4.2 Field trials and experimental plans

Three field trials were carried out in winter wheat in 2018, 2019 and 2020, respectively. The trials were conducted at Flakkebjerg research station (55.3253°N, 11.3913°E) on a fine clay loam soil. The experiment was set out as a completely randomised block design with four replications and a plot size of 22.5 m^2 (9.0 m length and 2.5 m width) and with 25 cm space between the plots. Plots were sown with a plot-sowing machine (modified Stegsted) at 2-4 cm depth aiming at 400 seeds per m^2. Crop management, including the application of fertilisers, herbicides, PGRs, insecticides, was conducted according to common crop practice, apart from

the different BCA and fungide treatment. The trials was in all 3 seasons irrigated 2-3 times depending on the water balance. In all 3 seasons the trials were irrigated 1 day ahead of ear treatments and fusarium inoculation to ensure good humitiy conditions for infection.

Detailed information on treatments and application timing is given in Tables 4.2.1 and 4.2.2, while information on sowing dates, dates for spraying at GS 37-39 and GS 61-65 and assessment time points are given in Table 4.2.3 and Table 4.2.4, respectively. In all three years, the cultivar Torp was used, known to be very susceptible to FHB (Jørgensen et al., 2021) and moderately susceptible to STB. The chemical products included were Proline EC 250 (prothioconazole 250 g/L, Bayer CropScience AG) and Propulse (prothioconazole 125 g/L+ fluopyram + 125 g/L, Bayer CropScience AG). The BCA products included were *Clonostachus rosea* IK726, Serenade ASO (*Bacillus velezensis* QST713*,* Bayer CropScience AG) and *Pseudomonas chlororaphis* MA342, BioAgri AB / Lantmännen BioAgri. BCAs were included alone or in combination with Proline EC 250. Chemical solutions were also tested as single treatments as well as an untreated control.

TABLE 4.2.1. Treatments applied in the field trial in 2018.

	Product(s)	**Dosage(s)**	**Growth stage**
1	Control	-	-
2	Proline EC 250	0.4 l/ha	37-39
3	Proline EC 250	0.4 l/ha	61-65
4	Proline EC 250	0.4 l/ha	37-39 + 61-65
5	Proline EC 250	0.8 l/ha	37-39 + 61-65
6	Proline EC 250	0.8 l/ha	37-39
7	*C. rosea* IK726	10^7 conidia/m^2	37-39
8	*C. rosea* IK726 + *P. chlororaphis* MA342	10^7 conidia/m^2 + 10^9 cells/m^2	37-39
9	*C. rosea* IK726+ *P. chlororaphis* MA342 + Proline EC 250	10^7 conidia/m^2 + 10^9 cells/m^2 + 0.4 l/ha	37-39
10	*C. rosea* IK726	10^7 conidia/m^2	37-39 + 61-65
11	*P. chlororaphis* MA342	10^9 cells/m^2	37-39 + 61-65
12	*C. rosea* IK726 + *P. chlororaphis* MA342	10^7 conidia/m^2 + 10^9 cells/m^2	37-39 + 61-65
13	*C. rosea* IK726 + *P. chlororaphis* MA342 + Proline EC 250	10^7 sporer/m^2 + 10^9 cells/m^2 + 0.4 l/ha	37-39 + 61-65
14	Propulse SE 250	1.0	37-39 + 61-65

TABLE 4.2.2. Treatments applied in the field trials in 2019 and 2020.

	Product(s)	**Dosage(s)**	**Growth stage**
1	Control	-	-
2	Proline EC 250	0.4 l/ha	37-39
3	Proline EC 250	0.4 l/ha	61-65
4	Proline EC 250	0.4 l/ha	37-39 + 61-65
5	Proline EC 250	0.8 l/ha	37-39 + 61-65
6	*C. rosea* IK726 Proline EC 250	10^7 conidia/m^2 0.4 l/ha	37-39 61-65
7	*C. rosea*	10^7 conidia/m^2	37-39
8	*C. rosea* IK726 + Serenade ASO	10^7 conidia/m^2 + 4 l/ha	37-39
9	*C. rosea* IK726 + Serenade ASO + Proline EC 250	10^7 sporer/m^2 + 4 l/ha + 0.4 l/ha	37-39
10	*C. rosea* IK726	10^7 conidia/m^2	37-39 + 61-65
11	Serenade ASO	4 l /ha	37-39 + 61-65
12	*C. rosea* + Serenade ASO	10^7 conidia/m^2 + 4 l /ha	37-39 + 61-65
13	*C. rosea* IK726+ Serenade ASO + Proline EC 250	10^7 sporer/m^2 + 4 l /ha + 0.4 l/ha	37-39 + 61-65
14a	*C. rosea* IK726[2]	10^7 conidia/m^2	61-65
14	Propulse SE 250	1.0 l/ha	37-39 + 61-65

[1)] Chemical control: Proline EC 250 (prothioconazole 250 g/l, Bayer CropScience AG) and Propulse SE 250 (prothioconazole 125 g/L+ fluopyram 125 g/L, Bayer CropScience AG). BCAs: Bacillus velezensis QST713 (Serenade ASO, Bayer Crop-Science AG) where 4L/ha corresponds to 109 sporer/m2. [2)] Treatment 14a was only applied in 2020.

TABLE 4.2.3. Main dates for foliar application of BCAs and fungicides, *Fusarium* inoculation of flowering wheat heads, disease assessments and grain harvesting in the three field trials.

Year	Sowing date	Application in GS 37-39 (dates)	Application in GS 61-65 (dates)	Fusarium inoculation	Disease assessments	Grain harvest
2018	23-9-2017	23/5, BCA 24/5, Proline EC 250	4/6, BCA 6/6, Proline EC 250	5-6	1/6, 11/6, 21/6, 28/6	28/7
2019	21-9-2018	20/5 BCA 21/5, Proline EC 250	11/6, BCA 12/6,,Proline EC 250	11-6	3/6, 14/6, 25/6, 28/6, 7/7	9/8
2020	24-9-2019	15/5, BCA 16-5, Proline EC 250	15/6, BCA 16/6, Proline EC 250	15-6	1/6, 10/6, 21/6, 1/7, 7/7	14/8

TABLE 4.2.4. Dates for sampling of leaves and ears for investigation of *Z. tritici* fungicide tolerance, CYP51 mutations, fungal community structure analysis and qPCR detection of *Clonostachys rosea*.

Year	Leaves AU[1)]		Leaves SLU[2)]+KU[3)]	Heads KU[3)]
	GS 31	GS 75	GS 61-65	GS 65
2018	May	28/6	8/6, 11/6	18/6, 25/6
2019	May	June	11/6, 17/6	11/6, 17/6
2020	May	21/6	15/6, 21/6	15/6, 21/6

[1)]Aarhus University, [2)]Swedish Agricultural University, [3)]University of Copenhagen

Treatments were initiated between growth stage (GS) 39 and 65 (BBCH, Lancashire et al., 1991). The products were applied with a self-propelled sprayer (Speedy 2500, Strøby Maskin Værksted), operating at a speed of 4.5 km/h and a boom height of 40 cm. The boom was fitted with Teejet 9504 nozzles, operating at a pressure of 2.4 bar and water volume rates varied from 150 to 200 L/ha.

Infection of STB developed naturally in the trials during the three seasons and the severity was low to moderate.

The infection of FHB was initiated artificially by applying a spore suspension with a mixture of *F. culmorum* and *F. graminearum* during flowering. Inoculation took place during flowering (GS 61-65), one day after treatment with fungicides and BCAs. In order to optimize the infection, the inoculum was applied in the evening. A concentration of 100 ml inoculum per plot (22 m^2) with a concentration of 10^4 spores/ml was applied. The inoculum was diluted and applied using a water volume of 200 L/ha. Ahead of inoculation, the crop was irrigated to ensure good humidity conditions in the crop.

Disease assessments were carried out visually as a percentage of Septoria tritici blotch (STB) coverage of green leaves evaluated on specific leaf layers at intervals of ten days, starting at the first application and finishing at senescence, following European Plant Protection Organization standards (EPPO/OEPP (2012) PP 1/26(4)). In each plot, a minimum of 4 x 1 m row was visually scored during the individual assessments. Leaf numbers (Leaf 1, Leaf, leaf 3 and Leaf 4) refer to the leaf layer assessed, where the lowest number indicates the top leaf layer at each growth stage.
Regarding assessments of Fusarium head blight, different scoring methods were used: 1) the number of attacked heads was counted in 4 × 1 m row per plot; 2) average attack of FHB on the assessed heads was scored using a 1-9 scale; 3) visual scoring of percent attacked heads per plot. The trials were harvested using a plot combine harvester (Dronningborg plot harvester). Yields in dt/ha (hkg/ha) were adjusted to 15% moisture. The five most common *Fusarium* mycotoxins (DON, NIV, ZEA, HT2 and T2) were measured from harvested grain

samples. The grain from each replication was milled to a fine powder and toxins quantified using HPLC MS/MS method as described by (Nicolaisen et al., 2009) Sampling of leaves and ears for further detection of *Z. tritici* fungicide tolerance, CYP51 mutations, fungal community structure and qPCR detection of *Clonostachys rosea* are given in Table 4.2.4. Weather data from 2018, 2019 and 2020 are shown in Appendix 1.

4.2.1 Fungicide resistance development in the *Z. tritici* population

Leaf samples with STB symptoms from three field trials were collected at GS 75-77 from specific treatments. In each trial, leaves were picked from each of the four replications (5×10 leaves per replication), kept separate, dried at room temperature and stored until further use. Single pycnidium isolates were produced from leaf samples collected in 2018/2019/2020. The leaves were kept in Petri dishes on moistened filter paper at high humidity for 24 h. With a sterile needle, cirrhus from a single pycnidium was transferred to PDA supplemented with 0.01% streptomycin, before incubation at 20°C in cycles of 12 h white light/12-h darkness for five days. A total of 487 *Z. tritici* isolates were produced in this manner. *Z. tritici* isolates were stored at -20°C. Samling from treatments and number of isolates sampled in each year are shown in Table 4.2.5.

TABLE 4.2.5. Isolates of *Z. tritici* collected and investigated for sensitivity of *Z. tritici* to fungicides.

2018	2019	2020
Treatments 1-14 247 isolates	Treatments 1, 4, 5, 10, 11, 12, 13, 14 160 isolates	Treatments 1, 5, 10, 13 80 isolates
Treatment numbers, see Tables 4.2.1 and 4.2.2 for specification.		

All isolates were *in vitro* tested for sensitivity to the SDHI fluxapyroxad and the azole prothioconazole-desthio (both Sigma-Aldrich, St. Louis, MO, USA) in microtitre in sterile demineralized water. The suspensions were vortexed in 10 ml centrifuge tubes for 10 min and adjusted to 2.5×10^4 spores/ml. Prothioconazole-desthio and fluxapyroxad were mixed with 2×PDB to obtain the following fungicide concentrations (mg/l): 10, 3.33, 1.11, 0.37, 0.12, 0.041, 0.014 and 0. A total of 100 µl spore suspension and 100 µl fungicide solution were added to Nunc™ 96-deep well microtitre plates (ThermoFisher, Roskilde, Denmark). Every isolate was duplicated and the sensitive isolates IPO323 and OP15.1 were used as references for both fungicides. The microtiter plates were then covered in alufoil and incubated in the dark at 20°C for six days. During the six days, plates were visually assessed for bacterial or fungal contamination before the analysis, which was performed in an iMark™ Microplate Absorbance Reader (Bio-Rad, Copenhagen, Denmark) at wavelength 620 nm.
The fungicide sensitivities were calculated as the concentration of fungicide, which inhibited fungal growth by 50% (EC_{50}) by non-linear regression (curve-fit) using GraphPad Prism (GraphPad Software, La Jolla, CA, USA).

4.2.2 Detection of CYP51 mutations from leaf samples

DNA extraction, cyp51 amplification and high-plex PCR

Leaves (20 leaves in each sample) from treatments with four biological replications from the field trails in 2018 and 2019 were sampled at GS61 and GS65 (see Tables 4.2.1 and 4.2.2). Necrotic lesions representing the symptoms caused by *Zymoseptoria tritici* were cut out from the leaves and used for DNA extraction. The samples were freeze dried for one week, ground in liquid nitrogen and DNA was extracted using the DNeasy® Plant Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's recommendations. The quality and concentration of DNA were checked using a NanoDrop™ spectrophotometer (Thermo Scientific, USA). For amplification of the *cyp51* gene, a gene-specific primer pair was used: CYP51 F 5'-GAAACAGCGTGTGTGAGAGC-3' and CYP51 R 5'- CTGCTGTAATCCGTACCCACCAC-3' (Leroux et al. 2007) added with heel sequences CTCTCTATGGGCAGTC and CTCGTGTCTCCGACT to the 5' end of CYP51 F and CYP51 R, respectively (Chaudhary et

al., 2020, Samils et al., 2021). First, a gradient PCR was performed to identify optimum primer annealing temperature and 60°C annealing temperature and 30 cycles were chosen for further runs. PCR was performed in a 2720 Thermal cycler (AB Applied Biosystems, USA) using Phusion Hot Start II High-Fidelity (HF) DNA polymerase in a 20 µl reaction volume using the following temperature cycle: denaturation cycle at 98°C for 30 s, followed by 30 cycles at 98°C for 15 s, annealing at 60°C for 30 s, extension at 72°C for 1 min and a final extension at 72°C for 7 min. The PCR result was checked on a 0.8% (w/v) agarose gel. To ensure primer specificity, amplicons were gel purified using the GeneJET Gel Extraction Kit (Thermo Scientific, USA) and were sequenced with Sanger sequencing. Amplicons were subsequently purified using Sera-Mag (Sigma-Aldrich, USA) following the manufacturer's instruction and the concentration was determined using Qubit (Invitrogen, USA). The samples were diluted to 3 ng/µl in deionised water and were used for tagging by adding indexing primers (Chaudhary et al., 2020, Samils et al., 2021) unique for each sample, using PCR. The PCR mixture (20 µl) consisted of 1X Phusion HF PCR buffer, 2 mM of each dNTP (Deoxynucleoside triphosphate), Phusion Hot Start II HF DNA Polymerase and 3 ng of amplicon template. Indexing primers of 2 µM were added to each PCR reaction mixture. The PCR conditions consisted of an initial denaturation at 98°C for 30 sec, 10 cycles of 98°C for 10 s, 62°C for 20 s and 72°C for 1 min followed by a final extension at 72°C for 5 min. The amplicons were pooled and then purified in two rounds, first with AMPure XP (Beckman Coulter, USA) and then with E.Z.N.A. Cycle Pure Kit (Omega Bio-Tek, USA). The amplicon concentration and size were analysed using a Qubit (Invitrogen, USA) and agarose gel electrophoresis, respectively.

PacBio Sequencing of CYP51 gene

Amplicon sequencing was performed at SciLifeLab, Uppsala (NGI, Sweden) using the PacBio platform (Pacific Biosciences, USA). PacBio employs the SMRT technology and circular consensus sequencing (CCS) to produce long reads.

Variant calling

Initial raw PacBio sub-reads were processed to generate unaligned CCS reads with the SMRT Link v9.0 tool / https://www.pacb.com/products-and-services/analytical-software/smrt-analysis/). Demultiplexing was performed to generate independent sample-based CCS reads using in-house scripts. The CCS reads from all samples were mapped to the selected CYP51 reference sequence with SMRT pbmm2 tool kit. Variant calling (SNP and Indels) was performed on aligned independent samples using GATK4 (McKenna et al., 2010). HaplotypeCaller and multi-sample variant calling was performed using CombineGVCFs and GenotypeGVCFs functions. Structural Variant calling was done on all ccs-aligned reads using SMRT pbsv tool and merged all samples into a multi-sample variant calling VCF file. Annotation of variants was processed with SnpEff v4.3t tool (http://snpeff.sourceforge.net/SnpEff.html) to predict protein changes and their impacts. For visualization of variants IGV desktop software (https://software.broadinstitute.org/software/igv/) was used.

4.2.3 Effects of BCAs on fungal communities on leaves

Wheat leaves were sampled at two time points (GS 61 and GS 65) in the field trial during the year 2018 and 2019 and were stored at -20°C until DNA extraction. In total, 64 samples (eight treatments with four biological replications) from each year were used for the study (Table 4.2.6).

TABLE 4.2.6. Selected treatments for microbial community analyses of wheat leaves sampled at GS 61 and GS 65, respectively.

Field trial 2018	Treatment number 1, 5, 7, 10, 11, 12 and 13 (see specifications in Tables 4.2.1)
Field trial 2019	Treatment number 1, 5, 7, 10, 11, 12 and 13 (See specifications in Tables 4.2.2)

DNA extraction. PCR amplification and PacBio sequencing

Wheat leaf samples (pools of 20 leaves per biological replications) were freeze dried for five days, ground in liquid nitrogen and DNA was extracted using the DNeasy® Plant Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's recommendations.

The ITS2 (internal transcribed spacer 2) region, which mainly amplify the basidiomycete and ascomycete phyla, was used as target for amplicon sequencing in this study (Karlsson et al., 2014, Karlsson et al., 2017, Castaño et al., 2020). The ITS region was amplified using the forward primer fITS7 (GTGARTCATCGAATCTTTG; (Ihrmark et al., 2012)) and the reverse primer ITS4 (TCCTCCGCTTATTGATATGC) (White et al., 1990). The ITS4 primer was tagged with an 8 bp barcode. PCR was run in 25-µl reaction mix containing 25 ng template, 200 µM of each dNTPS, 2.75 mM MgCl2, 500 nM fITS7, 300 nM ITS4 and 0.02 U/µl polymerase (DreamTaq Green, Thermo Scientific, MA, USA) in PCR buffer. Amplification was conducted in a 2720 Thermal cycler (AB Applied Biosystems, USA) using 94°C for 5 min, followed by 28-32 cycles at 94°C for 30 s, 57°C for 30 s, 72°C for 30 s and a final elongation step at 72°C for 7 min. PCR products were analysed by electrophoresis in a 1.5% (v/w) agarose gel. The number of PCR cycles was optimized for each sample to produce similar intensity bands on the agarose gel and was adopted with approximately the same strength for all samples to avoid oversaturation and distortion of the PCR pool. PCR products were purified using AMPure (Beckman Coulter, CA, USA) following the manufacturer's instructions. DNA concentration was measured on a Qubit Fluorometer (Thermo Fisher Scientific, MA, USA) using Qubit dsDNA assay Kit (Thermo Fisher Scientific, MA, USA) according to manufacturer's instructions. Purified PCR amplicons were pooled in equimolar amounts and concentrated by passing through the E.Z.N.A. Cycle Pure Kit (Omega Bio-Tek, USA). The quality of concentrated DNA sample was analysed using an Agilent 2100 Bioanalyzer system (Agilent Technology, Santa Clara, CA, USA). The sample pool was sent to SciLifeLab, Uppsala (NGI, Sweden) for library preparation and PacBio sequencing. The libraries were prepared using the SMRTbell Template Prep Kit 1.0 and sequenced on PacBio RS II SMRT cells (Pacific Biosciences, Menlo Park, CA, USA) using one SMRT cell.

Bioinformatics, taxonomic assignment and statistical analysis

The raw sequence data were analysed using the SCATA pipeline (http://scata.mykopat.slu.se) using the parameters described previously by Castaño et al. (2020). In brief, sequences were screened for tags (100% match) and primer sequences (requiring 90% match). Sequences shorter than 100 bases or with a missing 3' or 5' tag were discarded. For PacBio datasets, sequence quality scores (ranging between 0 and 40) indicate the probability of each base to be correctly called. Sequences with a mean amplicon quality score lower than 20 or with a score of lower than 7 at any position were also discarded. The sequences passing the quality control were clustered into operational taxonomic units (OTUs) using PROTAX with 50% probability (Somervuo et al., 2016, Somervuo et al., 2017). For statistical computing, the VEGAN package (Oksanen et al., 2015) in the R software environment (v.2.15.3; R Development Core Team, 2015) was used. QIIME2 was used to perform rarefaction (sampling depth 1,058), alpha diversity and beta diversity analyses (Bolyen & Rideout, 2019). ALDEX2 was used for differential abundance analysis.

4.2.4 qPCR detection of C. rosea in leaf and head samples

Analyses of the persistence of *C. rosea* under field conditions were conducted for leaf and head samples from plots sprayed with *C. rosea* alone or different combinations with Proline EC 250 and Serenade ASO. Samples were taken on the day of spraying (GS61; late spray) and six days later, respectively. Twenty leaves from each sample were cut in 2 cm pieces and transferred to paper bags and freeze dried for 6 days. Then samples were ground using zirconium oxide grinding balls (3 balls Ø 15 mm) on a 'SO-40a' shaker (Fluid Management Inc., Wheeling, IL, USA) until pulverised. Ground samples were stored at -20°C until use. The head samples were prepared as follows: 10 heads from each sample were selected and bracts and

kernel tissue were separated for each of the 10 heads. The bracts were ground in liquid nitrogen and samples were stored at -20°C.

For both leaf and head material, the DNA was extracted using the E.Z.N.A. Plant DNA Kit Plant (Omega Bio-tek, Norcross, GA, USA). Concentration and purity of DNA were measured by NanoDrop-1000 spectrophotometer (NanoDrop Technologies, Wilmington, USA). The specific amplification of *Clonostachys rosea* with TaqMan qPCR was performed using primers VTTact-F 5´ GGCCAGAGATTGTGTTGATGA 3´ and VTTact-R 5´ ACAGGTTAGGCTCAATGCTC 3´ and the hydrolysis probe VTTact probe 5´GAGGCTGGCAAGAGAGGTCAGTCAC 3' (Gimeno et al., 2019). All qPCR reactions were performed using an AriaMx Real-Time PCR System G8820A (Agilent Technologies, Santa Clara, CA, United States) in white Multiwell 96-well plates sealed with adhesive foil. The 15 µl volume reactions, containing 2×Prime Time Gene Expression MM (Integrated DNA Technologies, Inc), 6 pmol of each primer, 2 pmol of the hydrolysis probe and 2 µl template. The PCR programme consisted of pre-incubation for 10 min at 95°C, followed by 40 cycles of denaturation for 10 s at 95°C, annealing for 30 s at 62°C and extension for 60 s at 72°C, including signal detection. The run was finalised with a cooling period of 10 min at 40°C. For the standard curve, DNA was extracted from a pure culture of *C. rosea* IK726 and prepared in tenfold dilutions over a range of 0.001 ng DNA to 10 ng. Absolute quantification of *C. rosea* was achieved by comparing Ct values of test samples to a standard curve. A linear regression analysis of the data generated an equation, which was used to derive the *C. rosea* quantity of the test samples.

4.3 STB control in growth chamber experiments

The wheat cultivar Sevin Sejet was used for the bioassays as it is known to be highly susceptible to *Z. tritici* (Shetty et al., 2003, 2007, 2009). Seeds were sown in rectangular pots filled with sphagnum peat soil (Pindstrup substrate no. 2, Pindstrup Mosebrug A/S, Denmark), with at least 12 seeds in each pot.

The pots were placed in a growth chamber. Periods of 16 h light were supplied by fluorescent tubes (Philips Master IL-D 36 w/865, France, 200 $\mu Em^{-2}s^{-1}$) and the remaining 8 h per 24h cycle were in darkness. The daytime temperature was approximately 19°C with an RH of 50-60%, whereas night temperature was around 16°C with a RH of 80-90%. At 14 days after sowing, the second-developed leaf of 12 plants per pot were fixed to a bent Plexiglas plate with unbleached cotton strings, placing the leaves in a horizontal position (Figure 4.3.1). Watering of the plants took place every second day.

FIGURE 4.3.1. Setup for testing the effect of *Clonostachys rosea* on STB in growth chamber experiments.

Inoculum of biocontrol agents and subsequently *Z. tritici* was sprayed onto the fixed leaves until run-off, using a glass hand sprayer. The inoculated plants were sealed in plastic bags to secure 100% RH and subsequently placed in the dark in the same growth chamber as before. After 72 h, the bags were opened and light applied again.

Fungicide application

C. rosea treatments were combined with fungicide treatments prior to inoculation with *Z. tritici* in three experiments. The fungicide studied was Proline EC 250 in concentrations of 1 ppm (one experiment) and 3 ppm (two experiments). *C. rosea* inoculation took place at 4 days prior to inoculation with *Z. tritici.* Proline EC 250 was applied at 1 day prior to *Z. tritici* inoculation, using a paint gun (Varper Gravity Feed H.V.L.P. Touch Up Spray Gun with 1.0 mm nozzle, Star Asia-USA, Renton, Washington, USA).

Assessment of *Z. tritici* infection

Symptom expression (chlorosis and necrosis) of *Z. tritici* were studied at regular intervals after inoculation, from the time symptoms started to appear (usually from 10 days after inoculation, dai). Images of each pot were recorded until almost all leaves died. The images were analysed by Assess 2.0 Image Analysis Software for Plant Disease Quantification (The American Phytopathological Society, St. Paul Minnesota, USA), which calculates the percentage of diseased leaf area as a percentage of all leaves in a single pot.

Microscopy of the infection biology of *Z. tritici*

The infection biology of *Z. tritici* was studied by microscopy in an attempt to disclose by which mechanisms *C. rosea* caused inhibition of *Z. tritici*. The procedure was as described previously (Shetty et al., 2003, Shetty et al., 2007). Wheat leaves were inoculated with *C. rosea* or sprayed with water at 1 dai prior to *Z. tritici*. Thus, the experiment comprised two treatments: Water+*Z. tritici* and *C. rosea*+*Z. tritici*. Leaves were harvested at 3, 7, 11 and 15 dai. Four leaves were harvested at each sampling time for each treatment (one leaf from each of four pots).

The leaves were cleared on paper napkins saturated with a mixture of absolute ethanol and glacial acetic acid (3:1, v/v). When all chlorophyll was extracted from the leaves, they were transferred to a napkin saturated with water for about 30-60 min and subsequently transferred to napkins saturated with lactoglycerol (1:1:1 [v/v] mixture of lactic acid 85% glycerol : water) where they were kept until microscopy. Before the leaves were examined in the microscope, they were stained with 0.1% Evans blue in lactoglycerol for visualisation of fungal structures.

At 3 and 7 dai, 100 randomly chosen germinated spores were studied on each of the four leaves. For each germinating spore, it was recorded whether it caused penetration of stomata. Furthermore, the number of non-germinated spores were recorded.

At 11 and 15 dai, the total number of stomata was counted in 25 fields of vision per leaf (20× magnification). For each leaf, it was recorded whether each stoma was empty, if there was hyphal aggregation, pycnidial initials or fully developed pycnidia.

4.4 Fusarium head blight control and DON accumulation in greenhouse experiments

Greenhouse trials were performed to evaluate the efficiency of the BCAs to colonise wheat spikes and decrease FHB and DON accumulation *in planta*. Seeds of spring wheat cv. Diskett (Lantmännen SW Seeds AB) were sown in 2-l pots and plants were grown under greenhouse conditions until heading. Freezer stocks of the two BCAs suspensions and of *Fusarium graminearum* isolate WC-091-7 were adjusted with sterile MilliQ + 0.05% Tween 20 (v/v) (Sigma-Aldrich®) to 10^7 cells or spores/ml and 10^5 conidia/ml, respectively. At heading stage (BBCH 59), wheat heads were sprayed with *Clonostachys rosea* strain IK726 (3 experiments) or *Streptomyces griseoviridis* strain K61 (1 experiment). Approximately 1 ml of spores or cells suspension (10^7 cells or spores/ml) was applied for each individual head. Before the BCA treatments, spikes were pre-moistened by spraying all heads with sterile MilliQ water + 0.05% Tween (v/v).

At full flowering stage (BBCH 65), spikes were treated with a suspension of *F. graminearum* macroconidia (10^5 conidia/ml), 1 ml per individual spike. Autoclaved MilliQ water + 0.05% Tween 20 (v/v) (Sigma-Aldrich®) was applied as a water control treatment. All the treated

spikes were covered with pre-moistened plastic bags and sealed with adhesive tape. After 48 h, bags were removed. Severity of FHB was assessed at 5 days after inoculation with *F. graminearum* by counting the number of infected spikelets out of the total number of spikelets of each spike. At least 5 or 6 replications were included per treatment in each experiment.

DON quantification in spikes from greenhouse experiment

Wheat spikes were harvested at 5 days after *F. graminearum* infection. Plant material was finely ground with pestle and mortar in liquid nitrogen. The extraction procedure was performed as described by (Huang et al., 2019) using 15 ml centrifuge tubes, 0.6 g of frozen plant material were transferred and 4 ml ACN: water (85:15 v/v) were added. Tubes were mixed by vortexing for 30 sec and shaken horizontally at 200 rpm on a rotary shaker for one h. After this time, samples were left at room temperature for 20 min to settle the plant material. The supernatant was removed with a 1-ml syringe (Chirana®) and filtered through a 0.2 µm pore size filter (Advantec®). Samples were stored at -20°C until HPLC-MS/MS analysis.
Deoxynivalenol (Cat. Number 32943), 15-acetyldeoxynivalenol (Cat. Number 34133) and 3-acetyldeoxynivalenol (Cat. Number 32927) were purchased from Sigma-Aldrich. DON powder was dissolved in methanol and a 1000 ppm stock solution was prepared and stored in amber vials at +4°C. Mycotoxins were determined with a Waters Acquity UPLC equipped with a 2.1 × 50 mm BEH C18 column, particle size 1.7 µm (Waters, Milford, USA) and column temperature set at 40°C. The LC mobile phase was: A) water + 0.1% FA and B) acetonitrile + 0.1% FA. Gradient conditions were 0-3 min 10% B, 7 min 40% B, 7.10 min 90% B, 7.10-9 min 90% B. The column was equilibrated for 2 min before each run and the total run time was 11 min. Flow rate was 0.40 ml/min and injection volume was 5 µl. Detection was performed with a Waters Xevo TQD triple quadrupole mass spectrometer and electrospray ionization in positive ion mode. The exact mass of DON was m/z 296.12, 338.13 for 3ADON m/z and 338.13 for 15ADON m/z. The source temperature was 150°C, capillary voltage of 3.4 kV, cone voltage 20 V, desolvation temperature 400°C, desolvation gas flow 800 l/h and cone gas flow 20 l/h. Data processing was performed with MassLynx 4.0.2.3 (Waters, Milford, USA).

***Fusarium graminearum* biomass quantification in treated wheat spikes**

Wheat spikes from the whole-plant assays were harvested at 5 days after *Fusarium* infection. Each spike was finely ground with a pestle and mortar whilst continuously applying liquid nitrogen. DNA was extracted using the E.Z.N.A. Plant DNA Kit Plant (Omega Bio-tek, Norcross, GA, USA). Concentration and purity of DNA was measured on a NanoDrop-1000 spectrophotometer (NanoDrop Technologies, Wilmington, USA).
Primers for detecting the *Tri6* gene in *F. graminearum* in wheat samples were as described by Horevaj et al. (2011): Tri-10F 5′-TCTTTGTGAGCGGACGGGACTTTA-3′ and Tri6-4R 5′-ATCTCGCATGTTATCCACCCTGCT-3′. For plant DNA, primers for detecting the elongation factor gene *EF1*α in *Tritichum aestivum* were as reported by (Nicolaisen et al. (2009)): EF1α-F 5′-TCTCTGGGTTTGAGGGTGAC-3′ and EF1α-R 5′-GGCCCTTGTACCAGTCAAGGT-3′. Primer stock solutions (100 pmol/µl) were diluted to 4 pmol/µl. Final concentration for each primer in the wells was 200 nM. Reactions for the qPCR were prepared in a final volumes of 20 µl. Individual master mix was prepared for each set of primers, *Tri6* and *EF1*α, respectively. Template DNA concentration was adjusted with sterile MilliQ water to 100 ng/µl to achieve 5 ng/µl in the final reaction volume. Non-template controls (NTC) were included for each primer sets by addition of sterile MilliQ water in place of template DNA. All reactions were prepared in duplicates. Standard curves were established for each set of primers. For the specific primers of the pathogen, DNA was obtained from a pure culture of *F. graminearum*. DNA was extracted using DNeasy Plant Mini Kit (Qiagen, Hilden, Germany) and concentration was measured by NanoDrop-1000 spectrophotometer (NanoDrop Technologies, Wilmington, USA). Wheat reference DNA was extracted from untreated spikes. Ten-fold serial dilutions were performed from an initial DNA concentration of 50 ng, for both *Fusarium* and wheat DNA. In total, 7 concentrations were included: 50 ng/µl, 5 ng/µl, 0.5 ng/µl 0.05 ng/µl, 0.005 ng/µl, 0.0005 ng/µl and

0.00005 ng/µl. All PCR reactions were run using the AriaMx Real-Time PCR System G8820A (Agilent Technologies, Santa Clara, CA, USA).
Primer efficiencies were 90-110%. Cq values from the *Fusarium* standard curve were plotted against DNA concentration to obtain a slope coefficient applied for determining *Fusarium* DNA concentration in greenhouse samples, as described previously (Colombo et al., 2020). The same procedure was applied for calculating the content of wheat DNA in each sample. Subsequently *Fusarium* DNA was normalised to *T. aestivum* DNA for each sample.

4.5 Fungicide tolerance test of BCAs *in vitro*

The spore assays were performed on Czapek Dox agar (50g/l Sigma). A stock solution, 4000 ppm Prothioconazole-desthio (CAS number 120983-64-4), was prepared in 80% ethanol and stored at 4ºC. 3-fold dilutions of the Prothioconazole-desthio stock was added to the liquid agar 55ºC to obtain plates with 3 ppm, 1 ppm, 0,333 ppm, 0.111 ppm, 0,037 ppm, 0,012 ppm, 0,004 ppm. Czapek Dox agar without the fungicide served as control. Approximately 500 BCA spores were added to each plate by inoculating 10 µl water suspension (5×10^4 spores/ml) to the centre of the Petri dish with three replications per fungicide concentration. The Petri dishes were incubated in a growth chamber (conditions described in section 3.3). Germination of conidia was observed for 100 spores per replication (300 spores per concentration) after 1, 2 and 3 days of incubation, by placing the dish directly under a light microscope at x40 magnification. A spore was considered germinated if the length of the germ tube was longer than the length of the spore, while at day 2 and day 3, a spore with further hyphal growth were noted as an active spore. The experiment was conducted twice with *C. rosea* and furthermore, fungicide tolerance of two fungal strains isolated from apparently healthy wheat leaves were also tested i.e. *Penicillium olsonii* strain ML37 and *Acremonium* train ML38 (Latz et al., 2020).
The effect of Prothiozonazole-desthio on colony development was determined in two ways. Colony growth from spore suspensions was determined for IK726 and ML38 by measuring colony diameter after 2, 3, 4 and 9 days of incubation (conditions described in section 3.3). Colony growth from the mycelial plugs where no sporulation was observed was determined after 4 and 9 days of incubation. The inhibition of *Z. tritici* spore germination and activity was also tested using the set-up described above. This test included the prothioconazole sensitive isolate IPO323 and the field isolate ZT07.

4.6 Statistical analysis

Data from growth chamber experiments on *Z. tritici*, microscopy, greenhouse experiments on *F. graminearum* and field experiments were analysed using the statistical software package SAS 9.4 (SAS Institute, United States, North Carolina). Hypotheses were rejected at $P< 0.05$.
Growth chamber and greenhouse experiments: Data from the bioassays on disease reductions represent a continuous variable and were analysed by analysis of variance assuming a normal distribution. Variances were stabilised by appropriate transformation of data if necessary. Data from the microscopy of the infection biology of *Z. tritici:*represent discrete variables and were assumed to follow a binomial distribution since it was recorded whether an event occurred (e.g. if a *Z. tritici* spore caused penetration or not). Therefore, data were analysed by logistic regression. Any overdispersion was corrected by Pearson-scaling. *Fusarium* biomass quantification of grains was conducted using the amount of the *Fusarium* DNA normalized the wheat DNA of each sample. The normalized DNA values were analysed by analysis of variance, asuming normal distribution. Variance was stabilised by appropriate transformation of data if necessary.
Field experiments: Data from the field represent continuous variables and were analysed by analysis of variance as a completely randomised block experiment with four blocks, assuming a normal distribution. Variances were stabilised by appropriate transformation of data, if necessary. In appendix 2 any transformation used is indicated in the six supplementary data tables (Supplementary data table 1 to Supplementary data table 6). Bonferroni adjustments were applied to reduce the instance of false positives, declaring treatments incorrectly statistically significant.

5. Results

5.1 Disease development, DON content in grains and grain yield

Several combinations of two different BCAs (*C. rosea* and *P. chlororaphis* in 2018 and *C. rosea* and B. *velezensis* (Serenade ASO) in 2019 and 2020) were applied with and without reduced dosage of Proline EC 250 at two different spray time-points and some BCAs were applied alone once or twice. However, it was not possible to include all appropriate controls because all combinations of the variables would result in a very high number of treatments. Thus, due to both technical/statistical and economic constraints, this approach was not possible and therefore a subset of combinations were tested. The selection of treatments was focusing on testing effects of single applications of *C. rosea*, the combination of two BCAs with and without Proline EC 250 applied once or twice as well as on comparison of the BCAs with a reduced dose of Proline EC 250 (0.4 L/ha) applied once or twice or the full dose of Proline EC 250 (0.8 L/ha). The trials in all three seasons also included double treatments with 1.0 l Propulse SE 250 (in total 2 L/ha) as a control expected to provide the most efficient fungicide treatment of all. However, it should be noted that the maximum Propulse SE 250 rate permitted per season is only 1 L/ha. The Propulse SE 250 treatment was not included in the more specific analysis in Section 5.2.

Diseases were scored and named in tables as follows: Percentage STB coverage of green leaves (STB pct). Fusarium head blight was scored by three methods i) the number of attacked heads counted in 4 x 1m row per plot (Fusarium number), ii) average attack of FHB on the assessed heads was scored using a 1-9 scale (Fusarium scale), and iii) visual score of percent attacked heads per plot (Fusarium pct). In the following result part, spraying with BCAs and fungicides at GS 37-39 is also referred to as 'early' application whereas spraying at GS 61-65 is referred to as 'late' application. Results including all data and statistical analyses are available in tables in appendix 2 (Supplementary data table 1-3) whereas only data where statistical significant difference between treatments were found ($P<0.05$) are reported in the tables below (Table 5.1.1, Table 5.1.2 and Table 5.1.3).

Field trial 2018

The growth season of 2017/2018 was quite unusual. The autumn of 2017 was very wet and made sowing difficult. On the other hand, the summer of 2018 was very hot, dry and sunny and only in August (at the time of harvest) rain started again. Collectively, this weather meant that water-dispersed diseases like STB and FHB only became of little general significance. However, artificial irrigation of the trial meant that STB still reached 31% on the lowest leaf (leaf 3) on 21st June (Table 5.1.1), while attack stayed low to moderate at the upper leaves. The BCAs included were *C. rosea* and *P. chlororaphis,*

On 11th June, the STB severity (percent STB coverage of leaf area) on leaf 2 and 3 in the untreated control was 1.6% and 18.8%, respectively. On leaf 2, all treatments reduced STB significantly compared to the untreated control. In contrast, on leaf 3, the treatments where Proline EC 250 was applied at 0.4 or 0.8 L/ha in GS 37-39, the Propulse SE 250 treatment as well as the two combination treatments (*C. rosea* + *P. chlororaphis* + Proline EC 250 (0.4 L/ha) applied at GS 37-39 or both at GS 37-39 and GS 61-65 significantly reduced STB compared to the untreated control (62%-79% reduction). Surprisingly, for example Proline EC 250 0.4 L/ha and 0.8 L/ha applied at both GS 37-39 and GS 61-65 did not reduce STB significantly.

On 21st June, there was a considerable STB attack on leaf 2 and 3 and all 13 treatments reduced STB significantly. On leaf 2 (11.9% STB in the untreated control) the single BCA treatments reduced STB by 61%-68% whereas Proline EC 250 treatments alone or in combination with BCA(s) reduced STB by 76-92%. The pattern was the same on leaf 3 (31.9% STB in the untreated control) where the single BCA treatments reduced STB by 52-60% whereas Proline EC 250 treatments alone or in combination with BCAs reduced STB by 60-80%. The Propulse SE 250 treatment had the lowest STB severity at both assessment dates, but there was no statistically significant difference in STB level between BCAs alone, Proline EC 250 and Propulse SE 250.

Yellow rust (5% in the untreated control and brown rust (15% in the untreated control) was assessed on 21st June (Table 5.1.1). For yellow rust, all treatments reduced the attack except for *C. rosea* applied once in GS 37-39, the double treatment with *P. chlororaphis* and the double treatment with *C. rosea* + *P. chlororaphis*) applied twice. Brown rust was reduced significantly by all treatments except the two combination treatments (*C. rosea* + *P. chlororaphis*) applied either once or twice. Single treatments with *C. rosea*, applied either once or twice, reduced brown rust by 95-100%, double application of *P. chlororaphis* with 91% and the various fungicide treatments reduced the rust severity by 91-97%.

The *Fusarium* infection level (5.5 % in the untreated control on 28th June) was not significantly affected by any of the treatments and therefore the levels of mycotoxins was found too low for quantification. Furthermore, the yield was not significantly increased by any treatment either (Table 5.1.1).

Comparison of full Proline EC 250 dose (0.8 L/ha) sprayed either late or both early and late with the Propulse SE 250 treatment (1.0 L/ha) sprayed both early and late revealed no significant difference in STB control efficacy during the season even though the disease scores generally were lowest for the Propulse SE 250 treatment.

TABLE 5.1.1. Winter wheat field trial in 2018 at Research Center Flakkebjerg. The effects of the BCAs *Clonostachys rosea* and *Pseudomonas chlororaphis* on Septoria tritici blotch (STB), Fusarium head blight (FHB), yellow rust and brown rust were tested. BCAs were compared with Proline EC 250 in reduced dose (0.4 L/ha) and full dose (0.8 L/ha). Combinations of BCAs and Proline EC 250 (0.4 L/ha) were also tested.Treatment were applied in growth stage (GS) 37-39 and/or GS 61-65. Propulse SE 250[1)] (1.0 L/ha) applied twice was included as fungicide control.

Treatment	Dose	Growth stage[2)]	STB percent leaf 3 11th June		STB percent leaf 2 11th June		STB percent leaf 3 21st June		STB percent leaf 2 21st June	
Untreated		-	18.8	A	1.6	A	31.3	A	11.9	A
Proline EC 250	0.4	37-39	5.0	BCD	0.3	B	12.5	B	2.9	B
Proline EC 250	0.4	61-65	10.0	ABCD	0.4	B	11.9	B	2.8	B
Proline EC 250 Proline EC 250	0.4 0.4	37-39 61-65	17.5	ABCD	0.9	AB	12.5	B	2.8	B
Proline EC 250 Proline EC 250	0.8 0.8	37-39 61-65	11.3	ABCD	0.2	B	6.3	B	0.9	B
Proline EC 250	0.8	37-39	4.4	CD	0.1	B	12.5	B	2.1	B
C. rosea	1.0	37-39	12.5	ABC	0.4	B	14.4	B	4.0	B
C. rosea *P. chlororaphis*	1.0 1.0	37-39 37-39	13.8	ABC	0.4	B	13.8	B	3.3	B
Proline EC 250 *C. rosea* *P. chlororaphis*	0.4 1.0 1.0	37-39 37-39 37-39	6.9	BCD	0.2	B	7.0	B	0.9	B
C. rosea *C. rosea*	1.0 1.0	37-39 61-65	11.3	ABCD	0.5	B	12.5	B	3.9	B
P. chlororaphis *P. chlororaphis*	1.0 1.0	37-39 61-65	12.5	ABC	0.7	B	15.0	B	3.4	B
C. rosea *P. chlororaphis* *C. rosea* *P. chlororaphis*	1.0 1.0 1.0 1.0	37-39 37-39 61-65 61-65	8.8	ABCD	0.4	B	12.5	B	4.1	B
C. rosea *P. chlororaphis* Proline EC 250 *C. rosea* *P.chlororaphis* Pro-line EC 250	1.0 4.0 0.4 1.0 4.0 0.4	37-39 37-39 37-39 61-65 61-65 61-65	5.0	CD	0.2	B	10.6	B	3.0	B
Propulse SE 250 Propulse SE 250	1.0 1.0	37-39 61-65	3.8	D	0.1	B	10.6	B	0.6	B
P-value			<.0001		<.0001		<.0001		<.0001	

[1]Propulse SE 250 expected to be the most efficient fungicide treatment of all but it should be noted that the maximum rate permitted per season is only 1 L/ha, [2] BCAs applied 23/5 and 4/6 and fungicides applied 16/5 and 6/6. See supplementary data table 1 for information on statistical analyses.

TABLE 5.1.1 (continued). Winter wheat field trial in 2018 at Research Center Flakkebjerg. The effects of the BCAs *Clonostachys rosea* and *Pseudomonas chlororaphis* on Septoria tritici blotch (STB), Fusarium head blight (FHB), yellow rust and brown rust were tested. BCAs were compared with Proline EC 250 in reduced dose (0.4 L/ha) and full dose (0.8 L/ha). Combinations of BCAs and Proline EC 250 (0.4 L/ha) were also tested.Treatment were applied in growth stage (GS) 37-39 and/or GS 61-65. Propulse SE 250[1)] (1.0 L/ha) applied twice was included as fungicide control.

Treatment	Dose (L/ha)	Growth stage[2)]	STB percent leaf 1 21st June		Yellow rust whole plant 21st June		Brown rust whole plant 21st June		Yield Hkg/ha	
Untreated		-	2.3	A	5.0	A	15.0	A	83.9	A
Proline EC 250	0.4	37-39	0.5	B	0.0	D	0.4	B	83.1	A
Proline EC 250	0.4	61-65	0.4	B	0.4	CD	1.0	B	79.3	A
Proline EC 250 Proline EC 250	0.4 0.4	37-39 61-65	0.3	B	0.5	BCD	1.3	B	84.1	A
Proline EC 250 Proline EC 250	0.8 0.8	37-39 61-65	0.1	B	0.1	D	0.0[2]	B	84.4	A
Proline EC 250	0.8	37-39	0.3	B	0.0[1]	D	0.3	B	80.7	A
C. rosea	1.0	37-39	0.8	B	5.5	A	0.8	B	76.8	A
C. rosea *P. chlororaphis*	1.0 1.0	37-39 37-39	0.5	B	0.3	CD	2.8	AB	80.0	A
C. rosea *P. chlororaphis* Proline EC 250	1.0 1.0 0.4	37-39 37-39 37-39	0.2	B	0.1	D	0.1	B	81.7	A
C. rosea *C. rosea*	1.0 1.0	37-39 61-65	0.3	B	0.0	D	0.0*	B	81.2	A
P. chlororaphis *P. chlororaphis*	1.0 1.0	37-39 61-65	0.6	AB	1.0	ABC	1.3	B	79.7	A
C. rosea *P. chlororaphis* *C. rosea* *P. chlororaphis*	1.0 1.0 1.0 1.0	37-39 37-39 61-65 61-65	0.5	AB	6.0	A	1.8	AB	78.7	A
Proline EC 250 *C. rosea* *P. chlororaphis* Proline EC 250 *C. rosea* *P. chlororaphis*	1.0 4.0 0.4 1.0 4.0 0.4	37-39 37-39 37-39 61-65 61-65 61-65	0.3	B	0.3	CD	0.3	B	79.7	A
Propulse SE 250 Propulse SE 250	1.0 1.0	37-39 61-65	0	B	0.3	CD	0.4	B	88.0	A
P-value			<.0001		<.0001		<.0001		0.0.0821	

[1]Propulse SE 250 expected to be the most efficient fungicide treatment of all but it should be noted that the maximum rate permitted per season is only 1 L/ha, [2] BCAs applied 23/5 and 4/6 and fungicides applied 16/5 and 6/6. See supplementary data table 1 for information on statistical analyses.

Field trial in 2019

The weather in the season 2018/2019 can be categorized as normal, providing sufficient and average levels of precipitation and temperatures (see Appendix 1) and this resulted in considerable STB and FHB attacks during the growth season. The experimental plan was adjusted as compared with the 2018 plan. Treatments with the BCA product Serenade ASO replaced the *P. chlororaphis* treatments. Furthermore, the combination treatment '*C. rosea* at GS 37-39 + Proline EC 250 0.4 L/ha at GS 61-65' was included whereas the treatment 'Proline EC 250 at 0.8 L/ha at GS 61-65' was omitted from the 2019 trial (Table 5.1.2).

On 3rd June, STB level on leaf 1 was rather high (27.5% in the untreated control) and the only treatment reducing STB compared to the control was the triple combination treatment '*C. rosea* + Serenade ASO + Proline EC 250' applied in GS 37-39, resulting in 44% STB reduction (Table 5.1.2). There was a low attack of powdery mildew on leaf 1-4 (9% in untreated control) that was only significantly reduced by the combination treatment of '*C. rosea* at GS 37-39 + Proline EC 250 at 0.4 L/ha in GS 61-65' (61% reduction) and the Propulse SE 250 treatment (59% reduction).

On June14th, STB was reduced significantly by all treatments on leaf 1 (2.8 % in untreated control) except for the combination of *C. rosea* +Serenade ASO applied in GS 37-39 and GS 61-65. The same situation was seen for leaf 2 (17.0 % in untreated control). The Proline EC 250 treatment with reduced dose applied once or twice reduced STB by 46-58% whereas the best BCA treatment (*C. rosea* applied at GS 37-39) reduced STB by 66% (Table 5.1.2).

On June 25th STB on leaf 1 (13% in untreated control), only the treatments Proline EC 250 at 0.4 and 0.8 L/ha (applied twice at GS 37-39 and GS 61-65) and Propulse SE 250 at 2×1.0 L/ha reduced STB by 69, 66 and 85%, respectively. Leaf 2 was severely attacked by STB (47.5% in untreated control). Here, the single BCA treatments '*C. rosea* at GS 37-39' (42% reduction) and '*C. rosea* + Serenade ASO at GS 37-39' (44 % reduction) as well as all four pure fungicide treatments with Proline (40-50% reduction) and Propulse (76% reduction) reduced STB significantly (Table 5.1.2).

FHB was assessed on 28th June. All treatments significantly reduced number of *Fusarium* infected plants (50% reduction) except Serenade ASO sprayed twice (Table 5.1.2). Scoring *Fusarium* according to a 1-9 scale showed that all treatments treated with Proline EC 250 spray at GS 61-65 significantly reduced FHB (54-74%) as did Propulse SE 250 (74% reduction). Surprisingly, the pure BCA treatments '*C. rosea*' and '*C. rosea*+Serenade ASO' both sprayed once at GS 37-39 also reduced FHB significantly (44% reduction). The percentage of *Fusarium* infected plants on 7th July was significantly reduced in all treatment where Proline EC 250 was sprayed both at GS 37-39 and at GS 61-65, whether alone or in combination with BCAs (Table 5.1.2).

In the 2019 trial, an overall comparison of full dose of Proline EC 250 (2×0.8 L/ha) and Propulse SE 250 (2×1.0 L/ha), both sprayed at GS 37-39 and GS 61-65, revealed no significant difference in disease control efficacies of STB and FHB over the season even though the disease scores generally were lowest for the Propulse SE 250 treatment.
Yield (74.4 hkg in untreated control) was only significantly increased where Proline EC 250 (0.4 L/ha or 0.8 L/ha) was sprayed twice and by Propulse SE 250. These treatments resulted in 15%, 22% and 30% yield increase, respectively. On the other hand, it was only 'Proline EC 250 at 0.8 L/ha' applied twice that reduced the content of DON significantly as compared to the control (80% reduction). The levels of NIV and ZEA was unaffected by all treatments (Table 5.1.2).

TABLE 5.1.2. Winter wheat field trial in 2019 at Research Center Flakkebjerg. The effects of the BCAs *Clonostachys rosea* and *B. velezensis* (Serenade ASO) on Septoria tritici blotch (STB), Fusarium head blight (FHB) and powdery mildew were tested. BCAs were compared with Proline EC 250 in reduced dose (0.4 L/ha) and full dose (0.8 L/ha). Combinations of BCAs and Proline EC 250 (0.4 L/ha) were also tested.Treatment were applied in growth stage (GS) 37-39 and/or GS 61-65. Propulse SE 250[1)] (1.0 L/ha) applied twice was included as fungicide control.

Treatment	Dose (L/ha)	Growth stage[2)]	STB percent Leaf 1 3rd June		Powdery mildew leaf 1-4 3rd June		STB percent leaf 1 14th June		STB percent leaf 2 14th June		STB percent leaf 1 25th June		STB percent leaf 2 25th June	
Untreated		-	27.5	A	9.0	A	2.8	A	17.0	A	13.0	A	47.5	A
Proline EC 250	0.4	37-39	20.0	AB	4.5	AB	1.0	C	7.3	BCD	8.3	ABCD	28.8	B
Proline EC 250	0.4	61-65	27.5	A	6.3	AB	1.0	C	9.3	BC	9.0	ABCD	27.5	BC
Proline EC 250 Proline EC 250	0.4 0.4	37-39 61-65	18.8	AB	4.8	AB	0.5	C	7.3	BCD	4.0	CD	23.8	BC
Proline EC 250 Proline EC 250	0.8 0.8	37-39 61-65	17.5	AB	5.3	AB	0.5	C	5.3	CD	4.5	BCD	23.8	BC
C. rosea Proline EC 250	1.0 0.4	37-39 61-65	17.5	AB	3.5	B	0.9	B	8.0	BCD	8.8	ABCD	33.8	AB
C. rosea	1.0	37-39	18.8	AB	4.5	AB	0.6	C	5.8	CD	12.0	AB	27.5	BC
C. rosea Serenade ASO	1.0 4.0	37-39 37-39	17.5	AB	4.8	AB	1.0	C	7.8	BCD	10.0	ABC	26.3	BC
C. rosea Serenade ASO Proline EC 250	1.0 4.0 0.4	37-39 37-39 37-39	15.5	B	5.8	AB	0.8	C	8.3	ABCD	6.0	ABCD	32.5	ABC
C. rosea *C. rosea*	1.0 1.0	37-39 61-65	18.8	AB	5.8	AB	1.4	BC	8.8	BC	13.0	A	33.8	AB
Serenade ASO Serenade ASO	4.0 4.0	37-39 61-65	21.6	AB	5.8	AB	1.0	C	9.5	BC	10.0	ABC	31.3	ABC
C. rosea Serenade ASO *C. rosea* Serenade ASO	1.0 4.0 1.0 4.0	37-39 37-39 61-65 61-65	25.0	AB	5.8	AB	2.3	ABC	12.8	AB	11.3	ABC	35.0	AB
C. rosea Serenade ASO Proline EC 250 *C. rosea* Serenade ASO Proline EC 250	1.0 4.0 0.4 1.0 4.0 0.4	37-39 37-39 37-39 61-65 61-65 61-65	19.3	AB	5.3	AB	1.0	C	7.8	BCD	7.0	ABCD	27.5	BC
Propulse SE 250 Propulse SE 250	1.0 1.0	37-39 61-65	18.0	AB	3.7	B	0.2	C	2.5	D	2.0	D	16.2	C
P-value			0.0005		0.0457		<.0001		<.0001		0.0003		0.0014	

[1]Propulse SE 250 expected to be the most efficient fungicide treatment of all but it should be noted that the maximum rate permitted per season is only 1 L/ha, [2] BCAs applied 20/5 and 11/6 and fungicides applied 21/5 and 12/6. (See supplementary data table 2 for information on statistical analyses).

TABLE 5.1.2. (Continued). Winter wheat field trial in 2019 at Research Center Flakkebjerg. The effects of the BCAs *Clonostachys rosea* and *B. velezensis* (Serenade ASO) on Septoria tritici blotch (STB), Fusarium head blight (FHB) and powdery mildew were tested. BCAs were compared with Proline EC 250 in reduced dose (0.4 L/ha) and full dose (0.8 L/ha). Combinations of BCAs and Proline EC 250 (0.4 L/ha) were also tested. Treatment were applied in growth stage (GS) 37-39 and/or GS 61-65. Propulse SE 250[1)] (1.0 L/ha) applied twice was included as fungicide control.

Treatment	Dose (L/ha)	Growth stage[2)]	Fusarium number 28th June		Fusarium scale 1-9 28th June		Fusarium percent 7th July		STB percent 7th July		Yield 9th August		DON ppb	
Untreated		-	4.4	A	5.0	A	38.8	A	76.3	A	74.9	A	343.7	A
Proline EC 250	0.4	37-39	2.2	BC	3.0	AB	25.0	ABC	55.0	ABCD	81.6	ABC	211.0	AB
Proline EC 250	0.4	61-65	1.2	BC	2.3	B	18.8	BC	65.0	AB	83.5	ABC	126.0	AB
Proline EC 250 Proline EC 250	0.4 0.4	37-39 61-65	0.8	BC	1.5	B	18.8	BC	37.5	CDE	86.2	BC	123.3	AB
Proline EC 250 Proline EC 250	0.8 0.8	37-39 61-65	0.4	C	1.3	B	4.5	D	31.3	DE	91.4	CD	70.0	B
C. rosea Proline EC 250	1.0 0.4	37-39 61-65	0.7	BC	1.8	B	13.8	CD	52.5	ABCD	84.8	ABC	143.3	AB
C. rosea	1.0	37-39	2.2	BC	2.8	B	25.0	ABC	65.0	AB	81.6	ABC	225.7	AB
C. rosea Serenade ASO	1.0 4.0	37-39 37-39	1.7	BC	2.8	B	31.3	AB	65.0	AB	78.7	AB	225,7	AB
C. rosea Serenade ASO Proline EC 250	1.0 4.0 0.4	37-39 37-39 37-39	2.0	BC	3.0	AB	22.5	ABC	57.5	ABC	83.0	ABC	317,0	AB
C. rosea *C. rosea*	1.0 1.0	37-39 61-65	2.2	BC	3.0	AB	30.0	AB	66.3	AB	81.9	ABC	229.7	AB
Serenade ASO Serenade ASO	4.0 4.0	37-39 61-65	2.5	AB	3.3	AB	26.3	ABC	65.0	AB	76,8	AB	247.0	AB
C. rosea Serenade ASO *C. rosea* Serenade ASO	1.0 4.0 1.0 4.0	37-39 37-39 61-65 61-65	2.2	BC	3.0	AB	37.5	AB	72.5	A	80.8	AB	297.7	AB
C. rosea Serenade ASO Proline EC 250 *C. rosea* Serenade ASO Proline EC 250	1.0 4.0 0.4 1.0 4.0 0.4	37-39 37-39 37-39 61-65 61-65 61-65	1.4	BC	2.3	B	18.8	BC	45.0	BCDE	84.5	ABC	153.7	AB
Propulse SE 250 Propulse SE 250	1.0 1.0	37-39 61-65	0.5	C	1.3	B	4.5		23.8	E	97.4	D	120.0	AB
P-value			<.0001		<.0001		<.0001		<.0001		0.0001		0.0066	

[1]Propulse SE 250 expected to be the most efficient fungicide treatment of all but it should be noted that the maximum rate permitted per season is only 1 L/ha, [2]BCAs applied 20/5 and 11/6 and fungicides applied 21/5 and 12/6. (See supplementary data table 2 for information on statistical analyses).

Field trial in 2020

The weather of 2020 was generally warm and sunny, with several periods with high temperatures during the summer periode (Appendix 1) whereas the precipitation was quite average. The STB severity was very low (< 5.8 % in untreated controls) and no STB symptoms were observed after 10th of June. In contrast, a moderate FHB level was observed (Table 5.1.3).

STB was scored at very low levels on 1st June on leaf 3 (5.0% in untreated controls) and leaf 4 (1.5% in untreated controls) (Table 5.1.3). On leaf 4, the treatments with Propulse SE 250, the two double treatments with Proline EC 250 (0.4 L/ha and 0.8 L/ha) reduced infection as did the three pure BCA treatments ('*C. rosea*', '*C. rosea*+Serenade ASO' and 'Serenade ASO+Serenade ASO') and the treatment '*C. rosea*+Serenade ASO+Proline EC 250'applied twice at GS 37-39 and GS 61-65. On leaf 3, only the treatment '*C. rosea* + Serenade ASO + Proline EC 250' applied twice at GS 37-39 and GS 61-65 was able to reduce STB (92% reduction). On 10th June, the STB scored was still very low (<5.8% in untreated controls) and not significantly reduced by any treatment. The attack did not develop during June and early July (Appendix 2: Supplementary data table 3).

FHB was assessed 1st and 7th July. On 1st July, the number of *Fusarium* infected plants was significantly reduced (51% - 59% reduction) by three pure BCA treatments i.e. '*C. rosea*' applied either at GS37-38 or at GS 61-65 and by '*C. rosea*+Serenade ASO' sprayed once at GS 37-39. Likewise, all treatments where Proline EC 250 (0.4 L/ha or 0.8 L/ha) was sprayed at the late stage (GS 61-65) either alone or in combination with BCAs reduced FHB within the same range (54% - 69%) (Table 5.1.3). However, on 7th July, the number of *Fusarium* infected plants was significantly reduced only by the double treatment with full dose Proline EC 250 (59% reduction) (Table 5.1.3).

There were no significant differences in yield between the treatments (Table 5.1.3) even though the average yield differed considerably between treatments. Levels of the Fusarium mycotoxins NIV, ZEA, T2 and HT2 were unaffected by the various treatments. In contrast, all treatments sprayed with Proline EC 250 at GS 61-65, either alone or in combination with BCAs and irrespective of dose, reduced DON levels significantly (65-72% reduction) and so did the Propulse SE 250 treatment (70% reduction), Table 5.1.3.

TABLE 5.1.3. Winter wheat field trial in 2020 at Research center Flakkebjerg. The effects of the BCAs *Clonostachys rosea* and *B. velezensis* (Serenade ASO) on Septoria tritici blotch (STB) and Fusarium head blight (FHB) were tested. BCAs were compared with Proline EC 250 in reduced dose (0.4 L/ha) and full dose (0.8 L/ha). Combinations of BCAs and Proline EC 250 (0.4 L/ha) were also tested.Treatment were applied in growth stage (GS) 37-39 and/or GS 61-65. Propulse SE 250[1)] (1.0 L/ha) applied twice was included as fungicide control.

Treatment	Dose (L/ha)	Growth stage	STB leaf 4 1st June		STB leaf 3 1st June		Fusarium number 1st July		Fusarium number 7th July		Yield 14th August		DON ppb	
Untreated		-	1.5	A	5.0	A	9.8	A	14.5	A	71.2	A	1232.0	A
Proline EC 250	0.4	37-39	0.5	ABCD	2.0	AB	4.5	B	12.3	AB	74.4	A	1025.1	AB
Proline EC 250	0.4	61-65	0.6	ABCD	2.3	AB	4.0	B	11.0	AB	74.9	A	470.0	BC
Proline EC 250 Proline EC 250	0.4 0.4	37-39 61-65	0.0*	D	1.1	AB	4.5	B	11.3	AB	81.2	A	375.7	C
Proline EC 250 Proline EC 250	0.8 0.8	37-39 61-65	0.3	ABCD	2.8	AB	3.0	B	6.0	B	85.2	A	345.1	C
C. rosea Proline EC 250	1.0 0.4	37-39 61-65	0.4	ABCD	2.5	AB	4.5	B	10.8	AB	79.2	A	399.5	C
C. rosea	1.0	37-39	0.2	BCD	1.9	AB	4.5	B	15.0	A	77.9	A	870.8	ABC
C. rosea Serenade ASO	1.0 4.0	37-39 37-39	0.2	BCD	2.5	AB	4.0	B	12.0	AB	75.5	A	828.5	ABC
C. rosea Serenade ASO Proline EC 250	1.0 4.0 0.4	37-39 37-39 37-39	0.4	ABCD	3.3	AB	5.8	AB	14.0	A	67.2	A	797.8	ABC
C. rosea *C. rosea*	1.0 1.0	37-39 61-65	0.8	ABCD	1.8	AB	6.0	AB	16.8	A	75.2	A	977.8	AB
Serenade ASO Serenade ASO	4.0 4.0	37-39 61-65	0.1	CD	1.5	B	5.8	AB	12.8	AB	70.3	A	900.8	ABC
C. rosea Serenade ASO *C. rosea* Serenade ASO	1.0 4.0 1.0 4.0	37-39 37-39 61-65 61-65	1.1	AB	3.3	AB	5.0	B	15.5	A	75.9	A	1365.0	A
C. rosea Serenade ASO Proline EC 250 *C. rosea* Serenade ASO Proline EC 250	1.0 4.0 0.4 1.0 4.0 0.4	37-39 37-39 37-39 61-65 61-65 61-65	0.0	D	0.4	B	3.5	B	9.8	AB	74.5	A	344.0	C
C. rosea	1.0	61-65	1.0	ABC	4.5	A	4.8	B	13.3	AB	75.3	A	1027.0	AB
Propulse SE 250 Propulse SE 250	1.0 1.0	37-39 61-65	0.3	BCD	1.5	AB	3.5	B	10.3	AB	85.3	A	368.8	C
P-value			<.0001		0.0058		0.0024		<.0011		0.1052		<.0001	

[1]Propulse SE 250 expected to be the most efficient fungicide treatment of all but it should be noted that the maximum rate permitted per season is only 1 L/ha, [2] BCAs applied 15/5 and 16/5 and fungicides applied 15/5 and 16/6. (See supplementary data table 2 for information on statistical analyses).

Summary of main findings from three years field trials

Comparison of BCA treatments

During the three years, treatments with BCAs alone showed significant reductions of STB and FHB at various time points during the growth seasons.

- The single treatment with *C. rosea* applied at GS 37-39 was in general the most efficient BCA treatment followed by the mixtures '*C. rosea* + *P. chlororaphis*' applied GS 37-39 and '*C. rosea* + Serenade ASO' applied GS 37-39.
- There were no clear evidence of improved control efficacy by mixing two different BCAs.
    - Mixtures of *C. rosea* + *Pseudomonas chlororaphis* was tested in 2018, which was a very dry season which did not reveal strong data to support improved disease control by combined treatment.
    - Mixtures of *C. rosea* + Serenade ASO (*Bacillus velezensis*) were investigated 2019 and 2020, and again no clear benefits were seen from mixing these two organisms. In fact, in a few cases the mixture '*C. rosea* + Serenade ASO' applied at both GS 37-39 and GS 61-65 was less efficient than the BCAs applied singly.

Comparison of BCAs, BCAs+Proline EC 250 and Proline EC 250

- The STB and FHB control from '*C. rosea'* applied at GS 37-39 was generally at the same level as 'Proline EC 250 (0.4 L/ha) applied at GS 37-39 (see also Figure 5.2.1-5.2.3 for more detailed analyses).
- Mixtures of *C. rosea* + Serenade ASO and *C. rosea* + *P. cholororaphis* combined with 0.4 L/ha Proline EC 250 all applied at GS 37-39 were generally not superior to the BCA mixtures applied alone.
- The best treatments with BCA(s) alone were in generally controlling STB as efficient as treatments including application of Proline EC 250 when the STB attack was low to moderate (1.5% - 48% STB) during the growth periode from early heading (GS 51) until early to medium grain development (GS 73-75).
- Treatments with BCA(s) failed in controlling STB at late growth stage (GS 79) where weather conditions had favoured further STB development (>77% STB on flag leaf) - indicating less persistence control than Proline EC 250 with the BCA application strategies tested.
- Treatments with BCA(s) only, reduced FHB although the persistence of the effects were slightly less, as seen at later assessments where only treatments including Proline EC 250 0.4 and-0.8 L/ha at GS 61-65 (year 2019) and twice Proline 0.8 L/ha (year 2020) reduced FHB at GS 77.
- Mixtures of *C. rosea* + Serenade ASO and *C. rosea* + *P. cholororaphis* combined with 0.4 L/ha Proline EC 250 applied at GS 37-39 or applied both at GS 37-39 and GS 61-65 did not result in superior disease control in comparison with the corresponding treatments with Proline EC 250 applied alone (*i.e.* 'Proline EC 250 (0.4 L/ha) at GS 37-39' and 'Proline EC 250 (0.4 L/ha) applied both at GS 37-39 and GS 61-65'.

Comparison of Proline EC 250 dosages and full Propulse SE 250

- The effect of Proline EC 250 generally provided the best control of both STB and FHB with the high dosage of Proline EC 250 (0.8 L/ha) applied twice providing superior control as compared with the reduced dosage (0.4 L/ha) applied at both at GS 37-39 and GS 61-65.
- Propulse SE 250 was also included, which was seen as an overall more effective product for control of STB compared to Proline EC 250, but it did actually not differ significantly from Proline EC 250 applied both at GS 37-39 and GS 61-65.
- No clear evidence of loss of STB control efficacy due to azole resistance development in the *Zymoseptoria tritici* population was revealed based on comparison with Propulse

SE 250 (sprayed in twice the allowed dosis).

- Regarding control of FHB the efficacy of Proline EC 250 (0.4 L/ha and 0.8 L/ha) applied both at GS 37-39 and GS 61-65 and Propulse SE 250 (1.0 L/ha) also applied twice provided similar levels of control but only Proline EC 250 0.8 L/ha reduced the DON content as compared to the untreated control in both 2019 and 2020.

5.2 Detailed analyses of *C. rosea* and reduced Proline EC 250 dosis, alone and in combination

A subset of data consisting of all treatments receiving only *C. rosea* once or twice, *C. rosea* + reduced Proline EC 250 dosage (0.4 L/ha) and treatments with reduced dosages of Proline EC 250 once (either at GS 37-39 or at GS 61-65) or twice at both GSs were analysed. This enabled us to get a closer look at the integrated approach and it was possible to compare the effects of *C. rosea* on disease development, yield and mycotoxin accumulation with the fungicide treatments in more detail. The complete tables with new statistical analyses of all variables are shown in the supplementary tables (Supplementary data tables 4 – table 6). Only variables (STB, FHB, DON and grain yield) where statistical differences were detected are commented and shown below (See Fig. 5.2.1 - Fig 5.2.5).

In the 2018, field trial, *C. rosea* reduced the severity of STB significantly on all assessed leaves both on 11th and 21st of June to the same level as the three different Proline treatments. In fact, one early application with *C. rosea* was as efficient as Proline EC 250 0.4 L/ha applied once at GS 37-39 or at GS 61-65 as well as two application of the reduced Proline EC 250 even at moderately sever STB attack (18-30% STB in untreated control) (Fig 5.2.1). However, the STB development stopped developing and did not reach the flag leaf which probably was the reason why none of the *C. rosea* and Proline treatments resulted in an yield increase (Fig 5.2.5).

Similar results were seen in 2019 with more severe attacks of both STB and FHB; various *C. rosea* treatments resulted in significant reduction in severity of both diseases (Fig 5.2.2). For the STB assessments 14th and 25th June, the severe attacks leaf 2 (17% and 47.5% in untreated control, respectively) was reduced by *C. rosea* applied at GS 37-39 by (66% and 47 %) to the same level as Proline EC 250 applied either at GS 37-39 (57% and 41%) or at GS 61-65 (57% and 41%). At the last assessment (7th July) *C. rosea* applied once or twice as well as Prolin EC 250 applied at GS 37-39 had no significant effect on STB (reduction 15%, 13% and 15% respectively). On the contrary STB was reduced by '*C. rosea* at GS 37-39 + Proline at GS 61-65'(31%), 'Proline EC 250 at GS 37-39' (28%) and Proline EC 250 sprayed twice (51%).

In 2019, the early FHB assessment on 25th June showed that '*C. rosea* at GS 37-39' reduced FHB by 42%, which is the same level as Proline EC 250 applied twice (50%) (Fig 5.2.2). At 7th July, the same level of FHB control was obtained for *C. rosea* at GS 37-39' and 'Proline EC 250 at GS 37-39' (both 36% reduction) whereas the three treatments reciving Proline EC 250 in GS 61-69 reduced FHB by >50%. Interestingly, the combination treatment '*C. rosea* at GS 37-39 + Proline EC 250 at GS 61-65' had the lowest FHB severity of all treatments (64% reduction).

The disease reducing effects by *C. rosea* resulted in a yield increase not being significantly different from the fungicide treatments (Fig 5.2.5 and Table 5.2.1) and at the same time the DON content was also reduced significantly by all treatments except for the double *C. rosea* treatment (Fig 5.2.4 and Table 5.2.1). Surprisingly it was noted that *C. rosea* sprayed at GS 37-39 only generally, provided similar or slightly superior disease control as compared to *C. rosea* sprayed both at GS 37-39 and GS 61-65. This was observed for control of both STB and FHB (Fig. 5.2.2).

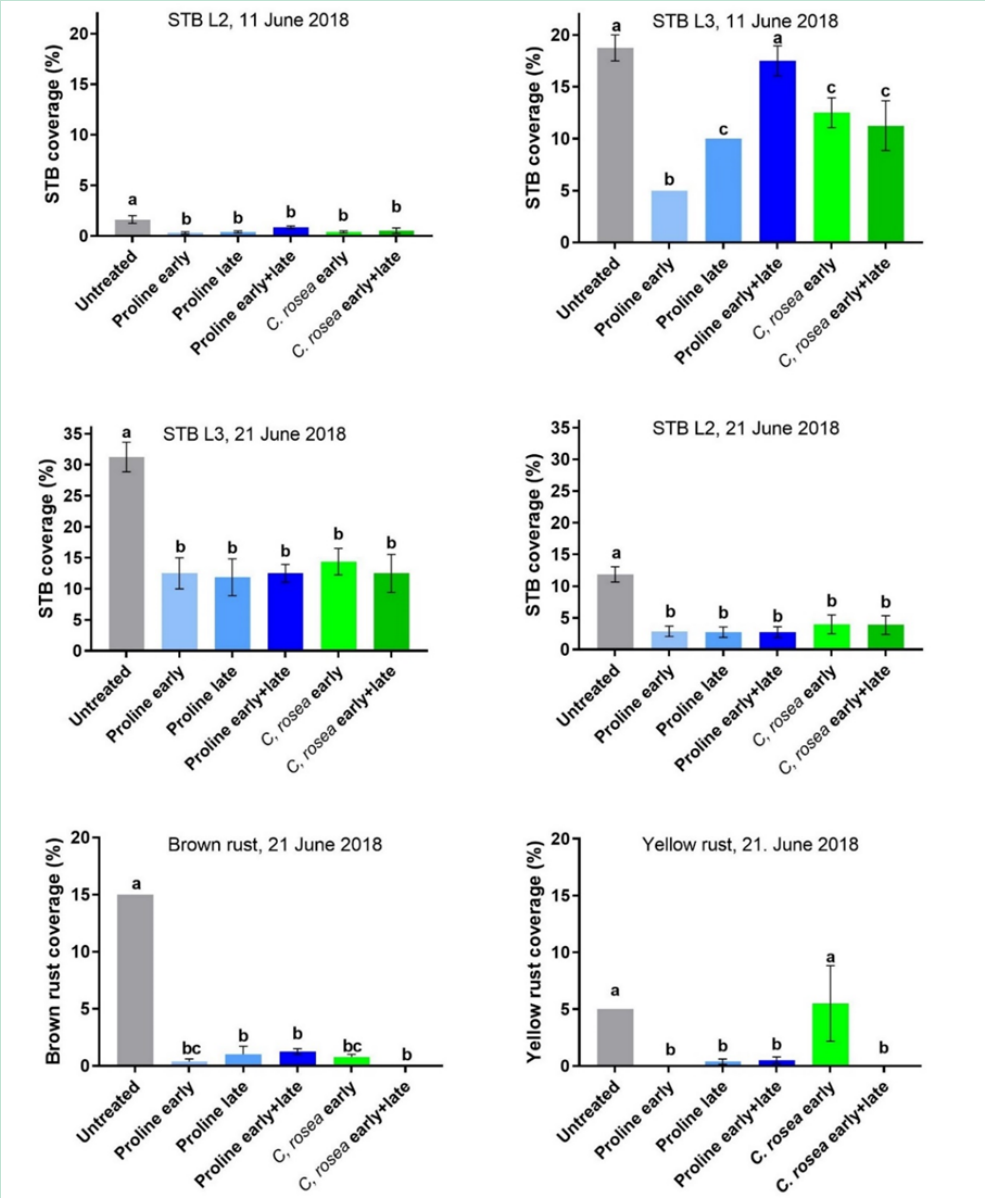

FIGURE 5.2.1. Effect of *Clonostachys rosea* and Proline EC 250 on STB infection and Brown rust in the field trial from 2018. Application once or twice as well as timing was tested. Application at GS37-GS39 (early) and application at GS61-GS65 (late). Proline EC 250 0.4 L/ha and *C. rosea* 1 x 107 pores/m^2. *C. rosea* applied 23/5 and 4/6 and Proline EC 250 applied 24/5 and 6/6. Means with different letters are significantly different (P ≤0.05).

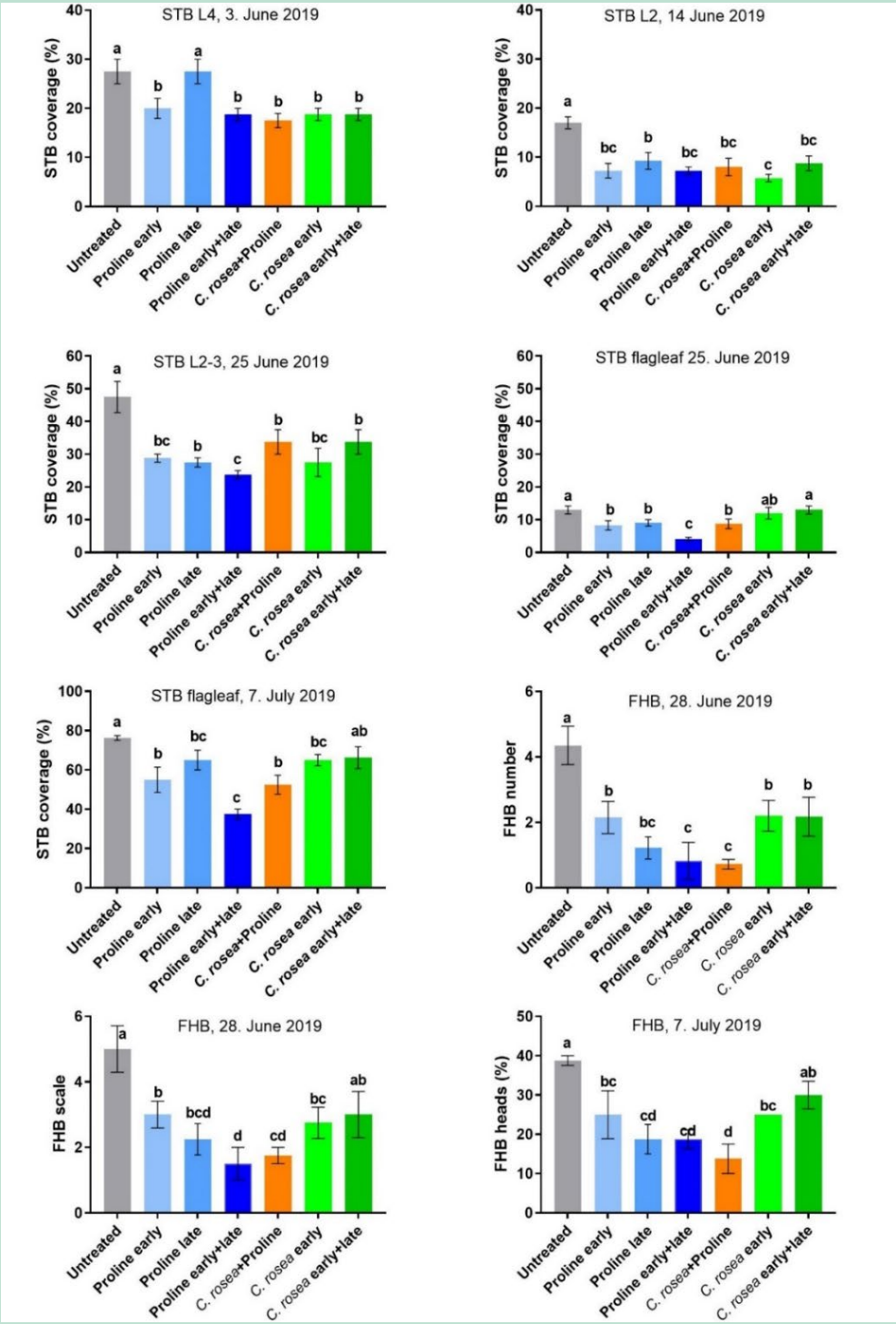

FIGURE 5.2.2. Effect of *Clonostachys rosea* and Proline EC 250 and a combination of the two agents on STB infection and Fusarium head blight (FHB) in the field trial from 2019. Application once or twice as well as timing was tested. Application at GS37-GS39 (early) and application at GS61-GS65 (late). Proline EC 250 0.4 L/ha and *C. rosea* 1 x 10^7 spores/m^2. *C. rosea* applied 20/5 and 11/6 and Proline EC 250 applied 21/5 and 12/6. Means marked with different letters are significantly different (P ≤0.05).

In the 2020 experimental set-up, a single application of *C. rosea* at GS61-65 was included in order to test if a late treatment was as effective as the single treatment at GS 37-39. In general, similar patterns of disease control as in 2019 was observed. At the 1st of June all treatment both fungicide and *C. rosea* reduced the minor STB symptoms (STB of untreated control < 1.5% on leaf 4 and <5% on leaf 3) significantly and to the same level except for *C. rosea* applied at GS 61-65 (Fig 5.2.3). This was not surprising since the late *C. rosea* treatment was applied at June 4th. From there on the STB attack did not develop further. Hence, at the latest STB assessment (10th June) where the STB attack was <5.8% in the untreated control none of the treatments reduced STB (table 5.2.3).

The FHB attack was low to moderate and was significantly reduced by approximately 50% by all treatments at the early assessments (1th July) whereas none of the treatments affected FHB on 7th July (Supplementary table 5.2.3). For the DON content in grains only treatments which included Proline at GS61-65 gave significant reductions of DON including '*C. rosea* at GS 37-39 + Proline EC 250 at GS 61-65' (Fig 5.2.4 and Table 5.2.1). The harvested grain yield was highly variable and therefore no significant yield increase could be detected (Fig. 5.2.5).

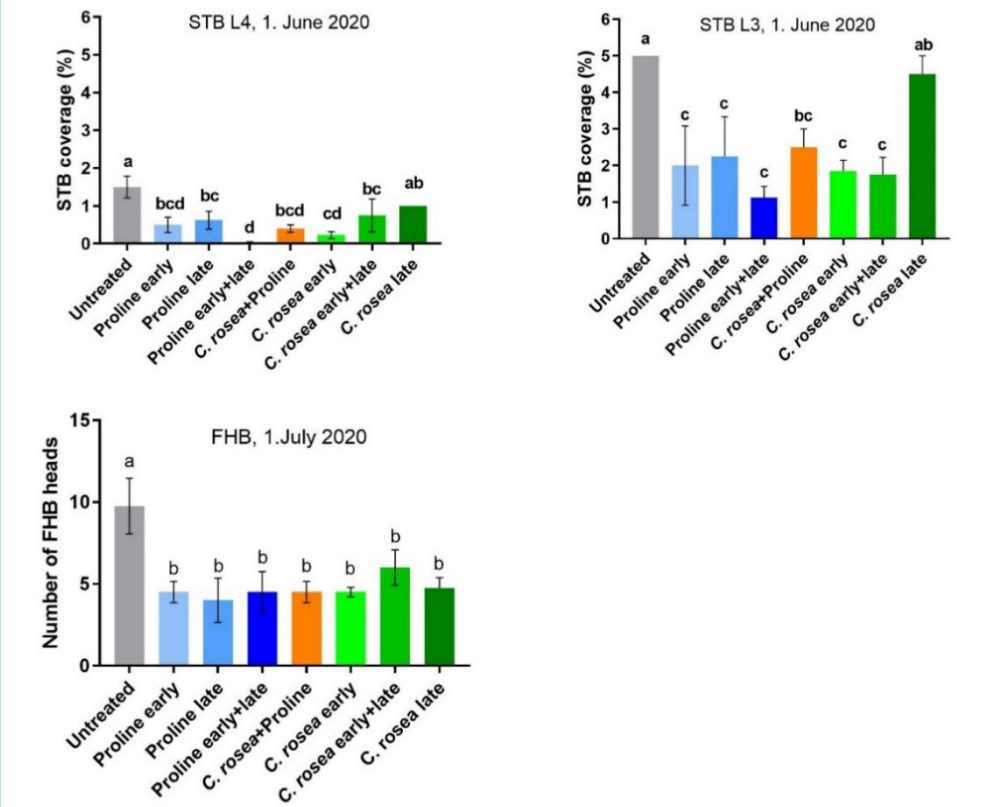

FIGURE 5.2.3. Effect of *Clonostachys rosea* and Proline EC 250 and a combination of the two agents on STB infection and Fusarium head blight (FHB) in the field trial from 2020. Application once or twice as well as timing was tested. Application at GS37-GS39 (early application) and application at GS61-GS65 (late application). Proline EC 250 0.4 L/ha and *C. rosea* 1 x 10^7 spores/m^2. *C. rosea* applied 15/5 and 15/6 and Proline EC 250 applied 16/5 and 16/6. Means marked with different letters are significantly different at $P<0.05$.

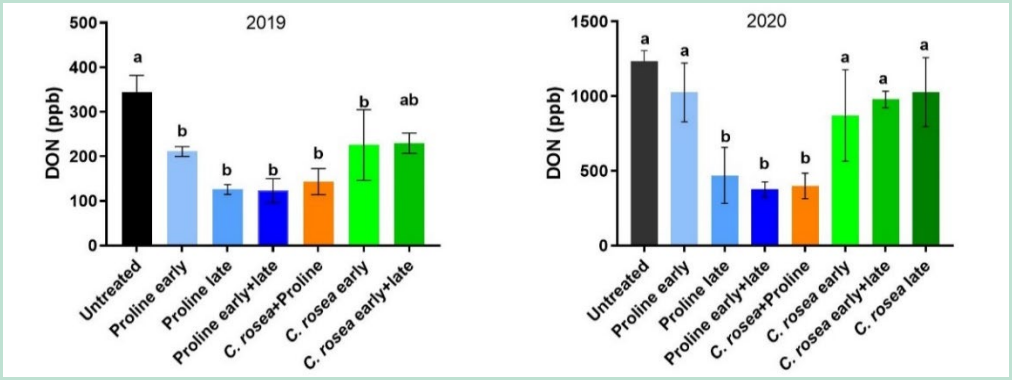

FIGURE 5.2.4. Effect of *Clonostachys rosea* and Proline EC 250 treatment on DON content in grains harvested in the field trial in 2019 and 2020. Means marked with different letters are significantly different at $P<0.05$.

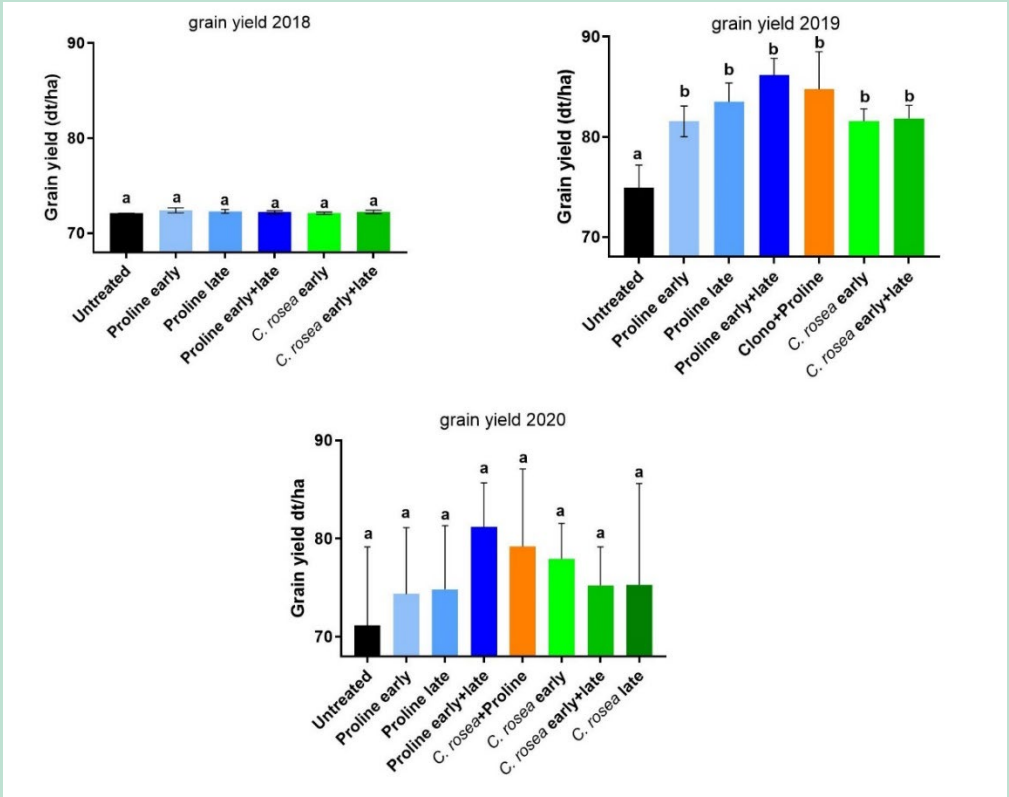

FIGURE 5.2.5. *Clonostachys rosea* and Proline EC 250 treatment on grain yield of field trials in 2018, 2019 and 2020. No significant effects of treatments on the yield in 2018 and 2020. Means marked with different letters in 2019 are significantly different at $P<0.05$.

Main results from 3 years testing for comparing effects of C. rosea and Proline EC 250 treatments

Overall, the 3 years of field testing has provided evidence that:

- *C.rosea* consistently gives moderate control of STB when applied at GS 37-39 (Table 5.2.1) despite that very different STB disease pressure was observed between years
- Both Proline 0.4 L/ha and *C. rosea* were applied as single treatments at GS 37-39 in all three years. Interestingly, the two treatment did not differ significantly in disease control of STB and FHB, yield and mycotoxin content in any of the years (except for STB on 11th June in 2018).
- *C.rosea* applied at GS 37-39 significantly reduced the DON content in one of the two seasons.
- Surprisingly the double treatment with *C.rosea* did not provide better FHB control than the single treatment applied only at GS 37-39, despite of the optimum timing normally having been proposed to be GS 61-65.
- Significant yield increase was only found in one of the three seasons. Here the yield improvement by *C. rosea* was not significantly different from the Proline EC 250 treatments.

In table 5.2.1 an overview of the effects of *C. rosea* and Proline EC 250 is given in terms of STB and FHB disease reduction, mycotoxin reduction and yield improvement.

TABLE 5.2.1. Overview of the effects of *Clonostachys rosea* and reduced dosis of Proline EC 250 (0.4 L/ha) on the STB, FHB and mycotoxin content (% reduction) as well as on grain yield (% increase). The number for STB and FHB represents the range of disease reduction at various assessments where ANOVA analyses revealed significant effects ($P < 0.05$) and likewise for DON and yield increase.

		Proline EC 250 GS 37-39	**Proline EC 250 GS 61-65**	**Proline EC 250 GS 37-39 Proline EC 250 GS 61-65**	***C. rosea* GS 37-39 Proline EC 250 GS 61-65**	***C. rosea* GS 37-39**
		STB, FHB and DON (% reduction) and yield (% increase)				
2018						
	STB	60-84	47-88	(7)[3] 44-87	-	34-75
2019						
	STB	28-64	(15)[4] 31-79	32-82	31-68	(8 and15)[5] 32-79
	FHB[1)]	36-50	52-73	74-91	64-84	36-50
	DON	39	63	64	58	34
	Yield[2)]	9 (6.7 hkg)	12 (8.6 hkg)	15 (11.3 hkg)	13 (9.5 hkg)	9 (6.7 hkg)
2020						
	STB	60-67	54-60	80-100	50-73	62-87
	FHB	50	59	50	50	50
	DON	17	62	70	68	29

[1)] all FHB assessment methods, [2)] Numbers in bracts represent the yield increase in (hkg/ha), [3)] STB severity reduction 11th June, [4)] STB severity reduction 7th July and [5)] STB severity reduction 25th June and 7th July.

5.3 Fungicide sensitivity of *Z. tritici* isolates

In general, there were no significant differences in the sensitivity of *Z. tritici* isolates exposed to the various spray treatments with BCAs and Proline EC 250. This was observed for leaves samples in 2018, 2019 and 2020. The *Z. tritici* isolates were tested for sensitivity prothioconazole-desthio and fluxapyroxad based on isolates collected from flag leaves in 2018, 2019 and 2020

- No effect of Proline EC 250 spray treatments (high and reduced dosages)
- No effect of BCAs alone
- No effect of combinations of BCA and reduced dosage of Proline EC 250

The EC_{50} values of *Z. tritici* isolates following the treatments showed a wide sensitivity range and that no treatment had an impact on fungicide resistance in the pathogen population in the year 2018 (Fig. 5.3.1), in the year 2019 (Fig 5.3.2) and in the year 2020 (Fig 5.3.3).

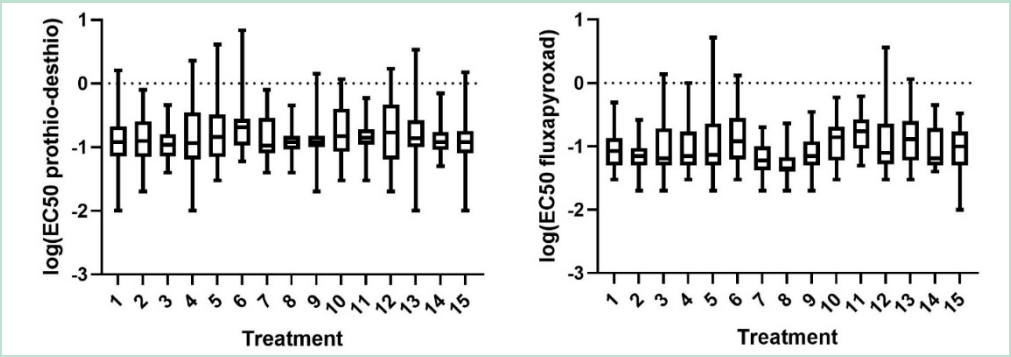

FIGURE 5.3.1. Box-whisker plot of EC_{50} (log-transformed) for prothioconazole-desthio (left) and fluxapyroxad (right) of *Z. tritici* isolates following fungicide and/or BCA treatment. Data from 2018.

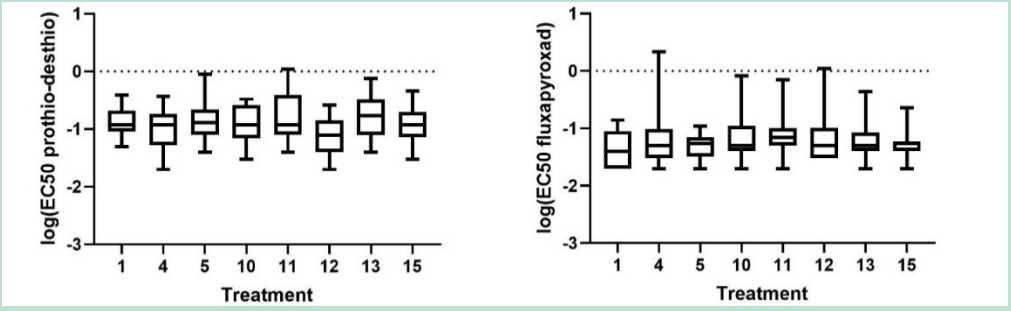

FIGURE 5.3.2. Box-whisker plot of EC_{50} (log-transformed) for prothioconazole-desthio (left) and fluxapyroxad (right) of *Z. tritici* isolates following fungicide and/or BCA treatment. Data from 2019.

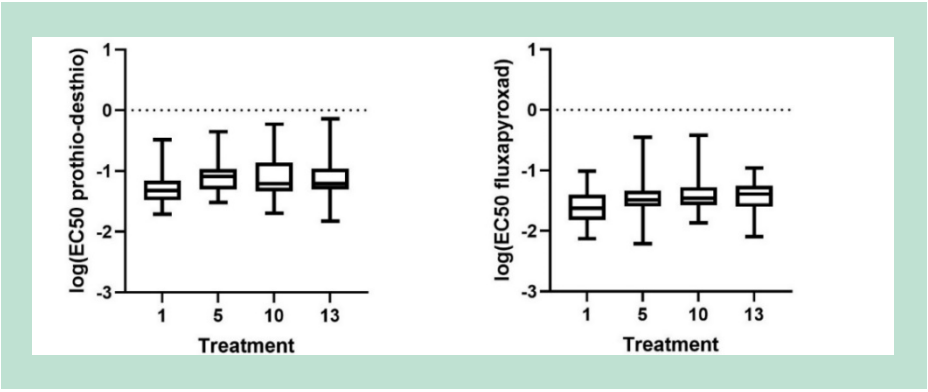

FIGURE 5.3.3. Box-whisker plot of EC_{50} (log-transformed) for prothioconazole-desthio (left) and fluxapyroxad (right) of *Z. tritici* isolates following fungicide and/or BCA treatment. Data from 2020.

5.4 PacBio sequence analysis of CYP51 mutations

A summary of PacBio sequencing is presented in table 5.4.1. PacBio sequencing produced a total of 116,930 and 135,544 circular consensus sequencing (CCS) reads with average read length 2150 bp and 2127 bp and Phred quality score above Q20 (99% base call accuracy) from the samples from the field trial 2018 and 2019, respectively (Table 5.4.1). After processing and quality analysis 96,323 (82.4%) and 86,778 (64.0%), CCS reads were accepted for sequence alignment. The overall alignment rate was 94.45% and 100%. The average number of aligned reads per treatment for the year 2018 and 2019 were 6065 and 5825 with a range between 5033 and 6852 and 4763 and 6800, respectively.

TABLE 5.4.1. Summary of PacBio sequencing of the CYP51 gene.

	Year 2018	**Year 2019**
Total CCS reads	145543	165496
≥Q20 Reads	116930	135544
≥Q20 Yield (bp)	251,494,046	288,358,989
≥Q20 Read Length (mean, bp)	2150	2,127
≥Q20 Read Quality (median)	Q34	Q36
Reads with tags	96323	86778
Aligned reads	90984	87382
Average aligned reads per treatment	6065	5825
Range of aligned reads	5033 – 6854	4763 - 6800

Using PacBio sequencing, full-length *CYP51* gene was successfully obtained from all treatment and replications in 2018 and 2019, respectively. Hence 120 samples were sequenced and analysed. Ten and nine non-synonymous mutations (a nucleotide mutation that alters the amino acid sequence of a protein) were detected in the samples from 2018 and 2019, respectively. To investigate the effect of the application of biocontrol agents on frequencies of specific mutations in *CYP51*, mutation frequency between treatments were analysed. Our results showed that all previously described CYP51 mutations were found. Not all mutations are of equal importance, and therefore the focus was on several 'marker' mutations to investigate the effect of BCAs on azole resistance. The most important mutations are D134G, V136A/C, A379G, I381V and S524T. All marker mutations were detected at different frequencies. The treatments of fungicides and BCAs did not have a clear effect on the selection of mutations, neither in 2018 nor 2019.

5.5 Community sequence analysis and taxonomic assignments of the leaf mycobiota

A summary of the output of sequence analysis is presented in table 5.5.1. As expected, more than half of the sequences passed quality control. We found 410 fungal operational taxonomic groups (OTUs, an operational definition used to classify groups of closely related individuals) in the pool of wheat leaves from the field trials 2018 and 2019. 253 OTUs were identified after rarefaction (a technique to calculate species richness for given number of samples), which excluded many OTUs present in low-to-non abundance. A taxonomic assignment of 29 most abundant OTUs, which is consisted mostly of fungal plant pathogens and yeasts are shown in table 5.5.2. We identified four OTUs (OTU1 *Blumeria graminis*, OTU2 *Z. tritici*, OUT3 *Puccinia striiformis*, OTU6 *Puccinia recondite*) as known fungal pathogens on wheat plant and one (OTU22 *Puccinia hordei*) as a fungal pathogen on barley (Table 5.5.2).

TABLE 5.5.1. Summary of PacBio sequencing of leaf samples from field trials in 2018 and 2019.

	Year 2018	Year 2019	Total
Total number of reads	372478	371458	743936
Sequences passing quality control	215598	203756	419354
Reads matching after reverse complement	104288	94554	198842
Number of reads truncated	0	0	0
Number of reads discarded	156880	167702	324582
Reads too short	39	135	174
Reads with low mean quality	6	0	6
Reads containing bases with low quality	15448	15684	31132
Mean read length	277	288	
Missing 5’ primer	52172	80310	132482
Missing 3’ primer	18982	12985	31967
Missing 5’ tag	40935	34455	75390
Missing 3’ tag	29298	24133	53431

OTU data were utilized to calculate the species richness and Shannon diversity indices (Fig. 5.5.1). Shannon diversity indices provide important information for diversity and evenness of sequence distribution among OTUs. Analysis showed no significant difference in species richness and Shannon index compared to untreated control treatment and between the treatments. However, the richness and Shannon index were higher in the samples from the year 2019 compared to 2018 (Fig. 5.5.1). Further, fungal communities' non-metric multidimensional scaling (NMDS) showed a significant year-to-year effect on fungal community composition on wheat leaves (Fig. 5.5.2).

TABLE 5.5.2. Taxonomic assignment of the 29 most abundant operational taxonomic units (OTUs) of the flag leaf (leaf 1) from wheat field trials in 2018 and 2019.

OTUs	Phylum	Order	Family	Genus	Species	Assignment
OTU 1	Ascomycota	Erysiphales	Erysiphaceae	Blumeria	*B. graminis*	Pathogen
OTU 2	Ascomycota	dummy order Zymoseptoria	Dummy family Zymoseptoria	Zymoseptoria	*Z. tritici*	Pathogen
OTU 3	Basidiomycota	Pucciniales	Pucciniaceae	Puccinia	*P. striiformis*	Pathogen
OTU 4	Basidiomycota	Sporidiobolales	dummy family Sporobolomyces	Sporobolomyces		Yeast
OTU 5	Ascomycota	Capnodiales				-
OTU 6	Basidiomycota	Pucciniales	Pucciniaceae	Puccinia	*P. recondita*	Pathogen
OTU 8	Basidiomycota	Tremellales				Yeast
OTU 7	Ascomycota	Pleosporales	Pleosporaceae	Alternaria		Pathogen Saprotroph
OTU 11	Basidiomycota	Tremellales	Tremellaceae	Dioszegia		Yeast
OTU 10	Basidiomycota	Tremellales				Yeast
OTU 12	Basidiomycota					Yeast
OTU 15	Ascomycota	Hypocreales				Antagonists, saprotroph
OTU 14	Basidiomycota	Cystofilo-basidiales	Cystofilo-basidiaceae			Yeast
OTU 13	Basidiomycota	Filobasidiales	Filobasidiaceae	Filobasidium		Yeast
OTU 16	Basidiomycota	Tremellales				Yeast
OTU 17	Ascomycota	Pleosporales	Didymellaceae			-
OTU 20	Basidiomycota					-
OTU 25	Ascomycota					
OTU 19	Ascomycota	Helotiales	Dermateaceae			Pathogens Saprotroph
OTU 18	Basidiomycota	Malasseziales	Malasseziaceae	Malassezia		Pathogens Saprotroph
OTU 21	Ascomycota					-
OTU 24	Basidiomycota	Leucosporidiales	Leuco-sporidiaceae	Leucosporidium		Yeast
OTU 26	Ascomycota	Pleosporales				Saprotrophs
OTU 27	Ascomycota	Pleosporales	Didymellaceae			Pathogens Saprotroph
OTU 22	Basidiomycota	Pucciniales	Pucciniaceae	Puccinia	*P. hordei*	pathogen
OTU 30	Basidiomycota	Entylomatales				Pathogen
OTU 34	Ascomycota	Helotiales	Sclerotiniaceae			pathogen
OTU 28	Ascomycota					-
OTU 32	Ascomycota	Pleosporales				-

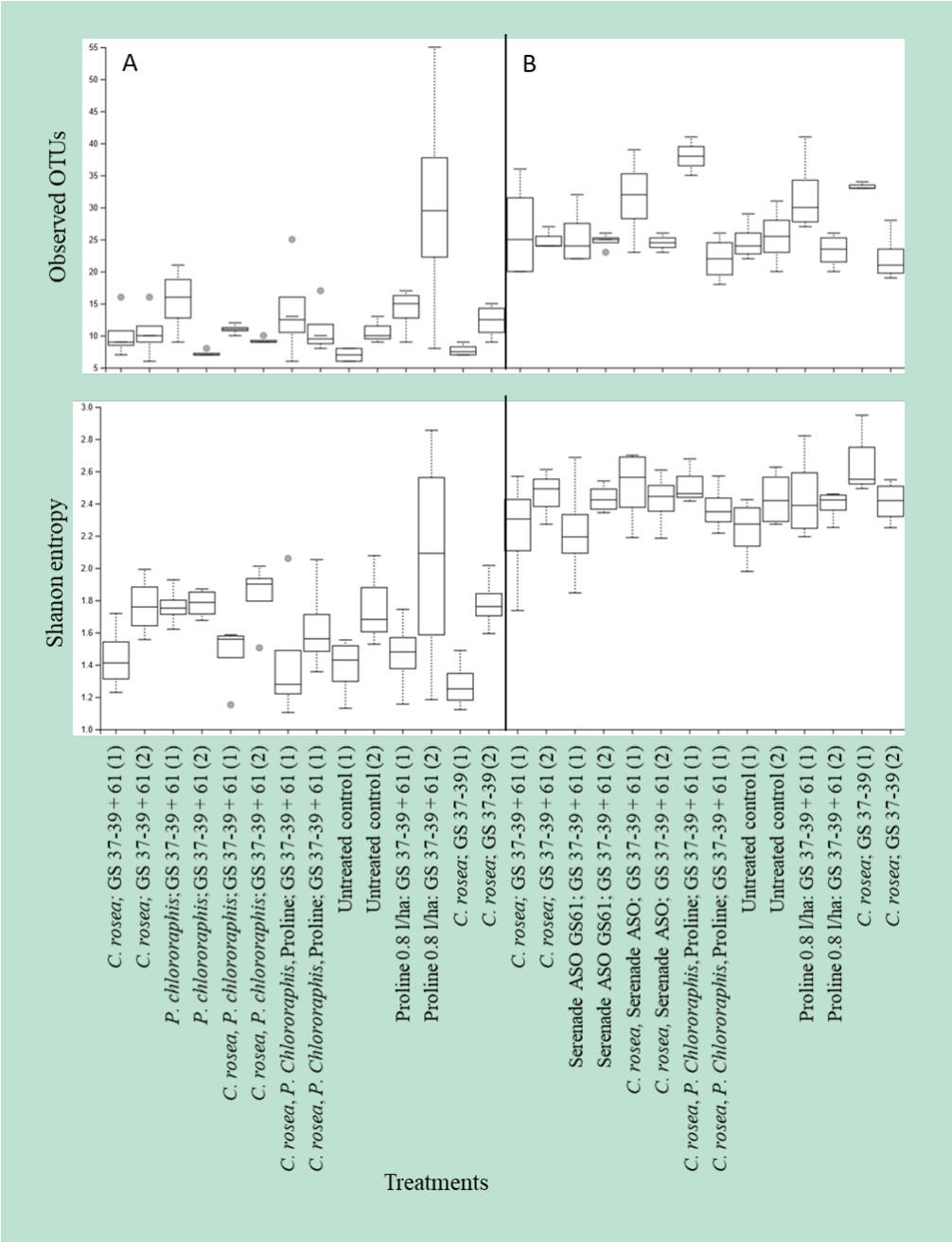

FIGURE 5.5.1: Richness of operational taxonomic units (OTUs) and community evenness. Boxplot showing A) OTU richness and B) community evenness grouped by treatment. (1), sampling points at GS 61. (2), sampling points at GS65.

The fungal community analysis showed significant year effect on fungal community. More variation in fungal community was observed in the samples from the field trial 2018 compared to year 2019 (Fig. 5.5.2). Furthermore, OUTs with relative abundance more than 1% were selected for further analysis to investigate the year effects (environmental conditions) on fungal community composition. Among the fungal pathogens, fungal community from the 2018 was dominated by powdery mildew pathogen *Blumeria graminis* followed by rust fungi *Puccinia striiformis* and *Puccinia recondita*. While *Zymoseptoria* spp and unidentified sporoblomyces spp were most and second most abundant spp in the 2019 samples (table 5.5.3). Furthermore, relative abundance of *P. recondita* was higher with 18% relative abundance at GS61 compared with 3.62% relative at GS63 in the year 2018. In contrast, relative abundance of *B. graminis*

(15.43%) and *P. striiformis* (9.36%) was higher at GS61 compared with 4.21% and 3.53%, respectively at GS 63 in the year 2019. However, the proportion of pink yeast Sporobolomyces was more at GS63 (25.50%) compared with GS 61 (7.06%) (Table 5.5.3).

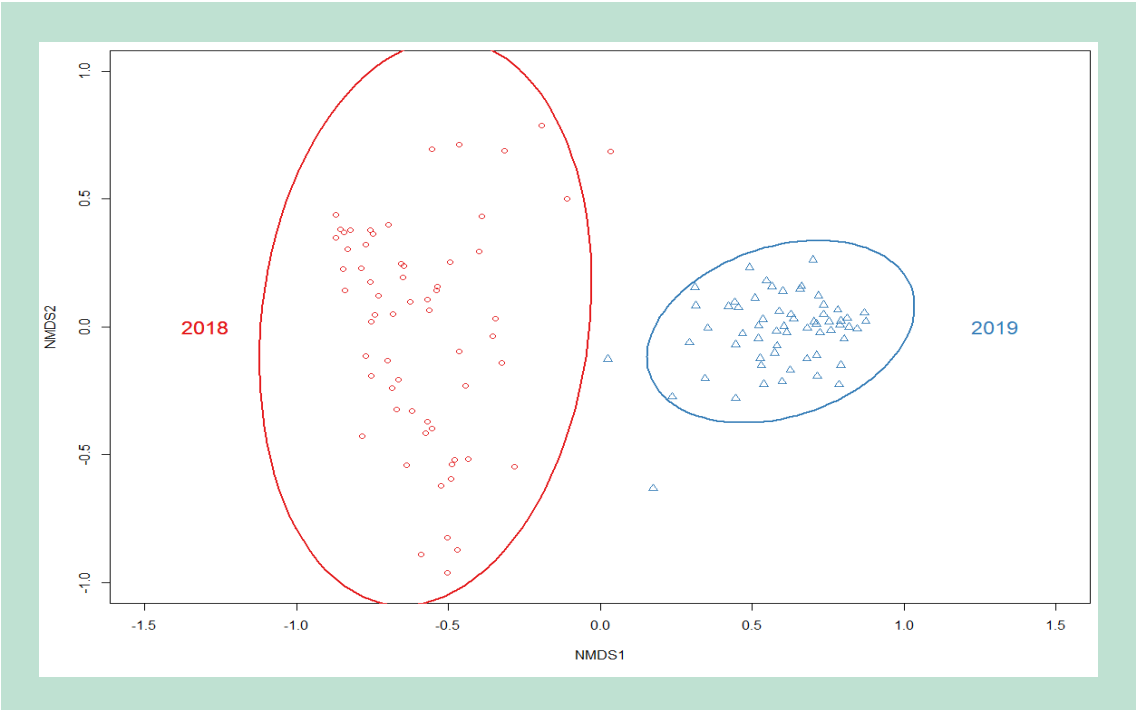

FIGURE 5.5.2. Non-metric multidimensional scaling (NMDS), showing variation in fungal community composition on wheat leaves from the year 2018 and the year 2019.

TABLE 5.5.3. Relative abundance of fungal species at two sampling time points at Growth stage (GS) 61 and GS 65 in 2018 and in 2019.

	Relative Abundance		Most abundant in:
	Sampling at GS 61	Sampling at GS 65	
2018			
Puccinia recondita	3.6%	18.0%	GS 65
unidentified_Hypocreales	0.1%	1.6%	GS 65
2019			
Blumeria_graminis	15.4%	4.2%	GS 61
Puccinia striiformis	9.4%	3.5%	GS 61
unidentified_Sporobolomyces	7.1%	25.5%	GS 65
unidentified_Capnodiales	12.6%	12.5%	GS 61
unidentified_Tremellales	4.4%	7.9%	GS 65
unidentified_Alternaria	3.1%	3.4%	GS 65
unidentified_Dioszegia	1.2%	2.4%	GS 65
unidentified	2.1%	2.6%	GS 65
unidentified_Cystofilobasidiales	0.8%	1.0%	GS 63

The application of fungicides or biocontrol agents showed no significant effect on fungal community composition on wheat leaves compared to control treatments in any of the two years. Furthermore, pairwise comparisons of treatments in relation to abundance of *Zymoseptoria* spp and *Blumeria graminis* revealed no specific differences between treatments (Fig 5.5.3). However, pairwise comparison between the treatments in 2018 showed that there was a higher relative abundance of *P. striiformis* in various treatment sprayed only with BCAs singly or in combination (*C. rosea* and *P. chlororaphis)* as compared to various treatments receiving

Proline EC 250 (Table 5.5.4). The lowest abundance of *P. striiformis* was found in the treatment '*C. rosea* + *P. chlororaphis* + Proline EC 250' applied at both GS 37-39 and GS 61(0.1%) while it was highest in the treatment '*C. rosea* at GS 37-39' (51.5%). Similarly, *P. recondita* was found in higher abundance in treatments only receiving *C. rosea* or *P. chlororaphis* compared to 'Proline EC 250 0.8 L/ha' applied at both GS 37-39 and GS 61. In summary, these data showed that the fungicide application reduced *P. striiformis* and *P. recondita* OTUs. However, this effect was not visible in the samples from the year 2019 (Fig. 5.5.3).

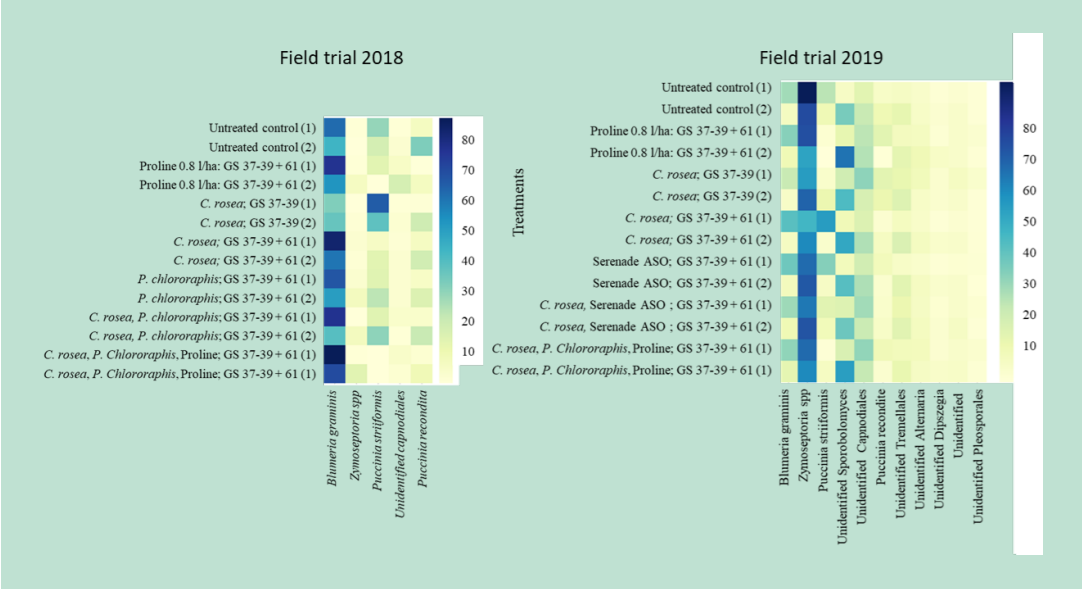

FIGURE 5.5.3. Effects of biocontrol agents or fungicides on fungal community composition on wheat leaves in the year 2018 and 2019, respectively. OTUs with relative abundance higher than 1% were used in the analysis. In the figure, (1) refers to sampling at GS 61 whereas (2) refers to sampling at GS65.

TABLE 5.5.4. Relative abundance of *P. striiformis* and *P. recondita* in various treatments sprayed only with BCAs singly or in combination as compared to various treatments receiving Proline EC 250 in the year 2018.

Treatments and fungal pathogens compared	**Relative Abundance (both time points)**	
P. chlororaphis GS 37-39 + 61 Vs Proline 0.8 l/ha GS 37-39 + 61	*P. chlororaphis*; GS 37-39+61	Proline 0.8 l/ha: GS 37-39 + 61
Puccinia striiformis	19.0 %	7.1 %
Puccinia recondita	10.5 %	3.5 %
P. chlororaphis; GS 37-39 Vs *C. rosea, P. chlororaphis,* Proline; GS 37-39 + 61	*P. chlororaphis*; GS 37-39	*C. rosea, P. chlororaphis*, Proline; GS 37-39 + 61
Puccinia striiformis	19.0 %	0.1 %
Proline 0.8 l/ha: GS 37-39 + 61 Vs *C. rosea*; GS 37-39	Proline 0.8 l/ha: GS 37-39 + 61	*C. rosea*; GS 37-39
Puccinia striiformis	7.1 %	51.5 %
Puccinia recondita	3.5 %	10.4 %
Proline 0.8 l/ha: GS 37-39 + 61 Vs *C. rosea*; GS 37-39 + 61	Proline 0.8 l/ha: GS 37-39 + 61	*C. rosea*; GS 37-39 + 61
Puccinia recondita	3.5 %	12.4 %
C. rosea; GS 37-39 Vs *C. rosea* , *P. chlororaphis,* Proline; GS 37-39 + 61	*C. rosea*; GS 37-39	*C. rosea, P. chlororaphis,* Proline; GS 37-39 + 61

Puccinia striiformis	51.5 %	0.1 %
C. rosea and P. chlororaphis; GS 37-39 + 61 Vs *C. rosea, P. chlororaphis*, Proline; GS 37-39 + 61	*C. rosea and P. chlororaphis*; GS 37-39 + 61	*C. rosea, P. chlororaphis*, Proline; GS 37-39 + 61
Puccinia striiformis	22.4 %	0.1 %

Since the fungal biocontrol agent *C. rosea* was sprayed onto the wheat leaves, we were interested in knowing whether *C. rosea* was only present only in treatment where *C. rosea* was applied either alone or in combination with other BCAs and Proline EC 250. Our initial result showed that there was no OTU assigned to the genus *Clonostachys* in any sample, however, we found five OTUs (OUT 15, OUT 81, OUT 133, OUT 204 and OUT 357) assigned to order Hypocreales (*C. rosea* belong to order Hypocreales) (Figure 5.5.5). After manual BLAST analysis, we found that OUT 15 is *Clonostachys rosea*. Surprisingly, our result showed that OTU 15 occurred in all treatments and that abundance was not associated to any specific treatment (Figure 5.5.4). However, we found that *C. rosea* was more abundant at the last sampling GS63 as compared to GS 61 time point B than at time point A and in year 2018 than in year 2019 (Figure 5.5.4).

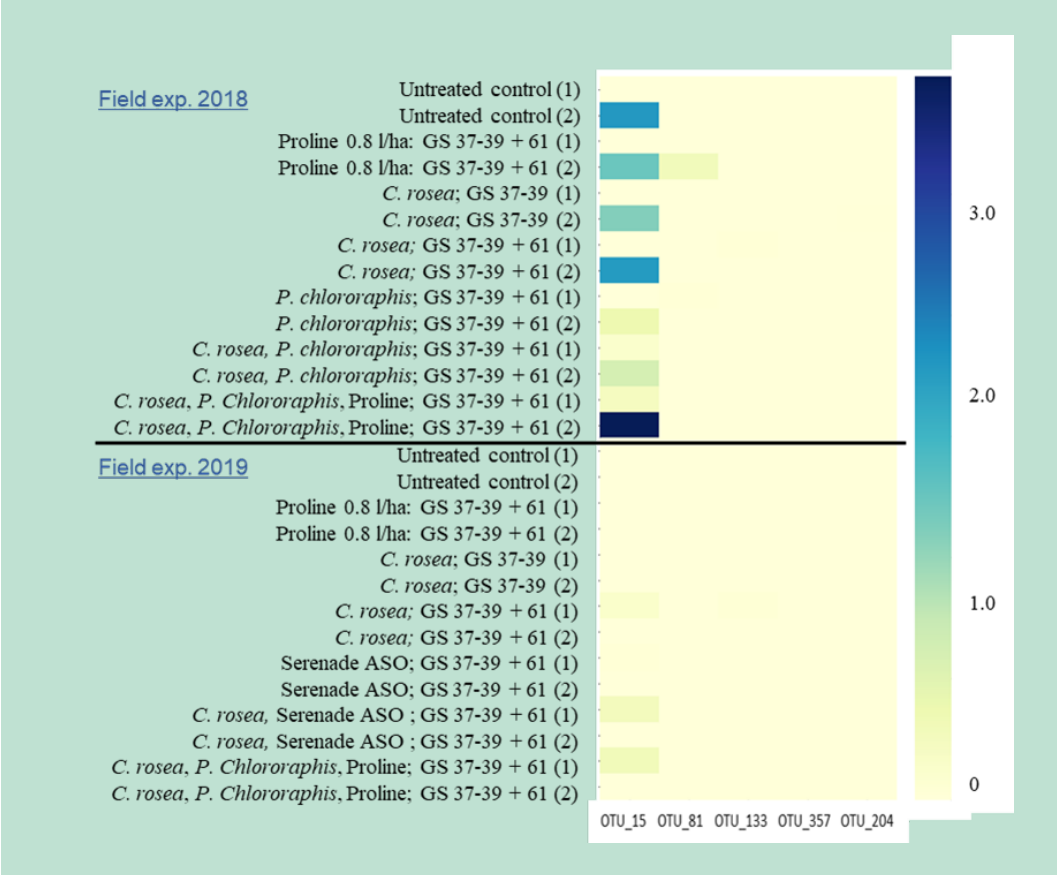

FIGURE 5.5.4. Heat map showing abundance of OUTs assigned to *C. rosea* (OUT_15) and other unassigned Hypocreales on wheat leaves in the year 2018 and 2019. (1), sampling points at GS 61. (2), sampling points at GS 65.

5.6 Persistence of *Clonostachys rosea* in leaf and head samples

Detection of *Clonostachys rosea* of both leaf and head samples strongly suggests that the TaqMan qPCR primers and the hydrolysis probe is highly species specific since there was no detection of *C. rosea* in any of the untreated leaf and head samples, respectively, from the three years (Table 5.6.1). However, it was also evident that the amount of fungal *C. rosea* DNA was very low and close to the detection limit. In 2018, *C. rosea* was detected in 4 out of

40 leaf leaf samples treated with *C. rosea*. In 2019, it was 9 out of 40 samples and in 2020, it was 10 out of 40 *C. rosea* treated samples. Furthermore, *C. rosea* was not detected in any leaves from the second sampling time point (GS65) in 2018 and 2019. It should also be noted that only one sample where *C. rosea* was applied once early at GS 37-39 was positive (Table 5.6.1).

For the wheat heads sampled at GS61, *C. rosea* was detected in 2, 4 and 2 out of 20 samples, respectively, in year 2018, 2019 and 2020. Clonostachys-positive samples were only detected in treatments receiving the late *C. rosea* spray treatment not in heads of the untreated control (Table 5.6.1).

TABLE 5.6.1. Persistence of *Clonostachys rosea* in samples of on leaves and spikes sprayed with *C. rosea* isolate IK726. The Clonostachys biomass was determined by TaqMan qPCR. Four replications were sampled at each time-point for all treatments. 0/4 = no detection of *C. rosea* in any of the 4 samples per treatment; 1/4 = one out of the 4 samples were *C. rosea* positive and 2/4 = two out of the 4 samples were *C. rosea* positive.

Treatments	Growth stage of treatments	Leaf sampling GS61	Leaf sampling GS65	Head sampling
Year 2018				
Untreated	-	0/4	0/4	0/4
C. rosea	37-39	0/4	0/4	**1/4**
C. rosea	37-39	**1/4**	0/4	**1/4**
C. rosea	61-65			
C. rosea + *P. chlororaphis*	37-39	0/4	0/4	0/4
C. rosea + *P. chlororaphis*	61-65			
Cr + *P. chlororaphis* + Proline EC 250	37-39	**1/4**	0/4	0/4
Cr+ *P. chlororaphis* + Proline EC 250	61-65			
Year 2019				
Untreated	-	0/4	0/4	0/4
C. rosea	37-39	0/4	0/4	0/4
C. rosea	37-39	**1/4**	0/4	**1/4**
C. rosea	61-65			
C. rosea + Serenade ASO	37-39	**2/4**	0/4	**1/4**
C. rosea + Serenade ASO	61-65			
C. rosea + Serenade ASO + Proline EC 250	37-39	**2/4**	0/4	**2/4**
C. rosea + Serenade ASO + Proline EC 250	61-65			
Year 2020				
Untreated	-	0/4	0/4	0/4
C. rosea	37-39	0/4	0/4	0/4
C. rosea	37-39	**2/4**	0/4	0/4
C. rosea	61-65			
C. rosea + Serenade ASO	37-39	**2/4**	**1/4**	**2/4**
C. rosea + Serenade ASO	61-65			
C. rosea + Serenade ASO + Proline EC 250	37-39	**2/4**	**1/4**	0/4
C. rosea + Serenade ASO + Proline EC 250	61-65			

5.7 *C. rosea* control of STB and combination with Proline EC 250 in growth chamber

A range of growth chamber experiments were conducted in order to study in details the effects of *C. rosea* on STB severity, the effect of timing as well as the potential of combining *C. rosea* and the fungicide Proline EC 250.

Application of *C. rosea* at 1 day before inoculation with the pathogen was tested in three experiments (Fig. 5.7.1a-c)). The *C. rosea* treatment resulted in lower disease severity as compared to the *Z. tritic*i control treated with water at 1 day before the pathogen in all experiments. Significant reductions were detected in two assays (Fig 5.7.1 b and c) on specific days after infection with the pathogen. However, when data from the experiments were pooled (no significant interaction between treatment and experiment), the *C. rosea* treatment reduced the STB severity when assessed at 14, 16 and 18 days after inoculation with *Z. tritici* (Fig 5.7.1d)

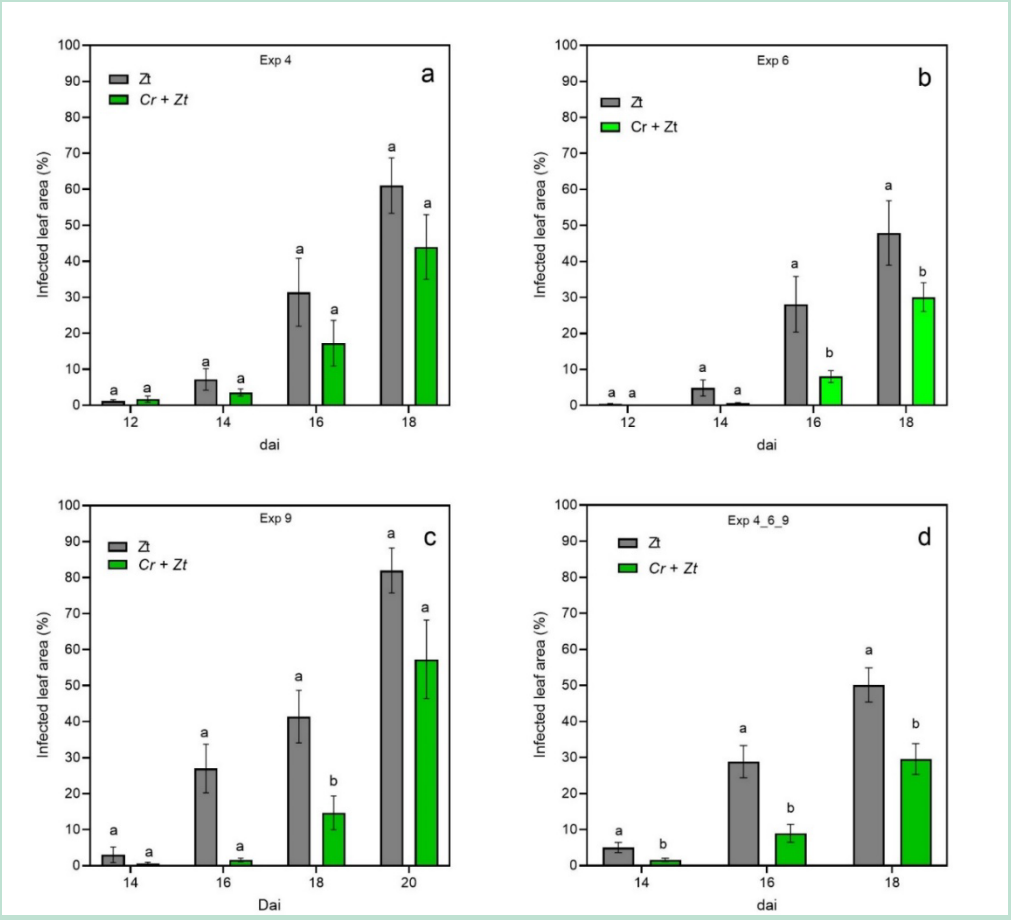

FIGURE 5.7.1. Effect of *Clonostachys rosea* on the STB (caused by *Z. tritici*) development on wheat seedlings. "*Zt*" = water applied 1 day prior to *Z. tritici* spores and "*Cr* + *Zt*" = *C. rosea* spores applied one day prior to *Z. tritici* spores. Means within each time point are comparable and bars marked with different letters are significantly different ($P \leq 0.05$).

The effect of applying *C. rosea* at four days before the pathogen was also tested in three assays. The average disease severity was lower at all assessments in all assays (Fig. 5.7.2). However, only in experiment 8, there was a significant reduction of *Z. tritici* infection at day 20 and 22 (Fig 5.7.2.). Due to interactions between treatment and experiment, data could not be pooled.

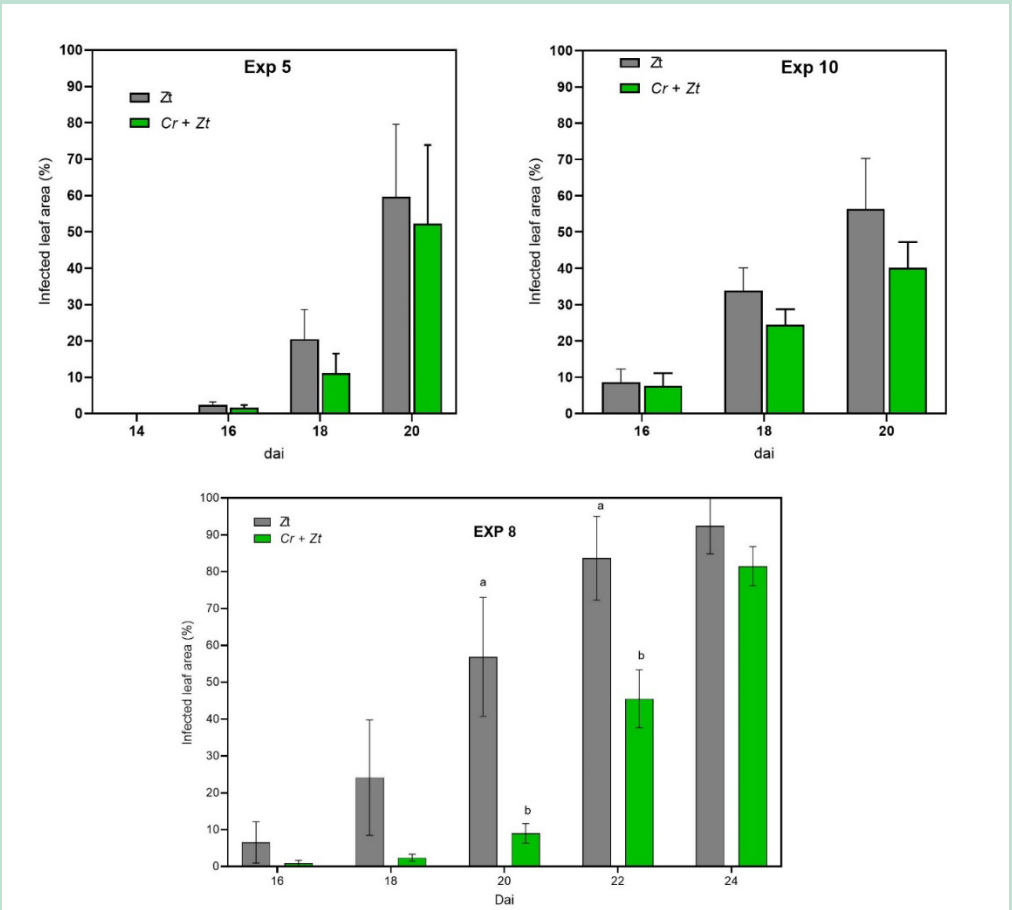

FIGURE 5.7.2. Effect of Clonostachys rosea on the disease development on wheat seedlings caused by *Z. tritici*. "Zt" = water applied 4 days prior to *Z. tritici* spores and "Cr+Zt" = *C. rosea* spores applied 4 days prior to *Z. tritici* spores. Means within each time point are comparable and bars marked with different letters are significantly different (P ≤0.05).

The effect of a combined treatment of *C. rosea* and Proline EC 250 were tested in three assays. In none of the assays treatment showed significant effects of *C. rosea*, Proline EC 250 or the combination of *C. rosea* and the fungicide (Fig. 5.7.3). However, microscopy of wheat leaves sprayed with increasing concentrations of Proline EC 250 from 0-500 ppm and spores of *C. rosea* D11 (gfp transformant of IK726) showed that the fungus was able germinate and colonise the leaf surface at 5 ppm Proline EC 250 (Fig. 5.7.4)

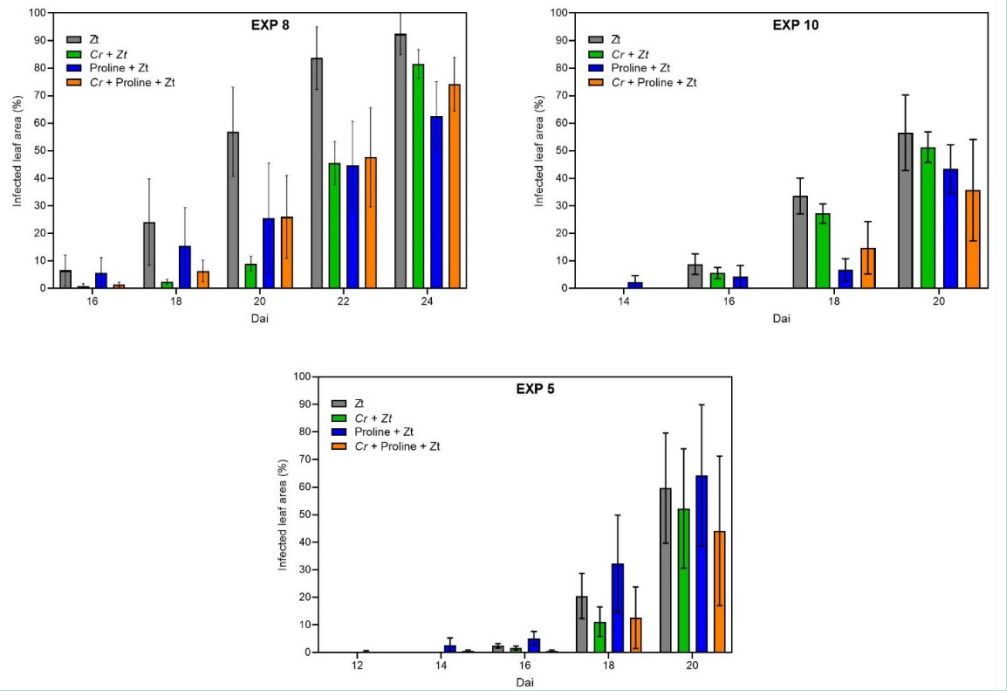

FIGURE 5.7.3. Effect of *C. rosea* and Proline EC 250 and a combination of the two on the percentage *Z. tritici* symptoms. *C. rosea* was applied at 4 days before the pathogen whereas Proline EC 250 was applied at 1 day before the pathogen. In two experiments, 3 ppm Proline EC 250 was applied (A and B) whereas 1 ppm Proline EC 250 was applied in the third experiment. Means within each time point are comparable and bars marked with different letters are significantly different ($P \leq 0.05$).

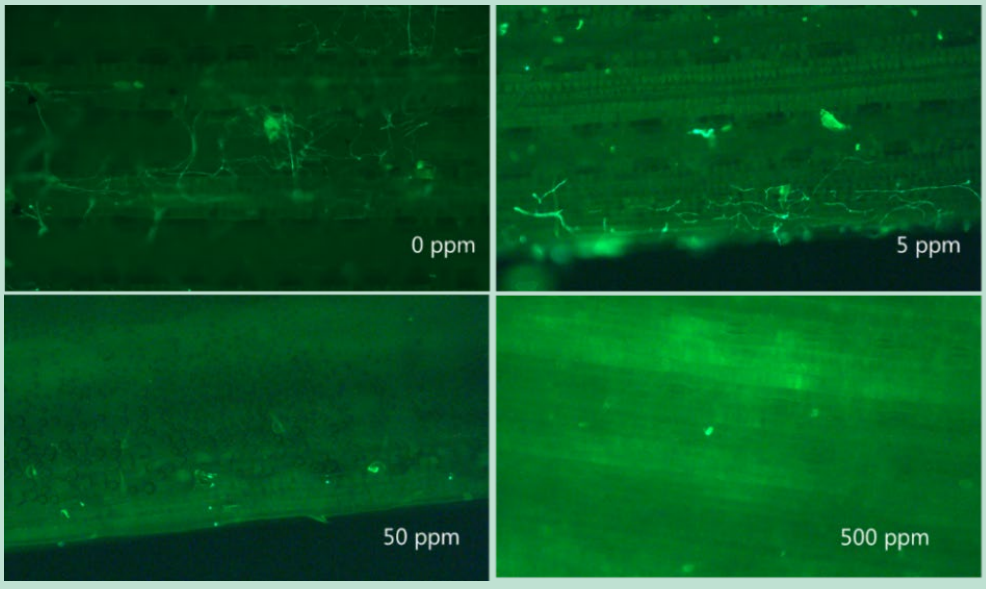

FIGURE 5.7.4. Leaf colonisation by *C. rosea* D11 (*gfp* transformant of IK726) on wheat leaves. Leaves were sprayed with increasing dosage of Proline EC 250 (dilutions in water) and inoculated with spores of *C. rosea*.

5.8 Mechanisms of *C. rosea* control of *Z. tritici* infection

In order to understand the mechanisms behind the *C. rosea* induced reduction of STB symptoms quantitative microscopy of the *Z. tritici* infection processes were performed on leaves sampled at 3, 7 and 15 days after inoculation with *Z. tritici* (dai). The frequencies of observed infection stages i.e. *Z. tritici* spore germination (Fig 5.8.1A), *Z. tritici* hyphae penetrating through stoma (Fig 5.8.1B), presence of hyphal aggregation (Fig 5.8.1C) and pycnidial initials and fully developed pycnidia (Fig 5.8.1D) were analysed.

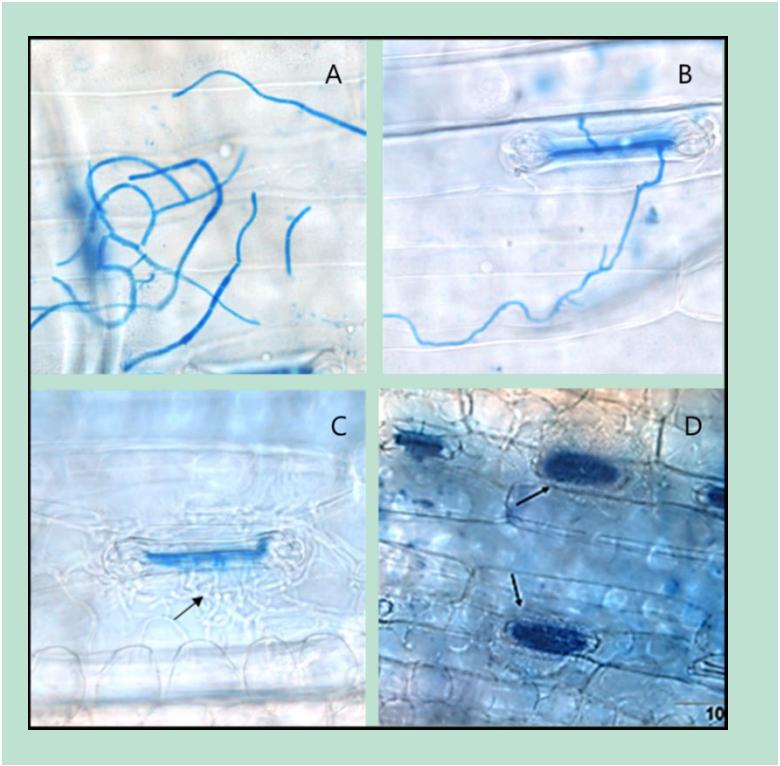

FIGURE 5.8.1. Infection structures of *Zymoseptoria tritici* observed on wheat leaves by microscopy. A) Germinated (arrows) and ungerminated spores, B) Penetration of stoma, C) aggregating hyphae (arrow) in substomatal cavity and D) Fully developed pycnidia (arrows). Photos A, B and C by Meike Latz and photo D by Annika Victoria Anine Slaatto & Katrine Bredahl Jacobsen.

The frequency of germinated spores was around 50% at 3 dai for both treatments whereas the penetration efficiency was significantly lower for the *C. rosea* treatment (Fig 5.8.2A). However, at 7dai, *C. rosea* reduced both germination of *Z. tritici* spores by 33% and the penetration efficiency by 92 % (Fig 5.8.2B). At the late infection stage (15 dai) the frequency of empty stomata without signs of infection was significantly higher for the *Cr* + *Zt* treatment than for the water control treatment (*Zt*). Furthermore, the frequency stomata with hyphal aggregation, pycnidia initials and developed pycnidia were significantly reduced by the *C. rosea* treatment (Fig 5.8.2C). Moreover, STB symptoms of seedlings from the same experiments showed that the *C. rosea* treatment significantly reduced the percentage leaf area infected by *Z. tritici* (Fig 5.8.2D).

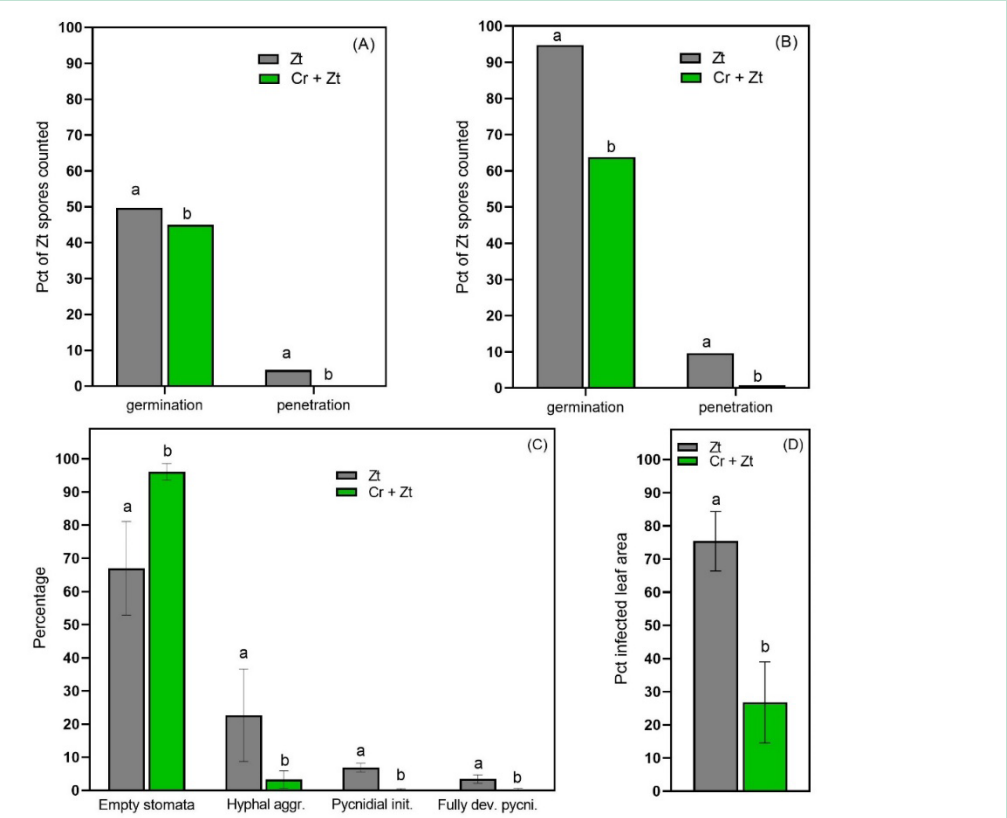

FIGURE 5.8.2. Effect of *Clonostachys rosea* IK726 on *Z. tritici* development on wheat seedlings. Quantitative microscopy of *Z. tritici* spore germination and penetration through stoma at 3 dai. (A) and at 7 dai (B), respectively. (C) Quantitative microscopy of *Z. tritici* colonisation and pycnidia development at 15 dai, counted as uncolonised (empty) substomatal cavities, hyphal aggregation, pycnicial initials and fully developed pycnidia in substomatal cavities. (D) Percentage leaf area infected with *Z. tritici*. All observation on plants from the same experiment. For each variable and each time-point, bars marked with different letters are significantly different at $P \leq 0.05$.

Since all fungal structures were stained in the specimen for microscopy, it was also possible to observe germination and growth of *C. rosea* on the infected leaves (Fig. 5.8.3). The spores often assembled around stomata and along anticlinal cell walls and germinated spores were easily identified at 3 dai (Fig 5.8.3 A and B). Furthermore, 11 dai it was also seen that *C. rosea* hyphae growing towards a stomata (Fig 5.8.3 C) occasionally grew in through stomata (Fig. 5.8.3 D).

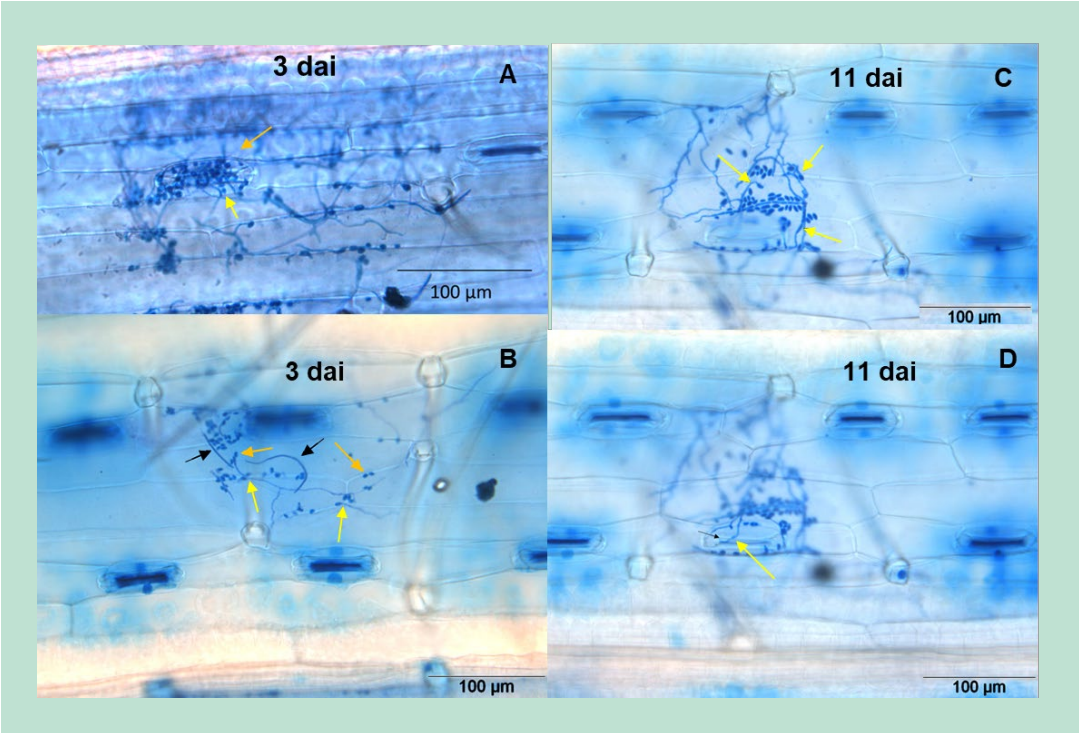

FIGURE 5.8.3. Microscopy of wheat leaves 3 and 11 dai with *Zymoseptoria tritici*, respectively. The leaves were inoculated with *Clonostachys rosea* IK726 one day before inoculation with *Z. tritici*. A) Many germinated and ungerminated spores of *C. rosea* around a stoma, B) Germinated Z. *tritici* spores, germinated and ungerminated spores of *C. rosea* closely together, C) Germinated *C. rosea* spores growing between and towards stomata and D) *C. rosea* hyphae penetrating the stoma. Yellow arrow = germinated *C. rosea* spore with hyphal growth, orange arrows = ungerminated *C. rosea* spores and black arrows = *Z. tritici* spores.

5.9 Control of FHB and DON accumulation in greenhouse experiments

A range of greenhouse trials were conducted in order to study in details the effects of *C. rosea* on FHB and DON accumulation in wheat heads.

Clonostachys rosea reduced FHB severity (Fig 5.9.1 and, Fig 5.9.2) as compared to the Fusarium water control in EXP 1 ($P<0.0048$) and in EXP 3 ($P<0.001$) whereas in EXP 2 (Fig 5.9.1) the difference was non-significant ($P<0.12$). However, in all three experiments the content of DON was reduced by 99%, 27% and 85% respectively in EXP 1 ($P<0.0013$), EXP 2 ($P<0.02$) and EXP 3 ($P<0.005$). Comparing the effects of *C. rosea* and *S. griseoviridis* K61 (bacterial organism of Mycostop) there were no significant difference in their efficacy against FHB and accumulation of DON (Fig 5.9.2). The ability of the two BCAs to reduce Fusarium infection was also confirmed by qPCR where they both significantly reduced the Fusarium DNA content in the spikelet for experiment 3 (Table 5.9.1).

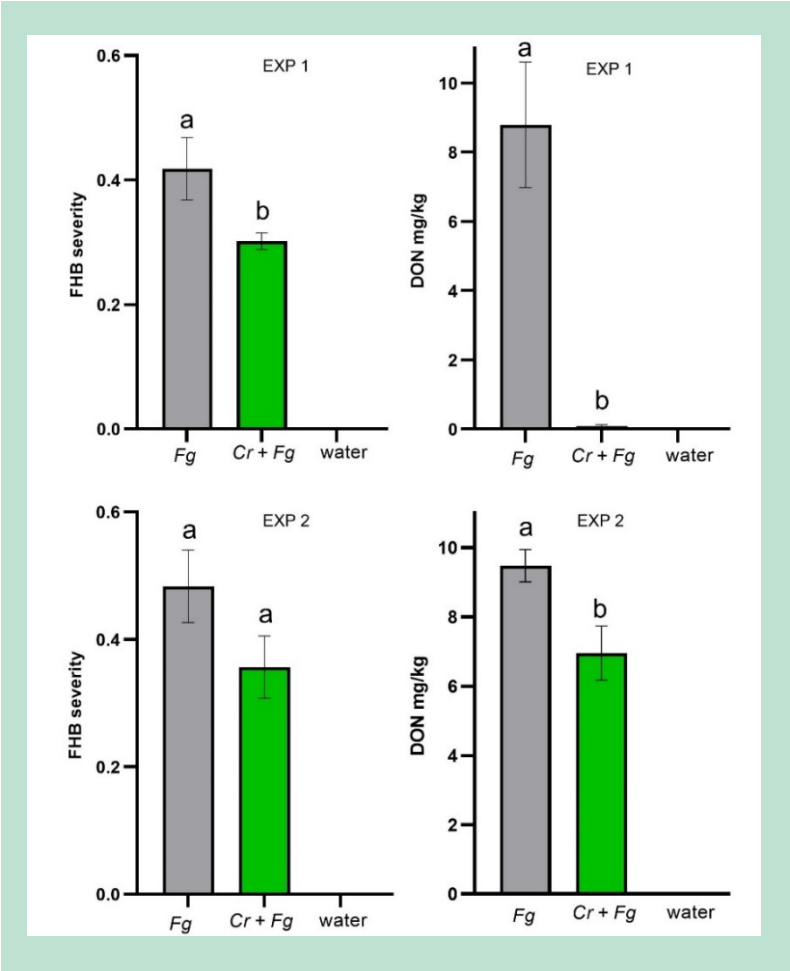

FIGURE 5.9.1. Biological control of Fusarium head blight (FHB) and DON accumulation caused by *Fusarium graminearum* WC-091-7 in two independent greenhouse experiments. *Clonostachys rosea* IK726 (1 x 10^7 spores/ml) was sprayed to spikes at heading stage of the wheat cultivar Diskett four days before inoculating the heads with *F. graminearum* (1 x 10^5 spores/ml). FHB severity was assessed 5 day after pathogen inoculation (incidence of symptomatic spikelets) and, the DON content of the spikes were quantified by HPLC MS/MS. "water": no inoculation with Fusarium and *C. rosea*. Means marked with different letters are significantly different ($P \leq 0.05$).

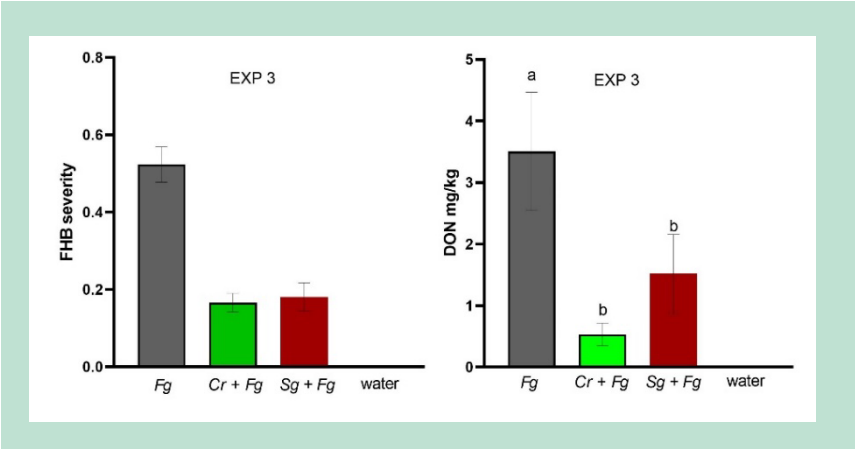

FIGURE 5.9.2. Biological control of Fusarium head blight (FHB) and DON accumulation caused by Fusarium graminearum WC-091-7 in two independent greenhouse experiments (EXP1 and EXP2). *Clonostachys rosea* IK726 (1 x 10^7 spores/ml) and *Streptomyces griseoviridis* (1 x 10^7 cells/ml) were sprayed to spikes at heading stage of the wheat cultivar Diskett

four days before inoculating the heads with *F. graminearum* (1 x 10^5 spores/ml). FHB severity was assessed 5 day after pathogen inoculation (incidence of symptomatic spikelets) and the DON content of the spikes were quantified by HPLC MS/MS. Means marked with different letters are significantly different ($P \leq 0.05$).

TABLE 5.9.1. Effect of. *Clonostachys rosea* IK726 (1 x 10^7 spores/ml) and *Streptomyces griseoviridis* (10^7 cells/ml) on *Fusarium graminearum* DNA in spikes at heading stage of the wheat cultivar Diskett (EXP3). The BCAs were applied four days before inoculating the heads with *F. graminearum* ((10^5 spores/ml). Mean values followed by different letters are significant different at P<0.05.

Treatment	Fusarium DNA/ wheat DNA (pg/ng)1)	Reduction (%)
FHB control	61.09±26.96a	-
Clonostchys. rosea IK726	17.89±13.63b	71
Streptomyces griseoviridis K61	29.60±21.45b	52
Water control	8.27±10.58c	86

The relationship between FHB severity and DON content of spike samples from experiment 1-3 is shown in Fig 5.9.3. For the pooled data of the *F. graminearum* water controls there were no significant correlation between FHB and DON whereas there was a positive correlation between FHB and DON for the *C. rosea* treated spikes(r=0.64, *P*=0.004).

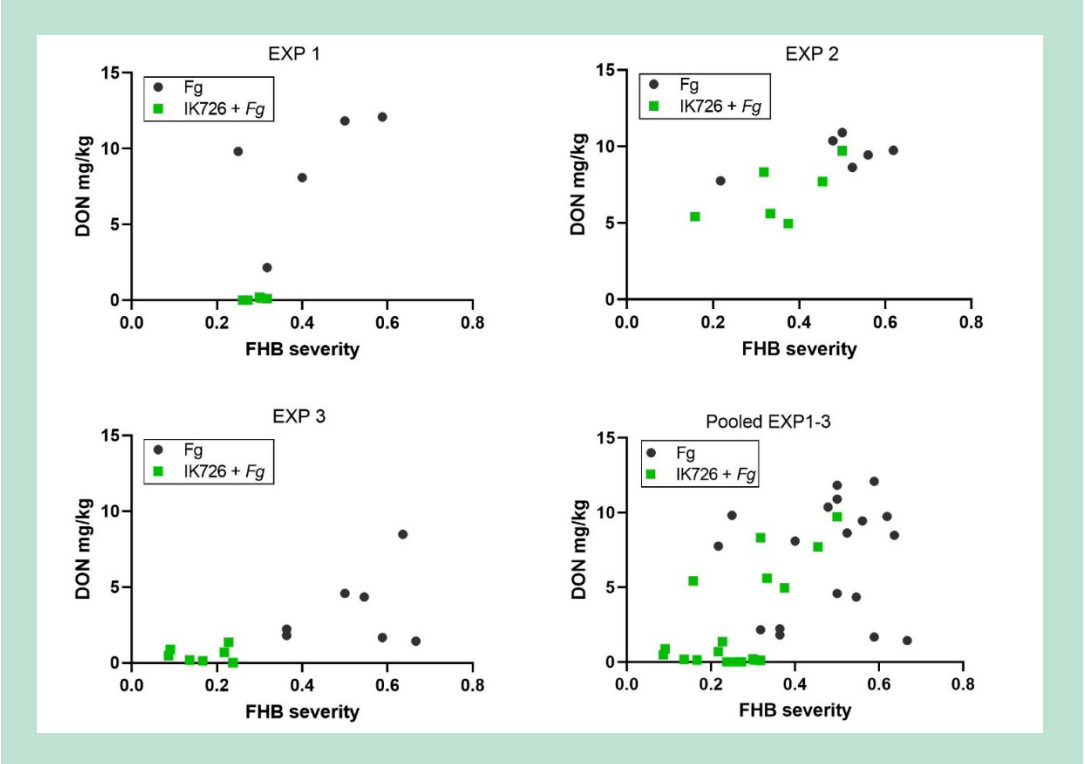

FIGURE 5.9.3. Biological control of Fusarium head blight (FHB) caused by *Fusarium graminearum* using *Clonostachys rosea* isolate IK726. Relationship between DON content in spikes (mg/kg) and FHB symptom severity in three independent experiments (EXP1, 2 and 3) and for pooled data of the experiments.

5.10 Fungicide sensitivity of fungal isolates

Germination of *C. rosea* and *A. alternatum* spores were unaffected at prothioconazole concentrations ≤0,012 and ≤ 0,037 ppm, respectively, whereas *P. olsonii* spores highly sensitive (Fig. 5.10.1). After 2 days of incubation the germination rate of all three isolates were ≥99%. Comparison of the germination of the three BCA after 1 day furthermore showed that *C. rosea* germinated faster than spores of the two other BCAs.

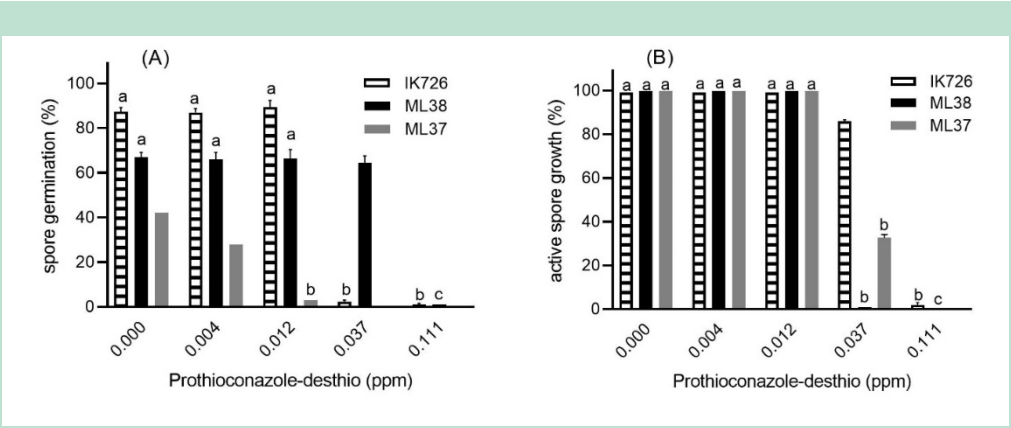

FIGURE 5.10.1. Effect of prothioconazole-desthio concentration (ppm) on Czapek Dox agar. (A) germination of fungal spores after incubation for 1 day and (B) continued hyphal growth of germinated spores after 2 or 3 days of incubation (active spores). Spores of the fungal biocontrol agents *Clonostachys rosea* (IK726), *Acremonium alternatum* (ML38) and *Penicillium olsonii* (ML37). Germination and hyphal growth were quantified by microscopy. Mean values within each of the three BCA treatment marked with different letters are significantly different (P <0.05).

The effect of prothioconazole-desthio on hyphal growth either from pure spores or from agar-plugs with established mycelial growth (no spores present) could grow at a higher fungicide concentration ≤ 0,111 ppm. This suggest that hyphae already established on agar are less sensitive to the fungicide than growth initiating from spores (Fig 5.10.2). The effect of increasing fungicide dosage on *C. rosea* colony morphology and radial growth can be seen in Figure 5.10.3.

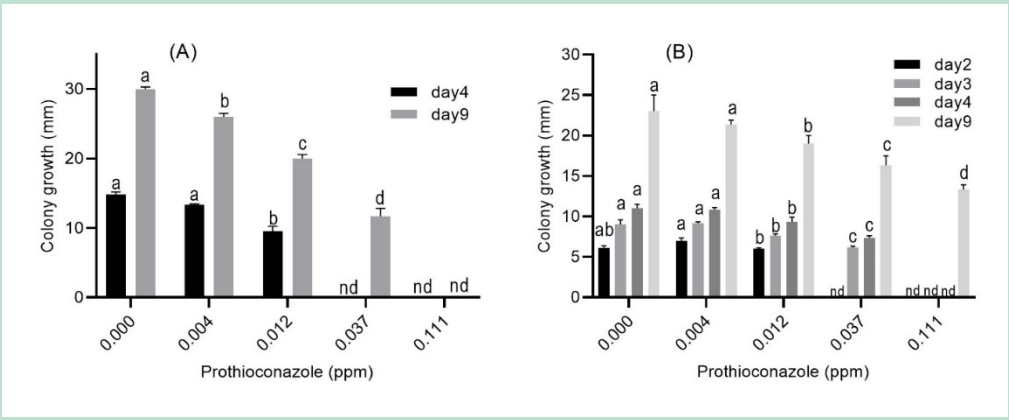

FIGURE 5.10.2. Effect of prothioconazole-desthio concentration (ppm) in Czapek Dox agar on colony growth of Clonostachys rosea IK726 measured as (colony diameter in mm after 2 to 9 days of incubation. (A) growth from spores and (B) growth from agar plugs. Mean values

within each of the four time points (days) and marked with different letters are significantly different (P <0.05).

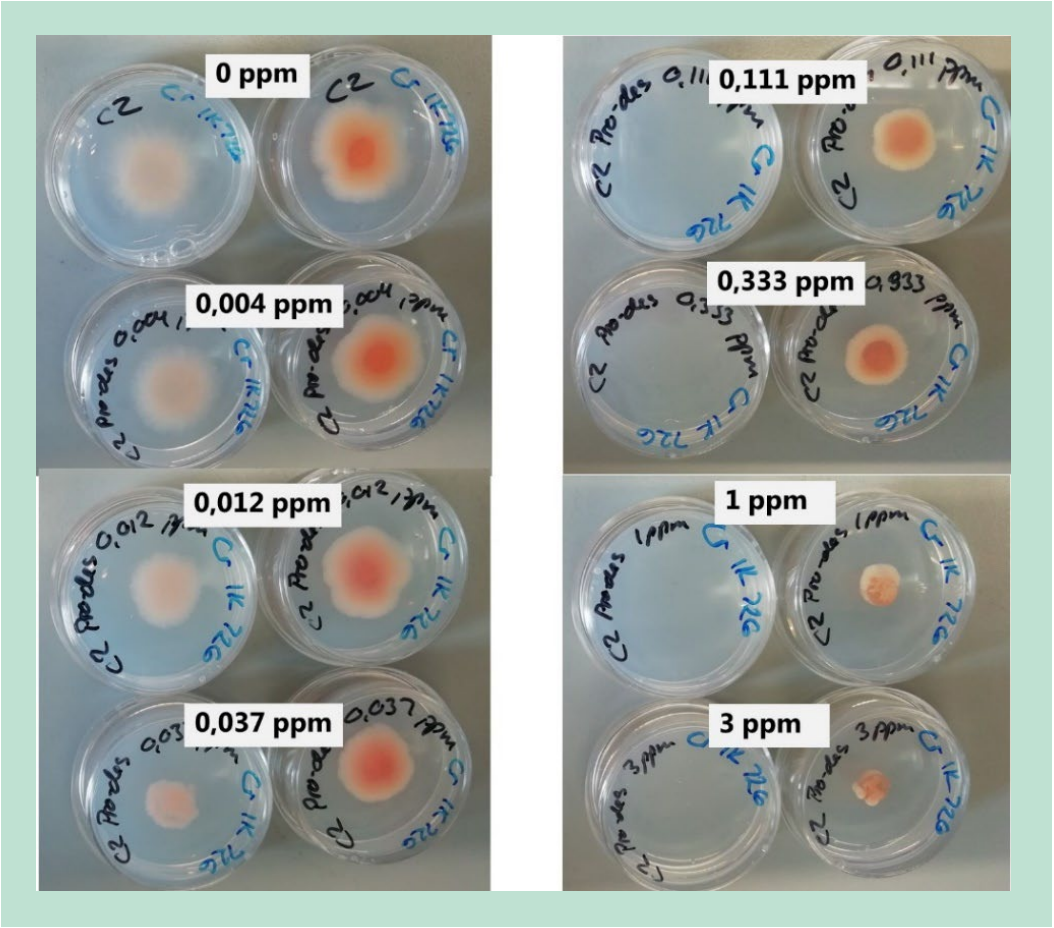

FIGURE 5.10.3. Effect of prothioconazole-desthio concentration (ppm) in agar medium on colony growth rate on Czapek Dox agar. Left side pictures at each concentration are from reverse side of petri dishes and right hand side pictures are from above.

6. Discussion and perspectives

The field trials in 2018, 2019 and 2020 were conducted under highly different weather conditions and this resulted in different development of STB and FHB between years. This is definitely a problem when testing the efficacy of biocontrol agents (BCAs) since statistically significant differences are often difficult to obtain at low disease levels. On the other hand, it can also give valuable information on the performance of BCAs under variable environmental conditions.

The DMI fungicide Proline EC 250 (prothiozonazole) was used as a reference fungicide mainly due to its very broad use, but also because of the problems with resistance associated with DMI use. Double treatments with Propulse SE 250 (2 x 1.0 /ha) was also included as a solution believed to provide a more effective control of both STB and FHB (L.N. Jørgensen pers com). However, it should be noted that the maximum Propulse rate permitted per season is only 1.0 l/ha and therefore the efficacy of Propulse as compared with all other treatments in the field trials represents an illegal solution in the Danish context. Other EU countries have a broader range of fungicides available providing more options for obtaining high levels of STB control and in this respect the double treatment with Propulse still represents a realistic scenario. Prothioconazole has showed a declining effect for particularly STB control over the last decade, due to increased fungicide resistance in *Z. tritici* populations. Other products (Balaya and Propulse SE 250) are now used in practice for control of *Z. tritici*. However, Proline EC 250 was chosen in order to look for possible changes in fungicide resistance and mutations in the *CYP51* gene of *Z. tritici.* This should enable us to test if the tested BCAs has potential to contribute to more sustainable disease control strategies.

Across the 3 seasons, several BCA treatments reduced the severity of STB significantly when applied either once or twice, providing moderate control depending on the timing of application. Indeed, at several assessments various BCA treatments gave disease control at similar levels as the fungicide Proline EC 250 when assessed during the growth period from early heading (GS 51) until early to medium grain development (GS 73-75). However, at later assessments (GS 79) the effect from BCAs was often reduced as compared to some fungicide treatments especially at high levels of STB attacks. This indicated a reduced persistence of BCAs when applied alone. In 2018, the efficacy of the best treatments with BCAs only were in the range of 50-75% STB reduction where relatively high STB attacks were seen at the early growth stages while infection of the flag leaf at later growth stages was insignificant. In 2019, STB was more severe throughout the season. Whereas the best BCA treatments reduced STB by 40-60% until the end of June, their effect failed at the last assessment in July where the flag leaf of the untreated control had reached approximately 80% STB. In comparison, Proline EC 250 treatments (0.4 L/ha) and (0.8 L/ha) applied both at GS 37-39 and GS 61-65 reduced STB by 50-70% at the first assessments and by 50-60% at the final assessment in 2019. For the 2020 trial, where the STB attack did not exceed 6% at any time point, the best single BCA treatments reduced STB in the range of 60-90%. Particularly, *C.rosea* consistently gave moderate control of STB when applied only one time at GS 37-39 irrespective of the differences in STB disease pressure between seasons. Very limited data exist regarding control of STB and FHB under field conditions. But low to moderate effects of Serenade ASO has previously been seen in field trials for control of yellow rust (Reiss & Jørgensen, 2017) and for control of powdery mildew in cereals (Matzen et al., 2019). However, in these experiments, the BCA was applied four times in order to get relatively consistent disease control.

Since *Z. tritici* can have several infection cycles throughout the growing season, it was surprising that the early single treatment with *C. rosea* had such a significant effect on STB. For example, in 2019, *C. rosea* applied 20th of May resulted in STB control 25th June on leaves that were not developed when the *C. rosea* spores were sprayed onto the canopy approximately 5 weeks earlier. A similar relationship was seen in 2018 where *C. rosea* treatment sprayed 23th May protected the two uppermost leaves 4 weeks later on 21st June (STB attack was minor but attacks were reduced by approximately 70% on leaf 1 and leaf 2). This points towards a systemic effect of the BCA treatment. This was further underlined by the microscopy investigation of the effect of *C. rosea* on the *Z. tritici* infection processes in a growth chamber experiment. Hence, the penetration efficiency of *Z. tritici* spores were lower and the pycnidia formation was impeded in leaves pre-treated with *C. rosea*. This indicates activation of the plant's own defence mechanisms (Fig 5.8.2).

There were only two seasons, 2019 and 2020, where BCA application for FHB control was tested since there was no FHB attack in 2018 due to the hot and dry weather. In 2019, *C. rosea* significantly reduced FHB when applied once at GS 37-39. Interestingly, the double treatment with *C. rosea* isolate IK726, applied at both GS 37-39 and GS 61 did not provide better control than the single treatment applied early despite the prediction that optimum application timing is usually being considered to be at GS 61-65 - when the spikelets are flowering. Significant control of FHB with *C. rosea* application at the late stage (GS 61) has, however, previously been observed in field trials (D. F. Jensen, unpublished). In support of the visual effects seen on FHB development as compared to the untreated control, *C. rosea* applied at GS 37-39 also reduced the level of DON significantly in 2019 by 34%. Field testing of the *C. rosea* isolate ACM941 in Canadian field trials has shown similar results with DON reduction from 21-33% (Xue et al., 2014, Hue et al., 2009). When applying *C. rosea* in combination with Proline EC 250, the DON reduction was also clear both in 2019 and 2020 with approximately 50% reduction as compared to the untreated control, although not significantly different from the corresponding single application of Proline EC 250. In addition, the ability of *C. rosea* to reduce *Fusarium* infection and DON accumulation was confirmed in three greenhouse experiments where the biomass of *F. graminearum* (pg *Fusarium* DNA/ng wheat DNA) was reduced by 50-86% and the DON content was reduced by 27-99% (Fig. 5.9.1 and 5.9.2, Table 5.9.1). Interestingly, Gimeno et al. (2021) recently showed that an oilbased formulation of *C. rosea* isolate SHA77.3 containing a sunscreen ingreidient reduced FHB in one of two tested cultivars and the DON content in both cultivars by 45-69%. However, the unformulated *C. rosea* SHA77.3 isolate (Tween added) had no significant effects on FHB or on DON in a field trial. Even though significant FHB reduction was obtained with unformulated conidia of isolate IK726 (Tween added) in the current trials, formulation is undoubtedly an important aspect, e.g. for UV protection of the spores as the work by Gimeno et al. (2021). This strongly emphasises that optimized formulation of a BCAs can improve field performance considerably.

The finding that single application of *C. rosea* at GS 37-39 can reduce FHB was further confirmed in the 2020 trial as both the single treatment at GS 37-39 and *C. rosea* single treatment at GS 61-65 (the latter treatment was only tested in 2020) reduced FHB significantly by approximately 50%. With these results in mind, one can speculate on how the early application of *C. rosea* can interact with the pathogen since *C. rosea* was applied more than three weeks before the *Fusarium* infection took place. This could indicate a systemic effect and one explanation could be that the BCA mediates an activation of the plants own defence system (induced resistance) and thereby indirectly control the disease. In line with this hypothesis, Rojas et al (2022) recently demonstrated that induced resistance probably played a role in biocontrol of FHB in wheat by the fungal BCA *Penicillium olsonii*. Results from the greenhouse experiments confirm the strong potential of *C. rosea* for reduction of FHB symptoms which coincided with a reduction of DON accumulation in spikes.

Detailed analyses of treatments only including *C. rosea*, Proline EC 250 at 0.4 L/ha and a combined treatment indicate that *C. rosea* has the potential to substitute the early Proline EC 250 application for control of STB and FHB. Furthermore, *C. rosea* applied early combined with Proline EC 250 (0.4 L/ha) applied at GS 61-65 gave significant reductions at the same level as a single reduced dose of Proline EC 250 applied either early or late. This combination could support control of both STB and FHB and suggests that inclusion of BCAs in disease control schemes can play a role in an IPM strategy, reducing the input of traditional fungicides. However further investigations are needed to examine whether this also will be the result in case of using more effective fungicides like Propulse SE 250 or newer active ingredients, when available.

The control achieved from both reduced rates of Proline EC 250 and the three different BCAs or combinations of these was in several cases less efficient than two treatments with full rates of either Proline EC 250 (0.8 L/ha) or with Propulse SE 250 (1.0 L/ha) (Table 5.1.1, 5.1.2 and 5.1.3). This could perhaps partly be related to insufficient compatibility with Proline EC 250 fungicides (Ons et al., 2020). For *C. rosea,* Prothioconazole-desthio (0.012-0.037 ppm in an agar medium) was inhibiting conidial germination (Fig 4.10.1) and mycelial growth (Fig. 4.10.2) even though dosages were incomparable with the field situation. However, generally little information on compatibility between microbial BCAs and fungicides are available in public. The co-formulation Propulse SE 250 (prothioconazole and fluapyram) provided better control of STB and similar control of FHB – in line with full rates of Proline EC 250. Double treatments with Propulse SE 250 are not legally accepted – but in practice, farmers include mixtures or co-formulations to achieve the most effective solutions. Farmers are particularly eager to pick the most effective solutions at flag leaf emergence – as this timing historically is seen as the most economical treatment. Therefore, one future step for integration of BCAs in disease management should e.g. be testing the combination of *C. rosea* with the current fungicide mixtures used.

It is an interesting hypothesis that a combination of two or more microbial species will improve the efficacy and stability of biological disease control. From a practical point of view, little is known, but much research has been done to select organisms that complement each other. The three organisms of this study are each well-known BCAs, but their efficacy in mixture against STB and FHB has not been tested previously. There was no clear evidence of improved control efficacy by mixing two different BCAs. Mixtures of *C. rosea* + *Pseudomonas chlororaphis* were tested in 2018, which was a very dry season resulting in low STB attack and no FHB. Data were therefore not strong enough to test the hypothesis of additive/synergistic effects of mixing the two BCA. In both 2019 and 2020, mixtures of *C. rosea* + Serenade ASO (*Bacillus velezensis*) were investigated and again, no consistent benefits from mixing *C. rosea* and the Bacillus strain were found. The results raise the question of whether it is actually a way to go. In fact, it is possible that there might as well be unpredicted interactions between the two microbial species decreasing the ability of one or both to display biocontrol activity. Xu et al. (2011a) and Xu et al. (2011b)) reviewed the difficulties in demonstrating additive or synergistic biocontrol effects as a result of mixing different BCAs. Their evaluations were based firstly on several published papers (Xu et al., 2011a) followed by a thorough analytical and theoretical investigation (Xu et al., 2011b).They found that there only were few examples published where synergistic effects by mixing two BCAs could be concluded based on sound statisitcs. A major result of their evaluation was that the effect of mixing more BCAs normally would not exceed the effect of single use of the best of the organisms included in a mixture. However, there seems in theory to be ways of optimizing the effect of mixing different BCAs if each work through a different biocontrol mechanism than the others in the mixture (i.e. antibiosis and mycoparasitism). Mixtures of *C. rosea* + Serenade ASO and *C. rosea* + *P. cholororaphis* combined with 0.4 L/ha Proline EC 250 applied both early and/or late did not result in superior disease control efficacy of STB and FHB as compared to the corresponding treatments with Proline EC 250 applied once, *i.e.* 'Proline EC 250 (0.4 L/ha) at GS 37-39' and 'Proline EC

250 (0.4 L/ha) or applied both at GS 37-39 and GS 61-65'. This strongly suggest that alternation between BCAs and fungicides would be a better strategy as shown by consistent effects obtained with the treatment *C. rosea* at GS 37-39 combined with Proline 0.4 L/ha at GS 61-65 (Fig. 5.2.1 - Fig 5.2.5 and Table 5.2.1).

As for the timing of BCA application, the question is whether the same application strategy should be used as for chemical fungicides? Hence, there are several considerations to make comprising timing, number and interval of repeated BCA applications and their compatibility with fungicides (Ons et al., 2020). For example should the BCAs be applied at earlier time points than the one used in this project? and what about pre-harvest application (during late ripening)? The application of BCAs have, contrary to fungicides no preharvest interval, and therefore can be applied after GS 65 during grain filling and early ripening. Such a strategy could have a positive effect on grain quality, for example, by reducing DON content, something which has not yet been documented. Strategies for BCA application may also depend on the mechanisms employed by the BCAs. Hence, induced resistance may be advantageous for the early BCA applications, whereas mycoparasitism may additionally be relevant for the late and pre-harvest applications. Therefore, it is important to establish which mechanisms BCAs use when controlling diseases on plants. Based on the infection studies on the effect of *C. rosea* on *Z. tritici* infection processes antibiosis and competition as well as induced resistance is likely involved. Therefore field trials with different timings of BCA application should be further investigated in order to identity the optimal application strategy which may not be similar to the one used for fungicide application. This would also be important for designing strategies for alternation between BCAs and fungicides.

Yield improvements from treatments were only statistically significant in 2019 while in 2018 and 2020 harvested yields of plots varied considerable and no significant differences were not seen. Only in 2019, all treatments including *C. rosea*, Proline EC 250 and *C. rosea* + Proline EC 250 increased yields significantly compared with untreated and no significant differences were measured between the core treatments. This may at least partly be reflected by the fact that disease levels varied considerably between years. It might also reflect the effect of Proline EC 250 was weakened and in recent years has dropped to ca. 40-50% control using a full rate (Jørgensen et al 2021). However, that may not be the case in the current field trials as the full dosis of Proline EC 250 (0.8 L/ha) applied twice gave yield increase not significantly different from Propulse SE 250 (1.0 L/ha) applied twice (total 2.0 L/ha). However, since only a total of 1.0 L/ha is allowed the effect of Propulse SE 250 may be overestimated as mentioned above. Thus, at low disease levels, it may be more difficult to observe differences between treatments since all may have more or less the same rather minor effect, whereas only very effective treatments may show an effect at high disease levels. Furthermore, drought occurred particularly in 2018, affecting both disease pressure from different diseases, but also the general yield levels

The variable results obtained raise the question of whether timing of BCA application needs to be different from traditional fungicides and whether optimisation can increase the general BCA effect. A closer analysis of the precise environmental conditions might also clarify whether particular environmental conditions can explain lack of effect. Earlier investigations have shown that BCA products are more challenged when it comes to outdoors than controlled conditions. Plant pathogens have variable environmental optima depending on e.g. temperature and humidity and likewise BCAs have specific requirements, which can challenge their use. Investigations of *B. velezensis* has shown that particularly severe epidemics of leaf diseases like yellow rust and powdery mildew also can limit the success from using BCAs compared to growth seasons with low to moderate attack (Reiss & Jørgensen, 2017, Matzen et al., 2019).Furthermore, it has also been demonstrated that high FHB resistance in wheat resulted in better biocontrol efficacy using *C. rosea* (Xue et al., 2014, Gimeno et al., 2021). These results point to also including other elements of IPM, with cultivar resistance being the most important.

C. rosea was only detected in low frequencies in leaf and spike samples by qPCR and most frequently from the first sampling time point at GS61 one day after application. Since the qPCR primers used are species but not isolate specific (M. Karlsson, pers.com) it is not possible to sort out if the detected *C. rosea* DNA originates from the applied *C. rosea* IK726 isolate. However, since none of the untreated samples revealed the presence of *C. rosea* DNA, it is highly likely that it was actually IK726 that was detected, but that the persistence of IK726 in the phyllosphere was very low. This was furthermore strengthened by the results of the amplicon sequence community analyses, which confirmed that *Clonostachys* amplicons were extremely rare but not more frequent in the *C. rosea* treated samples. This suggests a low indigenous phyllosphere colonisation by *Clonostachys* spp. Our results could imply that intensive colonisation of the phyllosphere by *C. rosea* is not a prerequisite for biocontrol by this BCA. If the mode of action is induction of host plant resistance, the BCA might only need to be actively interacting with the host plant for a short time after application to activate the resistance in the host plant. Even a constituent of a dead organism might be able to activate the induced resistance, acting as a MAMP (microbe-associated molecular pattern) or elicitor of defence (Haugaard et al., 2002, Shetty et al., 2009). Nevertheless, the inability to detect the BCA in high quantities can be considered as a positive trait since it indicates that the organism is not surviving and proliferating for a long time in the environment (low persistence), despite having an effect on disease control. Furthermore, the apparent low colonisation ability in the field is in line with the community analyses showing that *C. rosea* application did not affect the fungal community structure in the phyllosphere.

Application of BCAs in a crop protection scheme may also have another beneficial effect. Thus, by using different modes of action, resistance in the pathogens against traditional fungicides may be reduced. Results from the CYP51 mutations analyses did not give a clear picture despite some smaller differences in mutation frequencies between treatments. Furthermore, there were no apparent differences in the sensitivity to prothioconazole in *Z. tritici* isolates recovered from the different treatments. It may be necessary to follow the CYP51 mutation frequencies in *Z. tritici* populations over a longer time span, i.e. several years, to see an effect of relevance for preventing fungicide resistance. There might also already be a high frequency of some of the important CYP51 mutations in the natural pathogen populations at the experimental site at Flakkebjerg, where azole fungicides have been used over many years in efficacy tests. The current Danish *Z. tritici* population is highly adapted to prothioconazole (Proline EC 250) and strains tested already show high frequencies of marker mutations prior to the treatments (Heick et al., 2020). It can be assumed that more drastic changes in CYP51 mutations (e.g. introducing a new azole that selects in a different way) are needed in order to demonstrate any effects on CYP51 mutations using BCAs in IPM strategies. For research purposes, this could probably be done by increasing the number of DMI fungicide application to accelerate the build-up of fungicide resistance. In fact, Heick et al. (2017) showed that three applications of e.g. prothioconazole changed the frequencies of CYP51 alteration, but also single and split treatments of specific azoles have been demonstrated to impact CYP51 mutation rates (Wieczorek et al., 2015, Vestergaard et al. 202x (submitted 2022)). Alternatively, fungicides with different modes of action, which have currently not been selected for on the same level as for azoles, might be used to investigate the potential effect of BCAs in a spray programme with regard to fungicide resistance. This kind of field experiments could alternatively be supported by an experimental evolution approach, in which fungicide resistance development is provoked by exposing a wild-type strain to a fungicide at minimum inhibitory concentration (Ballu et al, BioRxiv 2021)

A relatively unexplored effect of the use of BCAs for crop protection in the phyllosphere is related to how the natural microbial communities present in the ecosystem are affected by such applications. It is envisaged that there may be an effect, but will it be detrimental, for example, by eradicating beneficial microorganisms naturally keeping the pathogens in check? Or will it

be beneficial by e.g. reducing mycotoxin producing fungi like *Alternaria*, *Aspergillus* and *Penicillium* being a beneficial community change? The fungal community of wheat leaves has often been shown to consist of 'pink' yeasts (*Sporobolomyces* and *Rhodotorula* producing carotenoid pigments), 'white' yeasts (*Cryptococcus*) and ascomycete saprotrophs (Fokkema & De Nooij, 1981, Magan & Lacey, 1986, Karlsson et al., 2014, Latz et al., 2021). We identified the wheat pathogens *Zymospetoria, Blumeria graminis, Puccinia striformis* and *P. recondita* as well *Sporobolomyces* spp. and *Alternaria spp.* among the 29 most abundant OTUs using PacBio amplicon sequencing. However, no major effects of BCA treatments on the mycobiome composition were seen, so the effects might only last for a short while. However, further mining and analyses of the sequencing data may reveal more details of interest. Furthermore, a long term effect could not be ruled out as we only investigated the community structure over a few weeks.

An obvious question is whether BCAs can become an economically viable tool for farmers? In order for the farmer to invest and use a BCA product he/she needs to have a relative good indication on the economical net-return. The current study has not revealed any clear the economic benefit, as only one of 3 trials gave a significant and positive yield increase. It is difficult to estimate the production costs, but there are clear intentions from large crop protection companies to develop biocontrol methods for control of FHB and STB, including the use of *C. rosea* (pending patent application). However, it is expected to become a valid option economically, if trial data can verify an economic benefit for the farmers. Currently, pesticides in Denmark are taxed based on their toxicity profiles and if and when BCA can match the efficacy- and yield responding profile of chemical fungicides or substitute e.g. one fungicide treatment, BCA products should have an advantage due to expected lower taxation following a better toxicity profile. Most BCAs are permitted for use in organic production. As this segment is increasing BCAs could become an important tool for future disease control in this production segment as well. The cost of using Serenade ASO - the only BCA authorized in wheat is approximately 500 kr pr treatment (4 l/ha). Which requires a return equal to min 3-4 hkg/ha. Similarly to this business scenario – *C. rosea* would probably require a similar size of return in order to be attractive to use for both conventional and organic farmers. Based on the field result from 2019 where *C. rosea* applied once at GS 37-39 resulted in 6.7 hkg/ha yield increase, the feasibility of using this BCA for disease control in wheat is promising.

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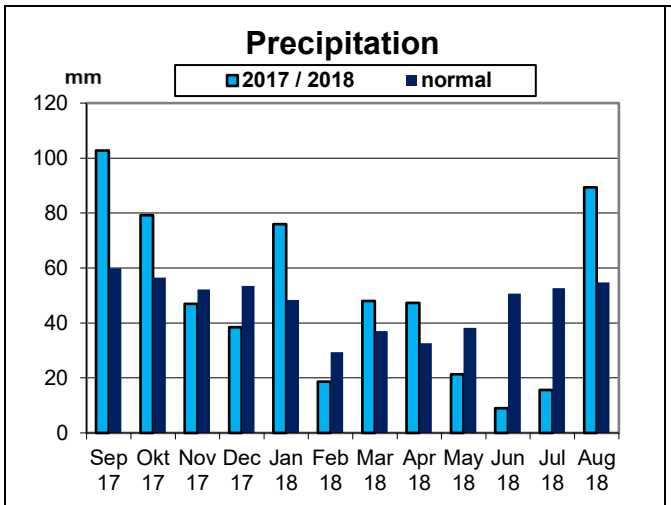
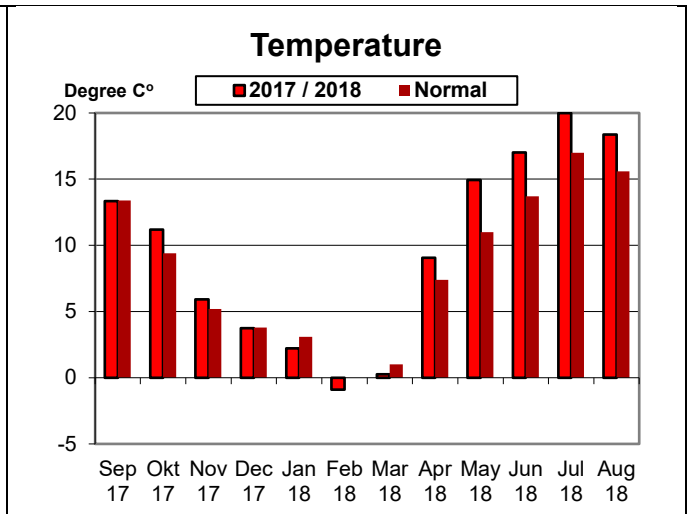
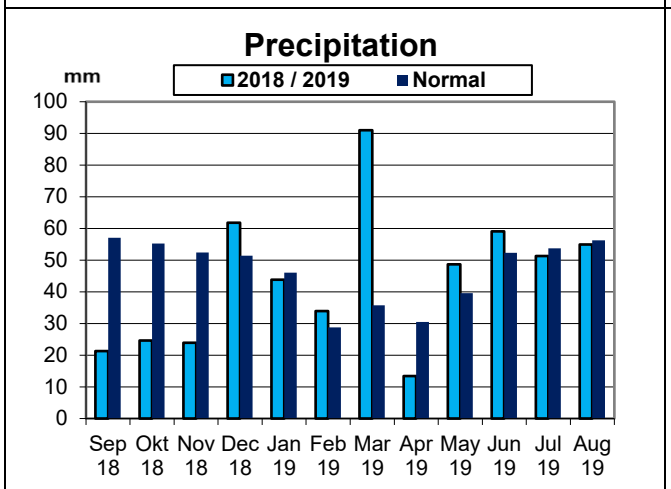
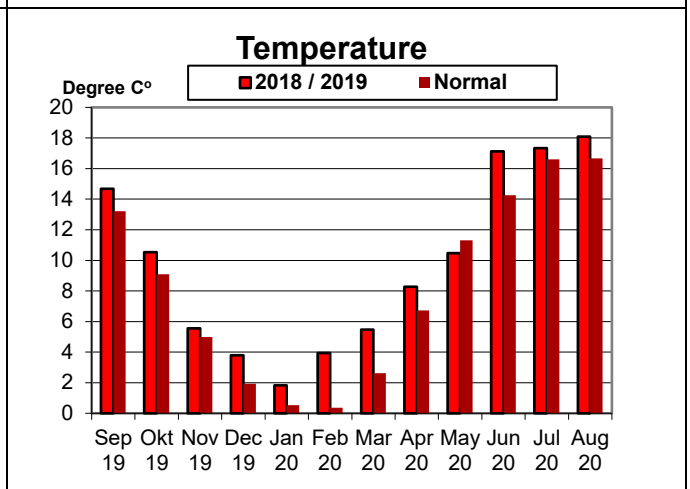
8. Appendix 1

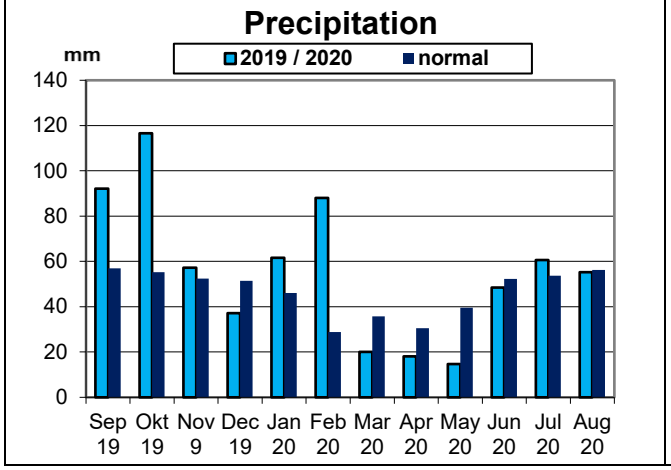
Appendix 1.1 Weather data from field experiment in 2018-2020 (Normal covers 40 year average 1973-2013)

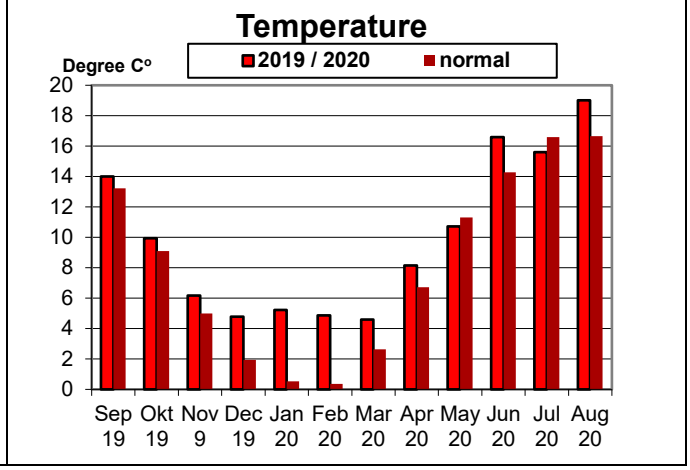












9. Appendix 2

Supplementary data table 1. Complete data set and analyses for the winter wheat field trial in 2018 at Research Center Flakkebjerg. The effects of the BCAs *Clonostachys rosea* and *B. velezensis* (Serenade ASO) on Septoria tritici blotch (STB) and Fusarium head blight (FHB) were tested. BCAs were compared with Proline EC 250 in reduced dose (0.4 L/ha) and full dose (0.8 L/ha). Combinations of BCAs and Proline EC 250 (0.4 L/ha) were also tested. Treatment were applied in growth stage (GS) 37-39 and/or GS 61-65. Propulse SE 250 (1.0 L/ha) applied twice was included as fungicide control.

Code	Treatment	Dose (L/ha)	Growth stage	STB leaf 4-6 18th May		STB leaf 4 24th may		STB leaf 4 1st June		STB leaf 3 1st June		STB leaf 3 11th June		STB leaf 2 11th June		STB leaf 3 21st June	
1	Untreated		-	15.0	A	20.0	A	35.0	A	0.1	A	18.8	A	1.6	A	31.3	A
2	Proline EC 250	0.4	37-39	15.0	A	20.0	A	35.0	A	0.1	A	5.0	BCD	0.3	B	12.5	B
3	Proline EC 250	0.4	61-65	15.0	A	20.0	A	35.0	A	0.1	A	10.0	ABCD	0.4	B	11.9	B
4	Proline EC 250 Proline EC 250	0.4 0.4	37-39 61-65	15.0	A	20.0	A	35.0	A	0.1	A	17.5	ABCD	0.9	AB	12.5	B
5	Proline EC 250 Proline EC 250	0.8 0.8	37-39 61-65	15.0	A	20.0	A	35.0	A	0.1	A	11.3	ABCD	0.2	B	6.3	B
6	Proline EC 250	0.8	37-39	15.0	A	20.0	A	35.0	A	0.1	A	4.4	CD	0.1	B	12.5	B
7	*C. rosea*	1.0	37-39	15.0	A	20.0	A	35.0	A	0.1	A	12.5	ABC	0.4	B	14.4	B
8	*C. rosea* *P. chlororaphis*	1.0 1.0	37-39 37-39	15.0	A	20.0	A	35.0	A	0.1	A	13.8	ABC	0.4	B	13.8	B
9	Proline EC 250 *C. rosea* *P. chlororaphis*	0.4 1.0 1.0	37-39 37-39 37-39	15.0	A	20.0	A	35.0	A	0.1	A	6.9	BCD	0.2	B	7.0	B
10	*C. rosea* *C. rosea*	1.0 1.0	37-39 61-65	15.0	A	20.0	A	35.0	A	0.1	A	11.3	ABCD	0.5	B	12.5	B
11	*P. chlororaphis* *P. chlororaphis*	1.0 1.0	37-39 61-65	15.0	A	20.0	A	35.0	A	0.1	A	12.5	ABC	0.7	B	15.0	B
12	*C. rosea* *P. chlororaphis* *C. rosea* *P. chlororaphis*	1.0 1.0 1.0 1.0	37-39 37-39 61-65 61-65	15.0	A	20.0	A	35.0	A	0.1	A	8.8	ABCD	0.4	B	12.5	B
13	*C. rosea* *P. chlororaphis* Proline EC 250 *C. rosea* *P. chlororaphis* Proline EC 250	1.0 4.0 0.4 1.0 4.0 0.4	37-39 37-39 37-39 61-65 61-65 61-65	15.0	A	20.0	A	35.0	A	0.1	A	5.0	CD	0.2	B	10.6	B
14	Propulse SE 250 Propulse SE 250	1.0 1.0	37-39 61-65	15.0	A	20.0	A	35.0	A	0.1	A	3.8	D	0.1	B	10.6	B
	P-value			1.0000		1.0000		1.0000		1.0000		<.0001		<.0001		<.0001	

Supplementary data table 1 (continued).

	Treatment	Dose (L/ha)	Growth stage	STB leaf 2 21st June		STB leaf 1 21st June		Yellow rust 21st June		Brown rust 21st June		Fusarium index 28th June		Fusarium pct. 28th June		Fusarium scale 28th June		Yield Hkg/ha	
1	Untreated		-	11.9	A	2.3	A	5.0	A	15.0	A	0.2	A	5.5	A	3.0	A	83.9	A
2	Proline EC 250	0.4	37-39	2.9	B	0.5	B	0.0	D	0.4	B	0.1	A	6.0	A	2.0	A	83.1	A
3	Proline EC 250	0.4	61-65	2.8	B	0.4	B	0.4	CD	1.0	B	0.1	A	3.5	A	3.0	A	79.3	A
4	Proline EC 250 Proline EC 250	0.4 0.4	37-39 61-65	2.8	B	0.3	B	0.5	BCD	1.3	B	0.1	A	2.5	A	2.5	A	84.1	A
5	Proline EC 250 Proline EC 250	0.8 0.8	37-39 61-65	0.9	B	0.1	B	0.1	D	0.0*	B	0.1	A	3.5	A	2.3	A	84.4	A
6	Proline EC 250	0.8	37-39	2.1	B	0.3	B	0.0[1]	D	0.3	B	0.2	A	4.5	A	3.0	A	80.7	A
7	*C. rosea*	1.0	37-39	4.0	B	0.8	B	5.5	A	0.8	B	0.1	A	4.0	A	2.8	A	76.8	A
8	*C. rosea* *P. chlororaphis*	1.0 1.0	37-39 37-39	3.3	B	0.5	B	0.3	CD	2.8	AB	0.1	A	2.5	A	2.5	A	80.0	A
9	*C. rosea* *P. chlororaphis* Proline EC 250	1.0 1.0 0.4	37-39 37-39 37-39	0.9	B	0.2	B	0.1	D	0.1	B	0.1	A	3.5	A	1.8	A	81.7	A
10	*C. rosea* *C. rosea*	1.0 1.0	37-39 61-65	3.9	B	0.3	B	0.0	D	0.0*	B	0.2	A	4.0	A	3.5	A	81.2	A
11	*P. chlororaphis* *P. chlororaphis*	1.0 1.0	37-39 61-65	3.4	B	0.6	AB	1.0	ABC	1.3	B	0.3	A	6.0	A	3.5	A	79.7	A
12	*C. rosea* *P. chlororaphis* *C. rosea* *P. chlororaphis*	1.0 1.0 1.0 1.0	37-39 37-39 61-65 61-65	4.1	B	0.5	AB	6.0	A	1.8	AB	0.1	A	3.0	A	2.3	A	78.7	A
13	Proline EC 250 *C. rosea* *P. chlororaphis* Proline EC 250 *C. rosea* *P. chlororaphis*	1.0 4.0 0.4 1.0 4.0 0.4	37-39 37-39 37-39 61-65 61-65 61-65	3.0	B	0.3	B	0.3	CD	0.3	B	0.1	A	5.5	A	2.5	A	79.7	A
14	Propulse SE 250 Propulse SE 250	1.0 1.0	37-39 61-65	0.6	B	0	B	0.3	CD	0.4	B	0.1	A	3.0	A	3.0	A	88.0	A
	P-value			<.0001		<.0001		<.0001		<.0001		0.8229		0.1958		0.8612		0.0.0821	

[1]0.03

Percentage STB coverage of green leaves (STB percent). Fusarium head blight was scored by three methods: i) the number of attacked heads counted in 4 x 1m row per plot (Fusarium number), ii) average attack of FHB on the assessed heads was scored using a 1-9 scale (Fusarium scale), and iii) visual score of percent attacked heads per plot (Fusarium pct). All data in the table were transformed before analysis by square root (FACTOR+2), except for Yellow rust and Fusarium index being transformed by 1/(square root (FACTOR+1)). Data for STB leaf 3 on June 11th was not transformed. BCAs applied 23/5 and 4/6 and fungicides applied 16/5 and 6/6.

Supplementary data table 2. Complete data set and analyses for the winter wheat field trial in 2019 at Research Center Flakkebjerg. The effects of the BCAs *Clonostachys rosea* and *B. velezensis* (Serenade ASO) on Septoria tritici blotch (STB) and Fusarium head blight (FHB) were tested. BCAs were compared with Proline EC 250 in reduced dose (0.4 L/ha) and full dose (0.8 L/ha). Combinations of BCAs and Proline EC 250 (0.4 L/ha) were also tested. Treatment were applied in growth stage (GS) 37-39 and/or GS 61-65. Propulse SE 250 (1.0 L/ha) applied twice was included as fungicide control.

	Treatment	Dose (L/ha)	Growth stage	STB percent Leaf 1 3rd June		Powdery mildew leaf 1-4 3rd June		STB percent leaf 1 14th June		STB percent leaf 2 14th June		STB percent leaf 1 25th June		STB percent leaf 2 25th June		Fusarium number 28th June	
1	Untreated		-	27.5	A	9.0	A	2.8	A	17.0	A	13.0	A	47.5	A	4.4	A
2	Proline EC 250	0.4	37-39	20.0	AB	4.5	AB	1.0	C	7.3	BCD	8.3	ABCD	28.8	B	2.2	BC
3	Proline EC 250	0.4	61-65	27.5	A	6.3	AB	1.0	C	9.3	BC	9.0	ABCD	27.5	BC	1.2	BC
4	Proline EC 250 Proline EC 250	0.4 0.4	37-39 61-65	18.8	AB	4.8	AB	0.5	C	7.3	BCD	4.0	CD	23.8	BC	0.8	BC
5	Proline EC 250 Proline EC 250	0.8 0.8	37-39 61-65	17.5	AB	5.3	AB	0.5	C	5.3	CD	4.5	BCD	23.8	BC	0.4	C
6	*C. rosea* Proline EC 250	1.0 0.4	37-39 61-65	17.5	AB	3.5	B	0.9	B	8.0	BCD	8.8	ABCD	33.8	AB	0.7	BC
7	*C. rosea*	1.0	37-39	18.8	AB	4.5	AB	0.6	C	5.8	CD	12.0	AB	27.5	BC	2.2	BC
8	*C. rosea* Serenade ASO	1.0 4.0	37-39 37-39	17.5	AB	4.8	AB	1.0	C	7.8	BCD	10.0	ABC	26.3	BC	1.7	BC
9	*C. rosea* Serenade ASO Proline EC 250	1.0 4.0 0.4	37-39 37-39 37-39	15.5	B	5.8	AB	0.8	C	8.3	ABCD	6.0	ABCD	32.5	ABC	2.0	BC
10	*C. rosea* *C. rosea*	1.0 1.0	37-39 61-65	18.8	AB	5.8	AB	1.4	BC	8.8	BC	13.0	A	33.8	AB	2.2	BC
11	Serenade ASO Serenade ASO	4.0 4.0	37-39 61-65	21.6	AB	5.8	AB	1.0	C	9.5	BC	10.0	ABC	31.3	ABC	2.5	AB
12	*C. rosea* Serenade ASO *C. rosea* Serenade ASO	1.0 4.0 1.0 4.0	37-39 37-39 61-65 61-65	25.0	AB	5.8	AB	2.3	ABC	12.8	AB	11.3	ABC	35.0	AB	2.2	BC
13	*C. rosea* Serenade ASO Proline EC 250 *C. rosea* Serenade ASO Proline EC 250	1.0 4.0 0.4 1.0 4.0 0.4	37-39 37-39 37-39 61-65 61-65 61-65	19.3	AB	5.3	AB	1.0	C	7.8	BCD	7.0	ABCD	27.5	BC	1.4	BC
14	Propulse SE 250 Propulse SE 250	1.0 1.0	37-39 61-65	18.0	AB	3.7	B	0.2	C	2.5	D	2.0	D	16.2	C	0.5	C
	P-value			0.0005		0.0457		<.0001		<.0001		0.0003		0.0014		<.0001	

Supplementary data table 2 (continued).

	Treatment	Dose (L/ha)	Growth stage	Fusarium scale 1-9 28th June		Fusarium percent 7th July		STB percent 7th July		Yield 9th August		NIV ppb		DON ppb		ZEA Ppb	
1	Untreated		-	5.0	A	38.8	A	76.3	A	74.9	A	35.7	A	343.7	A	24.0	A
2	Proline EC 250	0.4	37-39	3.0	AB	25.0	ABC	55.0	ABCD	81.6	ABC	18.0	A	211.0	AB	10.0	A
3	Proline EC 250	0.4	61-65	2.3	B	18.8	BC	65.0	AB	83.5	ABC	22.3	A	126.0	AB	10.0	A
4	Proline EC 250 Proline EC 250	0.4 0.4	37-39 61-65	1.5	B	18.8	BC	37.5	CDE	86.2	BC	27.0	A	123.3	AB	19.7	A
5	Proline EC 250 Proline EC 250	0.8 0.8	37-39 61-65	1.3	B	4.5	D	31.3	DE	91.4	CD	26.0	A	70.0	B	11.3	A
6	*C. rosea* Proline EC 250	1.0 0.4	37-39 61-65	1.8	B	13.8	CD	52.5	ABCD	84.8	ABC	21.3	A	143.3	AB	10.0	A
7	*C. rosea*	1.0	37-39	2.8	B	25.0	ABC	65.0	AB	81.6	ABC	23.0	A	225.7	AB	10.0	A
8	*C. rosea* Serenade ASO	1.0 4.0	37-39 37-39	2.8	B	31.3	AB	65.0	AB	78.7	AB	14.7	A	225,7	AB	10.0	A
9	*C. rosea* Serenade ASO Proline EC 250	1.0 4.0 0.4	37-39 37-39 37-39	3.0	AB	22.5	ABC	57.5	ABC	83.0	ABC	17.7	A	317,0	AB	19.0	A
10	*C. rosea* *C. rosea*	1.0 1.0	37-39 61-65	3.0	AB	30.0	AB	66.3	AB	81.9	ABC	31.7	A	229.7	AB	17.0	A
11	Serenade ASO Serenade ASO	4.0 4.0	37-39 61-65	3.3	AB	26.3	ABC	65.0	AB	76,8	AB	27.0	A	247.0	AB	11.7	A
12	*C. rosea* Serenade ASO *C. rosea* Serenade ASO	1.0 4.0 1.0 4.0	37-39 37-39 61-65 61-65	3.0	AB	37.5	AB	72.5	A	80.8	AB	17.7	A	297.7	AB	18.7	A
13	*C. rosea* Serenade ASO Proline EC 250 *C. rosea* Serenade ASO Proline EC 250	1.0 4.0 0.4 1.0 4.0 0.4	37-39 37-39 37-39 61-65 61-65 61-65	2.3	B	18.8	BC	45.0	BCDE	84.5	ABC	10.0	A	153.7	AB	12.3	A
14	Propulse SE 250 Propulse SE 250	1.0 1.0	37-39 61-65	1.3	B	4.5		23.8	E	97.4	D	22.3	A	120.0AB		10.0	A
	P-value			<.0001		<.0001		<.0001		0.0001		0.7739		0.0066		0.4456	

Percentage STB coverage of green leaves (STB percent). Fusarium head blight was scored by three methods: i) the number of attacked heads counted in 4 x 1m row per plot (Fusarium number), ii) average attack of FHB on the assessed heads was scored using a 1-9 scale (Fusarium scale), and iii) visual score of percent attacked heads per plot (Fusarium pct). Only data for Fusarium percent July 7th and DON were transformed before analysis by square root (FACTOR+2). BCAs applied 20/5 and 11/6 and fungicides applied 21/5 and 12/6.

Supplementary data table 3. Complete data set and analyses for the winter wheat field trial in 2020 at Research Center Flakkebjerg. The effects of the BCAs *Clonostachys rosea* and *B. velezensis* (Serenade ASO) on Septoria tritici blotch (STB) and Fusarium head blight (FHB) were tested. BCAs were compared with Proline EC 250 in reduced dose (0.4 L/ha) and full dose (0.8 L/ha). Combinations of BCAs and Proline EC 250 (0.4 L/ha) were also tested. Treatment were applied in growth stage (GS) 37-39 and/or GS 61-65. Propulse SE 250 (1.0 L/ha) applied twice was included as fungicide control.

	Treatment	Dose (L/ha)	Growth stage	STB leaf 4 1st June		STB leaf 3 1st June		STB leaf 3 10th June		STB leaf 2 10th June		Fusarium number 1st July		Fusarium scale 1-9 1st July		Fusarium number 7th July	
1	Untreated		-	1.5	A	5.0	A	5.8	A	1.4	A	9.8	A	3.0	A	14.5	A
2	Proline EC 250	0.4	37-39	0.5	ABCD	2.0	AB	5.5	A	0.6	A	4.5	B	3.0	A	12.3	AB
3	Proline EC 250	0.4	61-65	0.6	ABCD	2.3	AB	2.8	A	0.1	A	4.0	B	3.0	A	11.0	AB
4	Proline EC 250 Proline EC 250	0.4 0.4	37-39 61-65	0.0*	D	1.1	AB	3.3	A	0.4	A	4.5	B	3.0	A	11.3	AB
5	Proline EC 250 Proline EC 250	0.8 0.8	37-39 61-65	0.3	ABCD	2.8	AB	3.0	A	0.3	A	3.0	B	3.0	A	6.0	B
6	*C. rosea* Proline EC 250	1.0 0.4	37-39 61-65	0.4	ABCD	2.5	AB	4.0	A	0.7	A	4.5	B	3.0	A	10.8	AB
7	*C. rosea*	1.0	37-39	0.2	BCD	1.9	AB	4.5	A	0.6	A	4.5	B	3.0	A	15.0	A
8	*C. rosea* Serenade ASO	1.0 4.0	37-39 37-39	0.2	BCD	2.5	AB	2.8	A	0.1	A	4.0	B	3.0	A	12.0	AB
9	*C. rosea* Serenade ASO Proline EC 250	1.0 4.0 0.4	37-39 37-39 37-39	0.4	ABCD	3.3	AB	5.3	A	1.0	A	5.8	AB	3.0	A	14.0	A
10	*C. rosea* *C. rosea*	1.0 1.0	37-39 61-65	0.8	ABCD	1.8	AB	3.5	A	0.4	A	6.0	AB	3.0	A	16.8	A
11	Serenade ASO Serenade ASO	4.0 4.0	37-39 61-65	0.1	CD	1.5	B	3.0	A	0.6	A	5.8	AB	3.0	A	12.8	AB
12	*C. rosea* Serenade ASO *C. rosea* Serenade ASO	1.0 4.0 1.0 4.0	37-39 37-39 61-65 61-65	1.1	AB	3.3	AB	4.0	A	0.4	A	5.0	B	3.0	A	15.5	A
13	*C. rosea* Serenade ASO Proline EC 250 *C. rosea* Serenade ASO Proline EC 250	1.0 4.0 0.4 1.0 4.0 0.4	37-39 37-39 37-39 61-65 61-65 61-65	0.0	D	0.4	B	3.6	A	0.4	A	3.5	B	3.0	A	9.8	AB
14a	*C. rosea*	1.0	61-65	1.0	ABC	4.5	A	4.0	A	0.4	A	4.8	B	3.0	A	13.3	AB
14	Propulse SE 250 Propulse SE 250	1.0 1.0	37-39 61-65	0.3	BCD	1.5	AB	3.0	A			3.5	B	3.0	A	10.3	AB
	P-value			<.0001		0.0058		0.4424		0.1384		0.0024		1.0000		<.0011	

*0.03

*0.03

Supplementary data table 3 (continued).

	Treatment	Dose (L/ha)	Growth stage	Fusarium scale 1-9 7th July	Yield 14th August	NIV ppb	DON ppb	ZEA ppb	T2 Ppb	HT2 ppb
1	Untreated		-	4.0 A	71.2 A	18.2 A	1232.0 A	11.0 A	9.1 A	9.1 A
2	Proline EC 250	0.4	37-39	4.0 A	74.4 A	24.6 A	1025.1 AB	9.0 A	9.1 A	9.1 A
3	Proline EC 250	0.4	61-65	4.0 A	74.9 A	19.2 A	470.0 BC	10.0 A	10.3 A	10.2 A
4	Proline EC 250 Proline EC 250	0.4 0.4	37-39 61-65	4.0 A	81.2 A	17,9 A	375.7 C	9.1 A	9.1 A	9.1 A
5	Proline EC 250 Proline EC 250	0.8 0.8	37-39 61-65	4.0 A	85.2 A	32.3 A	345.1 C	9.1 A	9.2 A	10.8 A
6	*C. rosea* Proline EC 250	1.0 0.4	37-39 61-65	4.0 A	79.2 A	27.7 A	399.5 C	9.1 A	9.2 A	10.8 A
7	*C. rosea*	1.0	37-39	4.0 A	77.9 A	27.0 A	870.8 ABC	9.1 A	9.2 A	9.1 A
8	*C. rosea* Serenade ASO	1.0 4.0	37-39 37-39	4.0 A	75.5 A	31.4 A	828.5 ABC	9.1 A	9.2 A	9.1 A
9	*C. rosea* Serenade ASO Proline EC 250	1.0 4.0 0.4	37-39 37-39 37-39	4.0 A	67.2 A	27.7 A	797.8 ABC	9.1 A	9.2 A	9.1 A
10	*C. rosea* *C. rosea*	1.0 1.0	37-39 61-65	4.0 A	75.2 A	18.1 A	977.8 AB	9.1 A	9.2 A	9.1 A
11	Serenade ASO Serenade ASO	4.0 4.0	37-39 61-65	4.0 A	70.3 A	30.8 A	900.8 ABC	16.5 A	9.2 A	13.1 A
12	*C. rosea* Serenade ASO *C. rosea* Serenade ASO	1.0 4.0 1.0 4.0	37-39 37-39 61-65 61-65	4.0 A	75.9 A	18.1 A	1365.0 A	16.8 A	9.2 A	11.5 A
13	*C. rosea* Serenade ASO Proline EC 250 *C. rosea* Serenade ASO Proline EC 250	1.0 4.0 0.4 1.0 4.0 0.4	37-39 37-39 37-39 61-65 61-65 61-65	4.0 A	74.5 A	18.1 A	344.0 C	9.1 A	9.2 A	10.5 A
14a	*C. rosea*	1.0	61-65	4.0 A	75.3 A	27.8 A	1027.0 AB	9.2 A	9.2 A	9.2 A
14	Propulse SE 250 Propulse SE 250	1.0 1.0	37-39 61-65	4.0 A	85.3 A	19.8 A	368.8 C	9.2 A	9.2 A	9.2 A
	P-value			1.000	0.1052	0.0629	<.0001	0.1026	0.1318	0.0540

Percentage STB coverage of green leaves (STB percent). Fusarium head blight was scored by two: i) the number of attacked heads counted in 4 x 1m row per plot (Fusarium number), ii) average attack of FHB on the assessed heads was scored using a 1-9 scale (Fusarium scale). All data in the table were transformed before analysis: square root (FACTOR+2), except toxins, which were transformed: 1/square root (FACTOR+1). BCAs applied 20/5 and 11/6 and fungicides applied 21/5 and 12/6.

Supplementary data table 4. Complete data set and analyses for the winter wheat field trial in 2018 at Research Center Flakkebjerg where only data for the treatments *C. rosea*, and reduced dosage of Proline (0,4 l/ha) were included. Treatment were applied in growth stage (GS) 37-39 and/or GS 61-65.

	Treatment	Dose (L/ha)	Growth stage	STB leaf 4-6 18th May		STB leaf 4 24th may		STB leaf 4 1st June		STB leaf 3 1st June		STB leaf 3 11th June		STB leaf 2 11th June		STB leaf 3 21st June	
1	Untreated		-	15.0	A	20.0	A	35.0	A	0.1	A	18.8	A	1.6	A	31.3	A
2	Proline EC 250	0.4	37-39	15.0	A	20.0	A	35.0	A	0.1	A	5.0	C	0.3	B	12.5	B
3	Proline EC 250	0.4	61-65	15.0	A	20.0	A	35.0	A	0.1	A	10.0	B	0.4	B	11.9	B
4	Proline EC 250 Proline EC 250	0.4 0.4	37-39 61-65	15.0	A	20.0	A	35.0	A	0.1	A	17.5	A	0.9	B	12.5	B
7	*C. rosea*	1.0	37-39	15.0	A	20.0	A	35.0	A	0.1	A	12.5	B	0.4	B	14.4	B
10	*C. rosea* *C. rosea*	1.0 1.0	37-39 61-65	15.0	A	20.0	A	35.0	A	0.1	A	11.3	B	0.5	B	12.5	B
	P-value			1.0000		1.0000		1.0000		1.0000		<.0001		0.0005		0.0021	

Supplementary table 4 (continued).

	Treatment	Dose (L/ha)	Growth stage (GS)	STB leaf 2 21st June		STB leaf 1 21st June		Yellow rust 21st June		Brown rust 21st June		Fusarium index 28th June		Fusarium pct. 28th June		Fusarium scale 28th June		Yield	
1	Untreated		-	11.9	A	2.3	A	5.0	A	15.0	A	0.2	A	5.5	A	3.0	A	83.9	A
2	Proline EC 250	0.4	37-39	2.9	B	0.5	B	0.0	B	0.4	B	0.1	A	6.0	A	2.0	A	83.1	A
3	Proline EC 250	0.4	61-65	2.8	B	0.4	B	0.4	B	1.0	B	0.1	A	3.5	A	3.0	A	79.3	A
4	Proline EC 250 Proline EC 250	0.4 0.4	37-39 61-65	2.8	B	0.3	B	0.5	B	1.3	B	0.1	A	2.5	A	2.5	A	84.1	A
7	*C. rosea*	1.0	37-39	4.0	B	0.8	B	5.5	B	0.8	BC	0.1	A	4.0	A	2.8	A	76.8	A
10	*C. rosea* *C. rosea*	1.0 1.0	37-39 61-65	3.9	B	0.3	B	0.0	B	0.0*	C	0.2	A	4.0	A	3.5	A	81.2	A
	P-value			0.0024		0.0014		0.0032		<.0001		0.7982		0.6691		0.8957		0.0996	

*0.03

Percentage STB coverage of green leaves (STB percent). Fusarium head blight was scored by three methods: i) the number of attacked heads counted in 4 x 1m row per plot (Fusarium number), ii) average attack of FHB on the assessed heads was scored using a 1-9 scale (Fusarium scale), and iii) visual score of percent attacked heads per plot (Fusarium pct). All data in the table were transformed before analysis: square root (FACTOR+2).

Supplementary data table 5. Complete data set and analyses for the winter wheat field trial in 2019 at Research Center Flakkebjerg where only data for the treatments *C. rosea*, and reduced dosage of Proline (0,4 l/ha) were included. Treatment were applied in growth stage (GS) 37-39 and/or GS 61-65.

	Treatment	Dose (L/ha)	Growth stage	STB Leaf 1 3rd June		Pow. mildew leaf 1-4 3rd June		STB leaf 1 14th June		STB leaf 2 14th June		STB leaf 1 25th June		STB leaf 2 25th June		Fusarium number 28th June	
1	Untreated		-	27.5	A	9.0	A	2.8	A	17.0	A	13.0	A	47.5	A	4.4	A
2	Proline EC 250	0.4	37-39	20.0	B	4.5	BC	1.0	BC	7.3	BC	8.3	B	28.8	BC	2.2	B
3	Proline EC 250	0.4	61-65	27.5	A	6.3	B	1.0	BC	9.3	B	9.0	B	27.5	BC	1.2	BC
4	Proline EC 250 Proline EC 250	0.4 0.4	37-39 61-65	18.8	B	4.8	BC	0.5	C	7.3	BC	4.0	C	23.8	C	0.8	C
6	*C. rosea* + Proline EC 250	1.0 0.4	37-39 61-65	17.5	B	3.5	C	0.9	BC	8.0	BC	8.8	B	33.8	B	0.7	C
7	*C. rosea*	1.0	37-39	18.8	B	4.5	BC	0.6	BC	5.8	C	12.0	AB	27.5	BC	2.2	B
10	*C. rosea* *C. rosea*	1.0 1.0	37-39 61-65	18.8	B	5.8	BC	1.4	B	8.8	BC	13.0	A	33.8	B	2.2	B
	P-value			0.0041		0.0093		0.0002		<.0001		0.0010		0.0006		<.0001	

Supplementary table 5 (continued).

	Treatment	Dose (L/ha)	Growth stage	Fusarium scale 1-9 28th June		Fusarium percent 7th July		STB percent 7th July		Yield 9th August		NIV ppb		DON ppb		ZEA Ppb	
1	Untreated		-	5.0	A	38.8	A	76.3	A	74.9	A	35.7	A	343.7	A	24.0	A
2	Proline EC 250	0.4	37-39	3.0	B	25.0	BC	55.0	B	81.6	B	18.0	A	211.0	B	10.0	A
3	Proline EC 250	0.4	61-65	2.3	BCD	18.8	CD	65.0	AB	83.5	B	22.3	A	126.0	B	10.0	A
4	Proline EC 250 Proline EC 250	0.4 0.4	37-39 61-65	1.5	D	18.8	CD	37.5	C	86.2	B	27.0	A	123.3	B	19.7	A
6	*C. rosea* Proline EC 250	1.0 0.4	37-39 61-65	1.8	CD	13.8	D	52.5	B	84.8	B	21.3	A	143.3	B	10.0	A
7	*C. rosea*	1.0	37-39	2.8	BC	25.0	BC	65.0	AB	81.6	B	23.0	A	225.7	B	10.0	A
10	*C. rosea* *C. rosea*	1.0 1.0	37-39 61-65	3.0	B	30.0	AB	66.3	AB	81.9	B	31.7	A	229.7	AB	17.0	A
	P-value			<.0001		0.0015		0.0005		0.0162		0.8856		0.0131		0.4299	

Percentage STB coverage of green leaves (STB percent). Fusarium head blight was scored by three methods: i) the number of attacked heads counted in 4 x 1m row per plot (Fusarium number), ii) average attack of FHB on the assessed heads was scored using a 1-9 scale (Fusarium scale), and iii) visual score of percent attacked heads per plot (Fusarium pct).

Supplementary data table 6. Complete data set and analyses for the winter wheat field trial in 2020 at Research Center Flakkebjerg where only data for the treatments *C. rosea*, and reduced dosage of Proline (0,4 l/ha) were included. Treatment were applied in growth stage (GS) 37-39 and/or GS 61-65

	Treatment	Dose (L/ha)	Growth stage	STB leaf 4 1st June	STB leaf 3 1st June	STB leaf 3 10th June	STB leaf 2 10th June	Fusarium number 1st July	Fusarium scale 1-9 1st July	Fusarium number 7th July
1	Untreated		-	1.5 A	5.0 A	5.8 A	1.4 A	9.8 A	3.0 A	14.5 A
2	Proline EC 250	0.4	37-39	0.5 BCD	2.0 C	5.5 A	0.6 A	4.5 B	3.0 A	12.3 A
3	Proline EC 250	0.4	61-65	0.6 BC	2.3 C	2.8 A	0.1 A	4.0 B	3.0 A	11.0 A
4	Proline EC 250 Proline EC 250	0.4 0.4	37-39 61-65	0.0* D	1.1 C	3.3 A	0.4 A	4.5 B	3.0 A	11.3 A
6	*C. rosea* + Proline EC 250	1.0 0.4	37-39 61-65	0.4 BCD	2.5 BC	4.0 A	0.7 A	4.5 B	3.0 A	10.8 A
7	*C. rosea*	1.0	37-39	0.2 CD	1.9 C	4.5 A	0.6 A	4.5 B	3.0 A	15.0 A
10	*C. rosea* *C. rosea*	1.0 1.0	37-39 61-65	0.8 BC	1.8 C	3.5 A	0.4 A	6.0 B	3.0 A	16.8 A
16	*C. rosea*	1.0	61-65	1.0 AB	4.5 AB	4.0 A	0.4 A	4.8 B	3.0 A	13.3 A
	P-value			0.0039	0.0072	0.0639	0.4633	0.0074	1.0000	0.1004

*0.03 [1]GS = growth stage; [2] Proline EC 250 application 16/5 2020; [3]Proline application 16/6 2020; [4]*C. rosea* application 15/5 2020; [5]

Supplementary data table 6 (continued).

	Treatment	Dose (L/ha)	App code	Fusarium scale 1-9 7th July	Yield 14th August	NIV ppb	DON ppb	ZEA ppb	T2 ppb	HT2 Ppb
1	Untreated		-	4.0 A	71.2 A	18.2 A	1232.0 A	11.0 A	9.1 A	9.1 A
2	Proline EC 250	0.4	37-39	4.0 A	74.4 A	24.6 A	1025.1 A	9.0 A	9.1 A	9.1 A
3	Proline EC 250	0.4	61-65	4.0 A	74.9 A	19.2 A	470.0 B	10.0 A	10.3 A	10.2 A
4	Proline EC 250 Proline EC 250	0.4 0.4	37-39 61-65	4.0 A	81.2 A	17,9 A	375.7 B	9.1 A	9.1 A	9.1 A
6	*C. rosea* + Proline EC 250	1.0 0.4	37-39 61-65	4.0 A	79.2 A	27.7 A	399.5 B	9.1 A	9.2 A	10.8 A
7	*C. rosea*	1.0	37-39	4.0 A	77.9 A	27.0 A	870.8 A	9.1 A	9.2 A	9.1 A
10	*C. rosea* *C. rosea*	1.0 1.0	37-39 61-65	4.0 A	75.2 A	18.1 A	977.8 A	9.1 A	9.2 A	9.1 A
16	*C. rosea*	1.0	61-65	4.0 A	75.3 A	27.8 A	1027.0 A	9.2 A	9.2 A	9.2 A
	P-value			1.0000	0.6923	0.2735	<.0001	0.2262	0.1716	0.1148

Fusarium head blight was scored by two: i) the number of attacked heads counted in 4 x 1m row per plot (Fusarium number), ii) average attack of FHB on the assessed heads was scored using a 1-9 scale (Fusarium scale). All data in table were transformed before analysis by square root (FACTOR+1).

Microbial biocontrol agents in IPM strategies - reducing pesticide use in wheat and lowering the risk of fungicide resistance

The main questions of the project were: (i) Can Septoria tritici blotch and Fusarium head blight be efficiently controlled by microbial biological control agents alone or combined with traditional chemical fungicides applied in low dosages? (ii) Can the development of fungicide resistance in the Z. tritici pathogen population be reduced by the combined use of microbial biological control and fungicides as compared to repeated chemical fungicide applications? And (iii) can accumulation of mycotoxins in harvested grain be reduced by the use of microbial biological control agents?

The project included three microbial biological control agents. The versatile fungal Clonostachys rosea, IK726, isolated from barley roots in Denmark and the two registered bacteria-based products Cedomon (Pseudomonas chlororaphis, strain MA341) and Serenade ASO (Bacillus velezensis, syn. B. amyloliquefaciens, strain QST713). The azole fungicide Proline EC 250 (a.i. prothioconazole), belonging to the group of demethylase inhibitors, was chosen as a model fungicide in order to look for possible changes in fungicide sensitivity and mutations in the CYP51 gene of Z. tritici. In recent years, fungicide resistance to azoles in the Z. tritici population has developed in Denmark and the Nordic countries.

The current study has not revealed any clear economic benefit, as only one of three trials gave a significant and positive yield increase with fungicide and biocontrol treatments. In order for biological control agents to provide attractive solutions, trial data should verify an economic benefit for the farmers in a range of 3-4 Dt/ha in order to pay for the cost of treatments.

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