Combined chemical and biological control of Fusarium graminearum: co-application of antagonistic bacteria removed from winter wheat leaves

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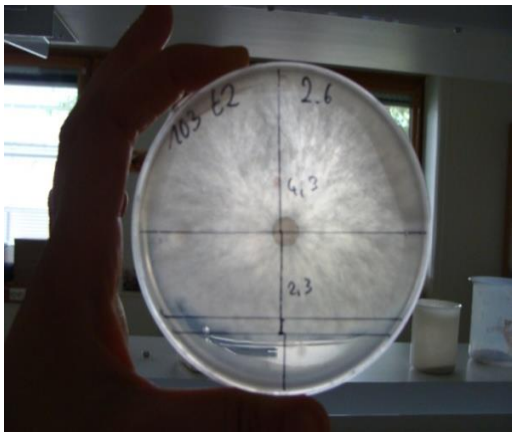
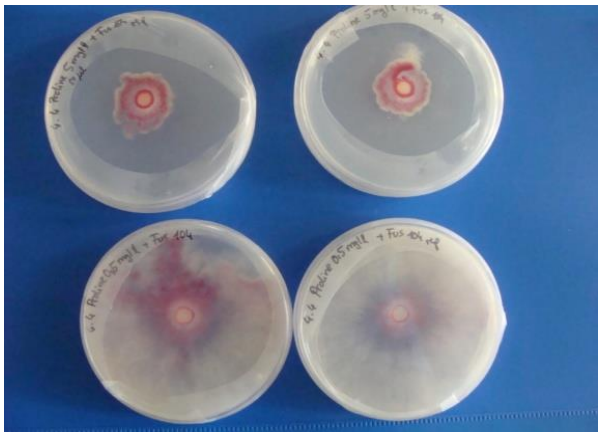
Submitted:
26.07. 2015
Published:
Uppsala, Sweden 2015
Department:
Crop Production Ecology; Swedish University of Agricultural Sciences
Master-Programme:
Environmental Science Soil, Water and Biodiversity (EnvEuro)





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Title:	*"Combined chemical and biological control of Fusarium graminearum: co-application of antagonistic bacteria removed from winter wheat leaves"*
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Year /Place:	Uppsala, Sweden 2015
Keywords:	Fusarium, wheat, prothioconazole, Integrated Pest Management, antagonists
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Course:	Independent Project in Environmental Sciences – Master´s Thesis (EX0431; 30hp)
Project Level:	Advanced, A2E

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Abstract

In the future Sweden needs to comply with EU directives which require the stronger implementation of Integrated Pest Management (IPM) which includes the reduction of the amount of pesticide applied in order to control plant pathogens such as *Fusarium graminearum* in wheat. The idea in this study was to compensate the reduced chemical control from the fungicide by the co-application of antagonistic bacteria which were isolated from winter wheat leaves. The aim was to test if a combination of antagonistic bacteria and a experimental dose (0.5 mg/l) of the fungicide Proline® provides an inhibition of fungal growth of *F. graminearum in vitro* (Dual culture assay) comparable to single fungicide application. The results showed that antagonistic bacteria are not affected by any of the used concentrations of the fungicide and provided additional inhibition of fungal growth. A experimental dose of the fungicide only reduced fungal growth rate during five days of incubation but did not stop it (mycelial area became equal to control after seven days incubation). The application of antagonistic bacteria however stopped fungal growth after seven days. The treatment effects were significant. From this it can be concluded that the co-application of fungicides and antagonistic bacteria might be promising to provide sufficient control of fungal growth of *F. graminearum* provided bacteria survive in the environment they are applied to in a high concentration. As the present study only assessed treatment effects in the lab on agar further field experiments are needed to test the findings of the present study under real life conditions. The bacteria used in the present study were removed from wheat leaves and therefore might be able to survive, inoculated as biocontrol agents, on wheat leaves and wheat heads.

Popular Summary

In Sweden the fungal plant pathogen *F. graminearum* in wheat and corn increases in relevance for agricultural production. *F. graminearum* produces mycotoxins which are harmful for human health and therefore decrease grain quality. In addition severe *Fusarium* infections can cause yield losses up to 40%. Farmers apply different agricultural practices to control fungal growth and the production of mycotoxins. One common practice is the application of fungicides which target *Fusarium*. But Sweden in the future needs to comply with EU directives to implement Integrated Pest Management strategies (IPM) in order e.g. to reduce pesticide use on fields to provide a more healthy and environmental friendly crop production. Reduced fungicide application to control *F. graminearum* poses the risk of insufficient chemical control of the fungal pathogen which needs to be compensated by more environmental friendly practices to avoid mycotoxin accumulation in grain and high yield losses. This lab study tested the possibility of co-application of a experimental dose of 0.5 mg/l of the fungicide Proline® (a concentration that does not kill the fungus) together with antagonistic bacteria isolated from wheat leaves which were able to inhibit fungal growth of *F. graminearum*. The fungus was incubated together with the antagonistic bacteria and the fungicide up to seven days. It was assessed if

the combined treatment (bacteria + fungicide) inhibited fungal growth of *F. graminearum* more effective than the single application of control measures (fungicide without bacteria addition and bacteria without fungicide addition). The hypothesis was that the combined control treatment would not yield a better inhibition of fungal growth than the single application of the fungicide because it was assumed that the bacteria might die or be affected in another way by the fungicide.

In this study it was found that the antagonistic bacteria survived fungicide application if at field dose level, because most of the antagonistic bacteria have been isolated from wheat leaves that received fungicide treatments and the bacteria quantity was not significantly affected by fungicide application. The antagonistic bacteria were tested for their survival ability by applying 0.5 mg/l and 500mg/l of the fungicide Proline® and no adverse effect was found. The combined application of the fungicide and the antagonistic bacteria always yielded the strongest inhibition of fungal growth compared to the single fungicide or bacteria application treatment. Therefore, it was concluded that the bacteria provided additional inhibition of fungal growth in comparison to the fungicide application without bacteria addition and the fungicide application in addition to antagonistic bacteria also provided additional control of fungal growth. The single fungicide application without bacteria only reduced the growth rate of the fungus but the antagonistic bacteria stopped fungal growth after seven days incubation. This result shows that antagonistic bacteria (if they survive in their new environment) are able to compensate a insufficient chemical control provided by a fungicide to stop fungal growth of *F. graminearum*. Therefore, antagonistic bacteria addition to fungicides might be promising for achieving a safe reduction of fungicide use in the field to control *Fusarium*. Further field experiments are necessary to test bacterial survival in the field. The isolated bacteria have been stored and are available for taxonomical identification which is needed to optimize their application in the field and to increase the chances of bacterial survival on wheat plants. When the hyphae of *F. graminearum* got in contact with the fungicide or the antagonistic bacteria it produced pink pigments. In the present study it was assumed that the pink pigment production might be correlated with the production of mycotoxins which help *F. graminearum* to compete with other microorganisms and fungi and to deal with environmental stress factors. Consequently further investigations are needed to understand if and how mycotoxin production is involved in stress responses of *F. graminearum*, if it is correlated to pink pigment production and how mycotoxin production is affected by different concentrations of a fungicide or different antagonistic bacteria.

1. Introduction

Worldwide, the infection of wheat(*Triticum aestivum*) by different species of the fungal pathogen Fusarium causes high yield losses and lowers the quality of grains by producing mycotoxins (Snijders 1990). The infection with pathogenic *Fusarium* species such as *Fusarium graminearum* and *F. culmorum* implies the risk for development of Fusarium head blight (FHB) for the mature crop and seedling and

stem blight for the early growth stages of wheat (Haidukowski et al. 2005).It is known that not just one *Fusarium* species cause *Fusarium* diseases in cropping systems but fungal complexes consisting of different *Fusarium* species which compete e.g. for nutrients during the infection of plant debris remaining on the soil surface (Xu et al. 2005, Leplat et al. 2012).The complicated competitive interactions within the fungal complex which mediates *Fusarium* disease development and severity makes it challenging to predict disease development and to plan control measures. Which fungal pathogen predominates in the complex differs between countries according to differential climatic regimes, cultivars used and their resistance to the dominant pathogen, crop rotations, pesticides applied and other cropping management practices (Xu et al. 2005).

The infection life cycle of *Fusarium* by Stephens et al. (2008) for wheat and *F. graminearum* has been identified in three different stages. The first stage is characterized by inoculation of *F. graminearum* caused by the germination of fungal spores on the wheat tissue which is followed by the formation of a superficial hyphal mat. Ascospores are produced and forcibly discharged by fungal fruiting bodies (perithecia). This is the start of the sexual life cycle of *F. graminearum* and has its origin in plant debris which contains overwintered fungal mycelium. Macroconidia spores belong to the asexual life cycle of *Fusarium* and occur in spring during anthesis (Talas 2011). The second development stage is characterized by adaxial colonization on the epidermis of the outer leaf sheath. It is accompanied by mycelia growth inside the leaf tissue (not necessarily visible) from the inoculation point to the crown. This means that the fungus first spreads systemic inside the plant at point of primary infection. The dispersal of the germinated spores (the spores germinate after they received enough nutrients taken up by the hyphae from the host) can cause secondary infection of the host especially during anthesis, the stage where the wheat plant is most susceptible to fungal infection through conidia and ascospores. The third stage is the extensive colonization of the internal crown tissue (Stephens et al. 2008). This study will focus on the primary stage of infection of the host tissue or substrate by *F. graminearum* and not on the infection and disease development of the mature plant.

It is important to distinguish between controlling grain, stem, leave or spikelet infection, general inhibition of fungal growth (hyphal growth) to avoid the development of asexual and sexual spores, control measures to lower FHB-infections and measures to lower the accumulation of mycotoxins in host material produced by some *Fusarium* species such as *Fusarium graminearum* and *F. culmorum* (Talas 2011). It is further important to keep in mind that wheat leaves show higher resistance to infections by *F. graminearum* than for example wheat anthers (Strange and Smith 1971).

F. graminearum was studied to be one of the most competitive *Fusarium* species associated with FHB worldwide (Xu and Nicholson 2009). The reason for its dominance among other *Fusarium* species have not been studied in a sufficient way

but it has been assumed that for the saprotrophic survival in soil the outstanding high amount of mycotoxin production by *F. graminearum* compared to other *Fusarium* species might be one reason for its dominance (Leplat et al. 2012).Other reasons for its predominance in Europe could be that *F. graminearum* through its production of ascospores increases its adaption ability to other climates, or the currently used wheat cultivars maybe were bred to be resistant to e.g. *F. culmorum* and hence are vulnerable to be attacked by *F. graminearum* which normally is suppressed by *F. culmorum* (Xu et al. 2005).*F. graminearum* is able to overwinter in soil, thus infected preceding crop residues are able to infect the following crop of the crop rotation if this crop is susceptible for *Fusarium* infection (Talas 2011). *F. graminearum* increases in concern for Sweden because of expected temperature rises caused by climate change. According to Roos et al. 2011 warmer and more humid climate than expected for especially the western parts of Sweden, was a reason for exceptionally high mycotoxin levels 2011 in this area. Roos et al. 2011 concluded that because of climate change pathogens like *F. graminearum* will find more optimal growth and spreading conditions in Sweden due to changing temperature and precipitation patterns. More precipitation and a milder climate imply a longer vegetation period for more northern regions in Sweden as the geographic temperate zone moves northward according to the model of Roos et al. (2011), which is based on model results published by the Intergovernmental Panel on Climate Change (IPCC). A higher infection risk of wheat by *F. graminearum* in Swedish regions, which is expected to experience a more humid climate and warmer mean annual temperatures, yields a higher risk for mycotoxin contamination of wheat grain and yield losses caused by FHB and seedling or stem rot.

1.1 Mycotoxins

The high mycotoxin production by *F. graminearum* is of high concern for the production of wheat, corn, barley, rice and oat as it yields grains that consist mycotoxin concentration that affect human and animal health. To protect human and animal health a threshold for mycotoxin contamination was integrated in the European legislation (Leplat et al. 2012). Mycotoxins are stable substances which survive thermal treatments. Because of that, mycotoxins can be present in processed or raw food and feeding stuff (El Khosht 2010). *F. graminearum* produces the mycotoxin deoxynivalenol (DON). DON is considered to be the most common mycotoxin contaminant associated with *Fusarium* infected wheat grains worldwide (Haidukowski et al. 2005). In this study the mycotoxin contamination of grains was not assessed but the potential for mycotoxin production during later infection stages makes it necessary to assess how to prevent infection with and growth of *F. graminearum* (Talas 2011).Therefore this study will not assess, as done by many studies, strategies for reduction of mycotoxin levels in wheat and the incidence and severity of FHB-disease but it will focus on inhibiting fungal growth at the early infection stage.

To control and reduce infections of wheat by *F. graminearum* several measures have been identified. These measures include the use of more resistant cultivars (which are very limited), to avoid *Fusarium* susceptible preceding crops in the crop rotation such as maize and the application of fungicides(Edwards 2004).

1.2 Chemical control of Fusarium graminearum

The success of chemical control measures by applying fungicides to reduce *F. graminearum* infection, growth and disease development is strongly dependent on amount, time, and frequency of fungicide application (Henriksen et al. 2005). Also the efficacy of the fungicide applied determines if all present pathogenic *Fusarium* species can be controlled. Sometimes the application of a fungicide which works excellent against some specific *Fusarium* species might not be effective against other species, which consequently become dominant. In this case *Fusarium* disease might still appear on the same level but it might be caused by another dominant *Fusarium spp.* species than in the previous year. For example the fungicide azoxystrobin in some cases have been reported not to be very effective to decrease the level of FHB mainly caused by *F. graminearum* (Cromey et al. 2001). These findings were supported e.g. by Xu et al. 2005 who stated that the complex of *Fusarium* species *"and their relative prevalence are affected by fungicides application regime since fungicides have differential effects against different FHB pathogens*" (Xu et al. 2005: 151). Henriksen et al. (2005) lists several possible reasons to explain this variability of efficacy of fungicides against different *Fusarium* species. This might be a too early application of the fungicide, or that the fungicide might have negative effects on saprophytic microflora growing on grains and the plant. This saprophytic microflora was described to show the potential to suppress e.g. the severity of FHB. Further reasons could be differences of cultivar resistance, fungicide coverage, timing and pathogen aggressiveness (Henriksen et al. 2005).

1.3 Biocontrol of Fusarium graminearum

European countries including Sweden need to comply with the EU directive 2009/128 which demands to strengthen Integrated Pest Management (IPM), which means as a consequence alia reduced usage of pesticides to minimize impact on human health and environment (Roos et al. 2011).To implement IPM in Sweden, new pest control strategies for *F. graminearum* in wheat will be needed. One option is to use microorganisms as active antagonists to inhibit the pathogenic fungal growth. The antagonistic interaction between the *Fusarium* and the antagonists are still under research. The application of biocontrol agents firstly calls for a good understanding how e.g. bacteria isolated from soil or from plant materiel are able to suppress *F. graminearum* and how their antagonistic performance might be affected by applied pesticides. Another problem is that not much is known under which conditions which *Fusarium* species becomes dominant. For example for the case, if *F. graminearum* is suppressed by the application of antagonistic bacteria as biocontrol agent or/and the application of a species specific fungicide, it might occur still high *Fusarium* infection

levels but caused by another composition of other *Fusarium* species which previously have been suppressed by *F. graminearum*. However, several bacterial species have been identified to act antagonistic against *F. graminearum* under field and lab conditions for example the bacteria species Sphingomonas S11(Wachowska et al. 2012), Bacillus subtilis strain 53 and 71 (Nourozian et al. 2005) and Pseudomonas spp.(Wachowska et al. 2012) and Pseudomonas fluorescens strain MKB 158 and 249 (Khan and Doohan 2009).

A better understanding of how biocontrol agent´s performance could be affected by the application of fungicides might be considerable to optimize fungicide application strategies (Karlsson et al. 2014).

1.4 Aim of the study

This study focused on assessing inhibitory interactions between *F. graminearum,* the fungicide Proline® and identified *Fusarium* antagonists isolated from winter wheat leaves and assessed on spring wheat leaves and as dual culture assay in petri dished on an agar substrate. The antagonistic performance of isolated bacterial species was evaluated with respect to *F. graminearum* because it represents one of the predominant species causing *Fusarium* diseases (Leplat et al. 2012).*F. graminearum* was also chosen because of its outstanding competitiveness against other *Fusarium* species and its increasing role in Sweden. This study does not include a taxonomic differentiation of the isolated antagonistic bacterial species. The winter wheat leaves used for isolation had received pesticide treatment and no pesticide treatment and were sampled in Västergötland near Skara in southwestern Sweden in 2011.The main research interest was grounded on the assumption that antagonistic bacteria, which normally suppress the development of *F. graminearum*, might be affected by the application of a non-lethal dose of fungicides. This presumption was supported by Henriksen et al. (2005) who studied the effect of fungicide application on *Fusarium* on wheat, barley and oat grain with natural infection sampled in plots where the spraying time had been too early to provide sufficient pathogen control as fungicides normally should be applied right before or during flowering. Their results showed that the application of some fungicides increased the level of *Fusarium* infection of wheat grain. Henriksen et al. (2005) assumed that the higher incidence of *Fusarium* infection in sprayed fields might have been caused by a too early application of the fungicides (=suboptimal application), which might have affected beneficial *Fusarium* inhibiting saprophytic microflora on grains but did not affect the target organisms *Fusarium*, which took competitive advantage from that. In addition, the present study builds on the research results of Karlsson et al. (2014) who found that fungicides applied to control diseases in wheat plants have moderate but significant adverse effect on the fungal community composition in the wheat phyllosphere. In the present study the application of fungicides might not only have adverse effects on the community composition of saprophytic fungal antagonists, which normally suppress *F. graminearum* in the

wheat phyllosphere as described by Karlsson et al. (2014), but also on the abundance of antagonistic bacteria species.

As a consequence, the first hypothesis aimed to be tested was that the application of a non-lethal (suboptimal) dose of the fungicide Proline® (active substance: 250g L^{-1} prothioconazole; Bayer Crop Science) combined with the application of biocontrol agents against *Fusarium* might result in failure of effective control of fungal growth of *F. graminearum* on spring wheat. This hypothesis was based on the second hypothesis that the failure of control of fungal growth of *F. graminearum* might be caused by the fungicide killing the applied antagonistic bacteria (artificial applied biocontrol agents) in the wheat phyllosphere. The inhibition of *F. graminearum* was assessed by measuring the extent of mycelia growth on agar plates and by measuring the length of lesions on spring wheat leaves caused by artificial inoculated *F. graminearum* in a detached leaf assay. The leaves and the agar plates contained dual culture assay of the *F. graminearum* isolate no. 104 and identified antagonistic bacteria exposed to a fungicide. The spring wheat variety “Vinjett” was grown in pots in a greenhouse.

In addition to the co-incubation experiments with the *F. graminearum* isolate no. 104 it also was tested antagonistic performance of the isolates against another isolate in order to find out if the antagonistic performance of bacteria is species specific. The antagonistic effect was also tested for fungal growth if a conidia suspension of *F. graminearum* was applied.

2. Material and Methods

Study area and sampling of antagonistic bacteria (biocontrol agents)

The leaf material analyzed in this study was sampled from pest surveillance plots from 13 fields. The leaf samples were representing seven different varieties of winter wheat used in Sweden. The 13 fields were subdivided in two surveillance plots. The inner plot (square in the middle of the field) received no fungicide treatment and the outer plot received fungicide treatment (1-3 fungicide and insecticide treatments (t)). Thus, through the subdivision it was taken wheat leaves samples from 26 plots in total. For each treatment 10 wheat leaves were taken and put together into one sample bag. The leaves were sampled below the flag leaf and randomly chosen.

Table 1: Overview of samples used for analyzing. “Northern area” refers to the samples taken from Skara in Västergötland Sweden. The “Southern Area” had not been analyzed. (from Karlsson et al. 2014)

Table 1. Distribution of wheat leaf samples between two geographical areas, fields and treatments, including weather data.

	Northern area	Southern area
Sampling date	20-June	27-June
No. of fields	13	5
No. of samples	26	16
No. of control samples	13	5
No. of fungicide-treated samples	13	11
Wheat developmental stage (DC)	61–69	61–83 nd
	06-June–20-June	13-June–27-June
Mean temp. (°C)	14.7	14.3
Mean rel. humidity (%)	81.6	82.6
Acc. rainfall (mm)	123.4	37.6
	13-June–20-June	20-June–27-June
Mean temp. (°C)	13.4	14.5
Mean rel. humidity (%)	80.5	81.4
Acc. rainfall (mm)	47.8	17.2
	19-June	26-June
Mean temp. (°C)	12.4	14.5
Mean rel. humidity (%)	93.5	78.2
Acc. rainfall (mm)	23.4	0.0

Mean temperature, mean relative humidity and accumulated rainfall[1] are given for two weeks, one week and the day before sampling.
DC = developmental stage according to the Zadoks scale, nd = not determined for all fields.
1 Weather data from the Lantmet weather stations in Skara and Anderslöv respectively.
doi:10.1371/journal.pone.0111786.t001

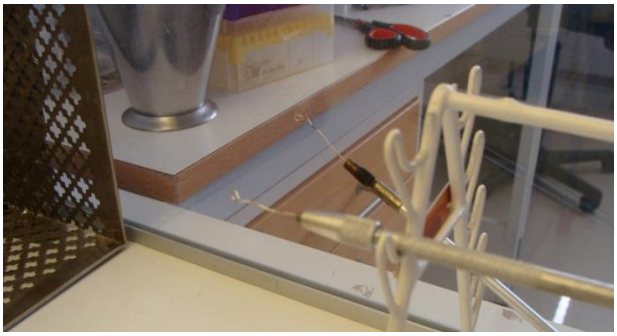
2.1 Removal of bacteria from winter wheat leaves

The wheat leave samples were used to isolate bacteria living on the surface of wheat leaves, to cultivate them and later determine which had antagonistic effects on *F. graminearum*. To remove bacteria for each sample, five leaves per treatment (“t” and “control”) were taken from the available 10 and cut with sterilized scissors into smaller pieces and put into a 50ml sterile plastic tube (Photo 1). Then each tube, containing the leaves, was filled with 25ml PBS buffer (phosphate buffered saline).Each tube was first manually shaken and to remove most bacteria from the leave surface the tube received following physical treatment: 3 x (45 sec. vortex and 45 sec.) in an ultrasonic bath (Photo 2). All equipment used for later experimental steps have been sterilized (Photos 3 and 4)

To provide countable single colony forming units (cfu) the PBS solution was diluted twice. It was prepared a 10 fold dilution series (0, -1, -2) with 3 replicates each.

Photo 1: winter wheat leaves in PBS solution

Photo 2: Ultrasonic bath



Photos 3 and 4: sterilization equipment (70% ethanol washing) and spreading equipment for fungicide and bacteria cultures

To cultivate the bacteria collected in the PBS-buffer solution, 70 µl of each PBS-bacterial solution with dilutions(Photo 20) was distributed evenly on non-species

selective half strength of nutrient agar plates (Nutrient Agar OXOID CM0003, which contained Lab-Lemco Powder, Yeast extract, Peptome, Sodium chloride and Agar and obtained a pH of 7.4;(half strength NA)). The agar plates were incubated at room temperature in the dark for up to six days.

2.2 Bacteria counting, isolation, identification of antagonists and storage

After three and six days of incubation the cfu from each plate were counted and the bacterial concentration [cfu/ml] on five wheat leaves for each treatment and field was calculated. The counting was conducted by dividing the plate in four equal parts, to count one quarter and to multiply it by four. It was assessed the differences between samples taken from the fungicide treated field parts of each and the not treated control part of the respective field. The species diversity was only determined by comparing shape and color to distinguish different species. For this purpose it was applied a LEICA Wild M3Z Stereozoom Microscope containing a camera LEICA IC80HD (Photo 5). The time frame of the project did not allow identifying different species by e.g. DNA-based methods.

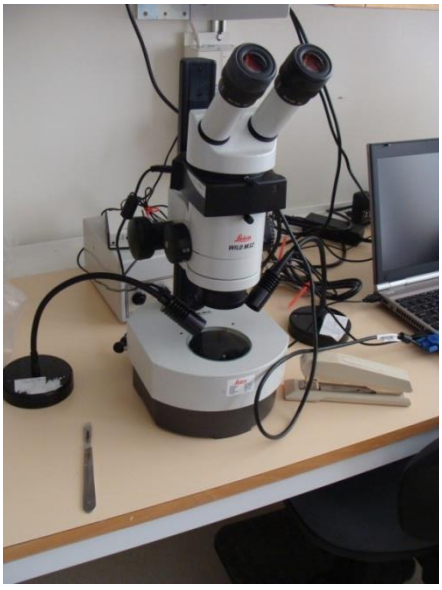


Photo 5: LEICA Wild M3Z Stereozoom Microscope including a camera LEICA IC80HD

For each bacterial sample (e.g. sample field “1 control”) in total 10 single and preferably morphological different bacterial colonies were removed from the nine agar plates prepared for all dilutions and their replicates, with sterilized equipment. Each single colony was transferred to its “own” single new half strength NA plate and spread by using a streaking technique to isolate “clean” single colonies. The first five colonies for isolation were taken three days after incubation (e.g. for part of the field untreated, dilution 0, replicate number one named as “Iso I – V 1control 0) and the other five after six days incubation (named as “Iso VI – X”). From this 260 isolation

plates in total have been obtained. If the isolation failed and still more than one species could be determined on the plate, the isolation was repeated. Each isolation plate was at least incubated for four days before a clean isolated colony was removed and “stored” with four other colonies on another half strength NA to be later tested for its antagonistic effect on *F. graminearum* (Photo 24). Each isolates got a number from 1 – 260.

Bacteria isolate testing for antagonistic effects against F.graminearum isolate no. 104

All 260 bacterial isolates were tested by co-inoculations on half strength NA plates with *F. graminearum* isolate 104for their antagonistic performance. Isolate 104 of *F. graminearum* was grown for 5-6 days on half strength NA plates before being applied in the co-inoculation. For the co-inoculation fungal mycelium was used, taken from the same area on the agar plate to obtain the same age of the *Fusarium* culture. For all bacterial isolates a piece of *F. graminearum* agar was cut out (diameter: 0.8 cm) (Photo 6)and transferred to a new half strength NA plate and placed in the center (Photo 7). For each plate one loopful of bacterial colonies was streaked on a line 1.5 cm away from the walls of the agar plate (Photo 7). It was assured that all pieces of *F. graminearum* had the same size to enable later comparison of the samples. It was prepared two replicates for each co-incubation of *F. graminearum* and isolated bacteria colony (520 isolate bacteria + *Fusarium* incubation plates in total for all fields and treatments) and incubated for five days. The bacteria isolates (1-260) taken from the storage plates were between four and five days old. After five days incubation the dual culture assay- plates were evaluated for the antagonistic performance of the bacteria.

The antagonistic effect of the tested bacteria on *F. graminearum* was assessed by measuring the distance (radius) from the agar plug of *Fusarium*. to the end of the mycelial mat. It was assumed that the shorter the mat was, the more successful the isolated bacteria colonies suppressed the growth of *F. graminearum* which was defined as their antagonistic performance(Photo 14).Normally *Fusarium* should grow as an almost uniform circle on agar plates (Photo 25), therefore if the bacteria were not antagonistic it was expected that the radius of the *Fusarium* should be similar in all directions(Photo 25).The incubation time of five days was ensured for all plates to achieve comparability. It was chosen five days of incubation because it was tested before as the time the *Fusarium* isolate needed to grow to a sufficient size, which means that it covers the whole plate if not disturbed and/or suppressed by any stress factors e.g antagonistic bacteria. To prepare a ranking of the antagonistic bacterial species it was measured three times the length of the mycelial mat (distance between agar pug and end of hyphae) in direction of the line where the antagonistic bacteria were grown. From these three measurements it was calculated a mean length (Photo 26). Bacterial isolates which showed the lowest length were considered to be most antagonistic.

Photo 6: Equipment for cutting out agar plugs of *F. graminearum*

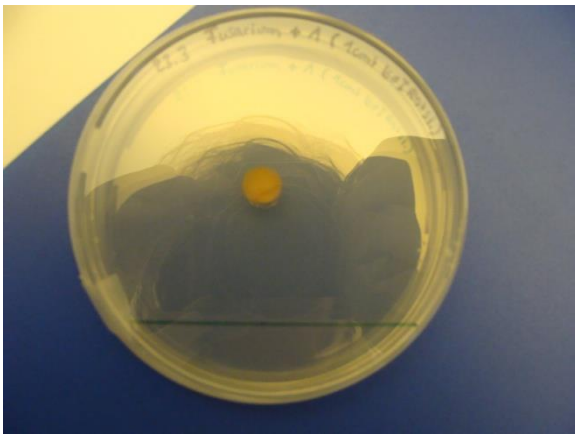


Photo 7: Set-up dual culture assay of *F. graminearum* (agar plug) + antagonistic bacteria isolates (on a line 1.5 cm distance from plate wall (+ fungicide later)

Storage of antagonistic bacteria

If a tested bacterial sample was identified to be antagonistic against *F. graminearum* all colonies of this isolate were taken with sterile equipment from the isolate's storage agar plate and stored by using Cyroinstant Cyrotubes (produced by VWR Chemicals, Prolab) (Photo 8). It contains Tryptone, Sodium Chloride, Meat extract, Yeast extract, L-Cysteine and Glycerol to provide a Maintenance Freeze Medium for bacteria cultures. The bacterial cultures were dissolved in the liquid of the tubes, which afterwards were shaken manually and stand for 1-2 minutes (Photo 9). Then, the liquid was removed with a pipette and the tubes were frozen at - 80^{o}C. The bacterial isolates were absorbed by the pearls.

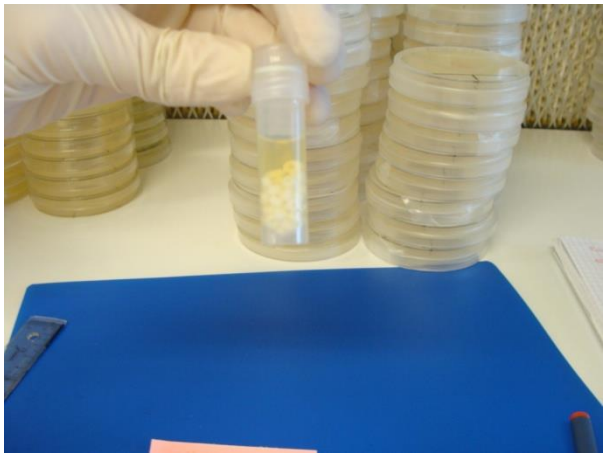


Photo 8: Cyroinstant Cyrotube

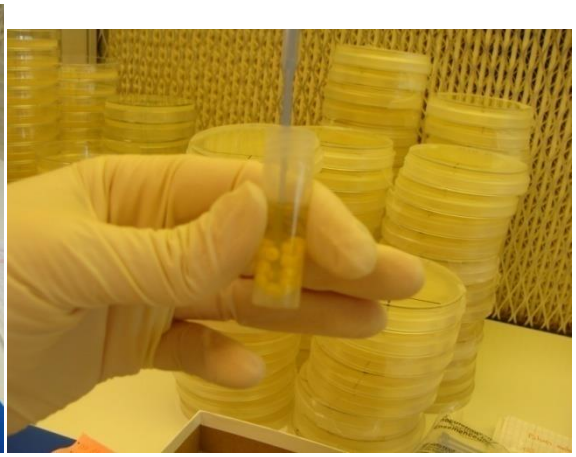


Photo 9: Transfer of antagonistic bacteria isolates to Cyrotube

2.3 Sowing of spring wheat plants

For testing the antagonistic bacteria´s performance to suppress *F. graminearum* on wheat plants spring wheat (Cultivar "Vinjett") was sown with seven seeds per pot (Photo 10). Each pot had a size of 9 cm diameter and a height if 7.5 cm. The seeds

were covered by 1 – 1.5 cm soil. The wheat plants were grown in the greenhouse at 15°C – 17°C with 50% relative humidity.

Photo 10: spring wheat grain arrangement (day of sowing)

The wheat plants were transplanted to bigger pots (height: 25cm, upper diameter: 19 cm, bottom diameter: 15cm) when the fifth leave emerged (Photo 11).

Photo 11: Transplanted spring wheat plants

For the **detached leaf assay** (Chapter 2.7) leaves of the same age were harvested at the “heading” stage before flowering (Zadoks stage 55; Zadoks et al. 1974).

2.4 Test antagonistic performance by applying fungicide on *Fusarium graminearum* isolate 104 on agar plates (Dual culture assay)

The ten most antagonistic bacterial isolates (according to ranking, Table 4) were selected and tested for their response to a fungicide treatment (0.5 mg/l) on half strength NA-plates. The bacteria were tested for their response to fungicide treatment with focus on their survival and antagonistic performance.

The fungicide Proline® (Bayer Crop Sciences, Sweden; Photo 12) with the active substance prothioconazole (250g /l) was chosen to test its interaction with the *F. graminearum* isolate 104 co-inoculated with the ten selected bacteria.

Calculation of field dose Proline® ha^{-1}:

V1 * C1 = V2* C2

V1= volume of pesticide used ha^{-1} [liter]

C1= concentration of pesticide emulsion before dilution [g/l]

V2 = V1 + volume of dilution water ha^{-1}

C2 = concentration of diluted pesticide applied on field [g/l]

C2 is unknown, V1 =0.6 l of the emulsion Proline®, C1= 250 g/l, V2= 300.8 liter (maximum dilution volume recommended by Bayer Crop Science 300l water + 0.6l Proline)

C2= (0.6l *250g/l)/300.8l = 0.5 g/l

Photo 12: Emulsion Proline® (active substance: prothioconazole 250 g/l)

First the effect of different concentrations of the Proline® fungicide was tested against *F. graminearum* isolate 104, in a tenfold dilution series of the fungicide of 500mg/l (field dose), 50mg/l, 5mg/l and 0.5 mg/l (Photo 13). The concentration, that showed a striking reduced inhibition of fungal growth of *F. graminearum*, compared to the field dose concentration (500 mg/l), after 5 days incubation, was chosen to be tested together with the ten selected antagonistic bacteria. Reduced inhibition means that the fungus did not get killed but fungal growth rate was just reduced **(= sub-lethal dose).** If the fungus can continue growing because of insufficient chemical control, it is possible to assess the beneficial inhibitory effect of bacteria antagonists applied together with the fungicide in the dual culture assay.

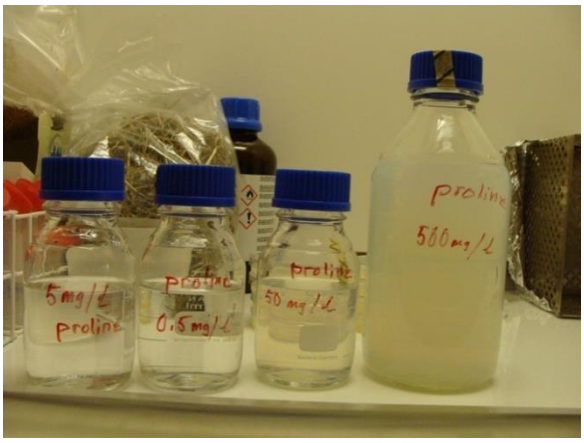


Photo 13: Tenfold dilution series of Proline®. Field dose (500 mg/l), dilution 1/10 (50mg/l), dilution 1/100 (5 mg/l) and dilution 1/1000 (0.5 mg/l)

In addition to the sub-lethal dose testing (0.5 mg/l) it was also tested antagonistic bacteria survival if the field dose of the fungicide is applied (500mg/l; only for treatment t3). The term sub-lethal was defined as an effective dose of the Proline® fungicide that lies between ED_{50} and ED_{90} for *F. graminearum*.

The fungicide treatment aimed to assess its effect on applied biocontrol agents and not its effects on native microbial community on the wheat leaves. Therefore, the bacteria colonies isolated from winter wheat leaves were applied as biocontrol agents on half strength NA-plates.

For the dual culture assay with and without Proline® treatment 25µl of antagonistic bacteria (concentration: 10^4-10^5 cfu/ml; Appendix: Table 9) was applied with a pipette on a line with 1.5 cm distance from the wall of the half strength NA-plate. In advance, the agar plates for pesticide treatment received 50µl Proline®, with a concentration of 0.5 mg/l, which was distributed uniformly on the agar surface. The fungicide was sterilized by filtering (PALL Life Sciences Acrodisc® Syringe Filter Supor® Membrane 0.2 µl), in order to avoid bacterial contamination in the dual culture assay on agar plates. After that a 0.8 cm agar plug with *F. graminearum* was placed in the

middle of the agar plate. The dual culture plates were incubated at room temperature in the dark for five days.

It was prepared four different treatments with three replicates each except of the control:

- Treatment 1 (t1) = antagonistic bacterial isolate + *F. graminearum* isolate 104 + Proline fungicide
- Treatment 2 (t2) = antagonistic bacterial isolate + *F. graminearum*
- Treatment 3 (t3) = antagonistic bacterial isolate + Proline fungicide
- Treatment 4 (t4) = *F. graminearum* isolate 104+ Proline fungicide
- Control = *F. graminearum* isolate 104

“Treatment 1 (t1)” aimed to assess how the fungicide Proline® affects the antagonistic performance of the bacteria isolate by comparing it with “treatment 2 (t2)” (in this case t2 was the control treatment). “Treatment 3 (t3)” and “4 (t4)” aimed to test if the Proline® fungicide affects the antagonistic bacterial isolates (t3) and how effective the used Proline® concentration was to inhibit fungal growth of *F. graminearum* isolate 104.

Evaluation of fungal inhibition by antagonistic bacteria

The inhibition of fungal growth was assessed by measuring the size of the mycelial mat after five days incubation. The mycelial mat showed non-circular growth patterns because of the antagonistic performance of bacteria. Therefore, the area of the each semicircle was calculated and summed with the other half. To quantify the antagonistic performance of the bacteria isolates it was measured the difference between the areas of the two semicircles.

Photo 14 shows the schematic subdivision of the mycelial mat on the agar plates:

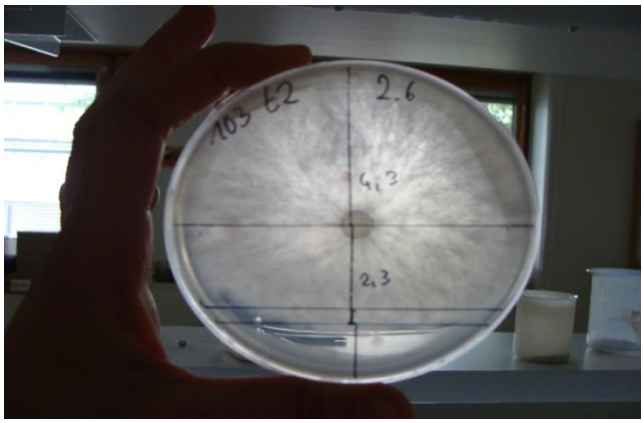


Photo 14: Subdivision of mycelial mat to determine its area (A); yellow: line on which bacteria are growing; radius blue [cm] – radius green [cm] = mean radius difference [cm] = antagonistic performance

The area (A) of a circle half was calculated as follow

$$A = \left(\frac{r(blue)^2 * \pi}{2}\right) + \left(\frac{r(green)^2 * \pi}{2}\right)$$

Equation 1: Mycelial area (A) [cm^2]

Bacteria concentration applied

It was considered necessary, for the later evaluation of the experiment, to apply a known concentration of bacteria [cfu/ml] on the agar plates and on the leaves. For that, the 10 selected antagonistic bacteria were re-grown on half strength NA plates by streaking one storage-pearl taken from frozen bacterial cultures. After four days incubation at room temperature in the dark one loopful bacterial colonies (Photo 15) was taken from the plate and dissolved in 1ml PBS solution and vortexed. From the original suspension 100µl were removed and transferred to another tube and dissolved by 900µl PBS-buffer. A 10-fold dilution series was prepared from the original suspension (0, 1:10 (-1), 1:100 (-2), 1:1000 (-3), 1:10000 (-4) and 1:100000 (-5)) for seven randomly chosen antagonists (isolate number: 177, 155, 33, 53, 103, 134 and 2) of the 10 re-grown bacterial isolates. For each bacterial dilution series 70µl of each bacterial dilution was spread on a half strength NA and incubated at room temperature. After four days the bacterial colonies were counted. For each dilution of each bacterial isolate the absorbance at 280nm (1µl sample; NanoDrop spectrophotometer; Photo 16) was measured and put in relation with the counted bacteria colonies per ml. From this data a calibration curve was prepared for the 10 bacterial isolates. The calibration curves were used to determine the concentration of bacteria applied together with *F. graminearum* and the fungicide by measuring its absorbance at 280nm. For the bacterial isolate 33 it was prepared a separate calibration curve (Figure 12) as it was characterized by a different color than the other antagonistic isolates. For the isolates 177, 155, 53, 103, 134 and 2 it was prepared three different calibration curves (Appendix: Figure 10, 11, 13), because for all isolates together the coefficient of determination (R^2) of the linear regression was very low (Appendix: Figure 10). Therefore, in order to determine a more reliable mean concentration (cfu/ml) for applied bacteria isolates it was calculated the mean concentration derived from the three different calibration curves (Appendix: Table 9).

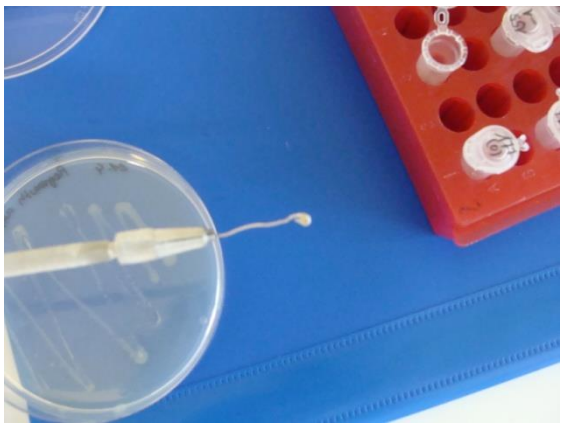


Photo 15: one loopful bacterial colonies taken to prepare bacterial suspension for NanoDrop calibration

Photo 16: NanoDrop spectrophotometer

2.5 Test antagonistic performance of 5 antagonists against another F. graminearum isolate 40

To test how the interaction of found antagonistic bacteria with other isolates of *Fusarium graminearum*, isolate number 40 (Photo 17) was incubated with five randomly selected antagonists (isolates no. 180, 108, 97, 134 and 164). The method for the co-inoculation was the same as described in paragraph 2.4.

Photo 17:*F. graminearum* isolate 40 after five days incubation on PDA (control treatment)

2.6 Test conidial fungal growth inhibition by 5 antagonists

To test if the fungal growth of *F. graminearum* from macroconidia is affected by the presence of antagonistic bacteria, macroconidia and antagonists were co-inoculated on half strength NA. Macroconidial spores from frozen samples with a concentration of 2.25 x $10^5$of conidia spores ml^{-1} were grown on Potato Dextrose Agar (PDA) for five days (Photo 18 and 19). Pieces of agar (with conidia) were cut out and placed in the middle of a half strength NA-plate and co-incubated with antagonistic bacteria spread on a line 1.5 cm away from the plate wall. It was prepared two replicates for each of the five selected antagonistic bacterial isolates.

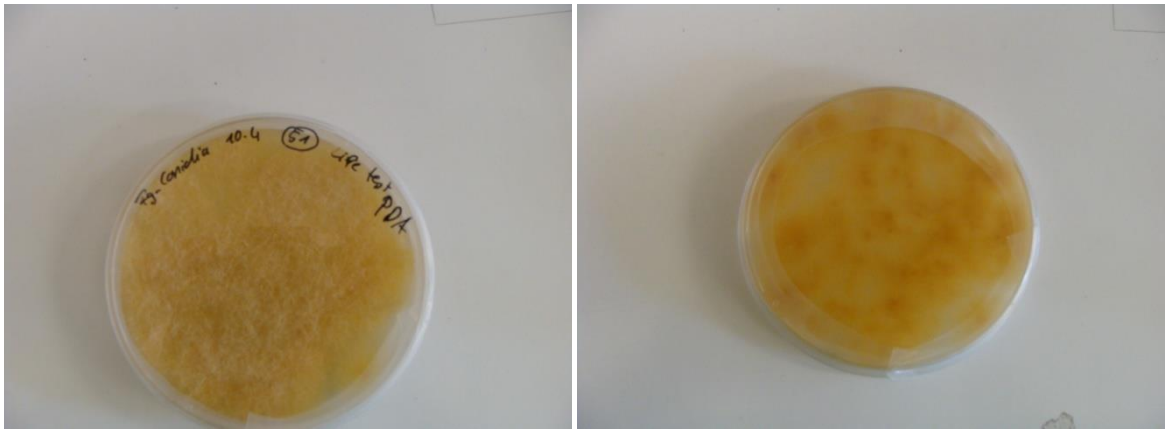


Photo 18 and 19: Conidia isolate 51 of *F. graminearum grown* on PDA after 5 days incubation

2.7 Detached leaf assay

To assess the effects of biocontrol agents application and/or fungicide treatment on living spring wheat leaves it was carried out an *in vitro* detached leaf assay. The leaves were detached from the wheat plants by using surface sterilized scissors, cut into 4 cm pieces and placed on a moist filter paper (tap water).

The leaves received the same treatments as described for the dual culture assay on agar plates (three replicates each except of control 1 and 2).

- Treatment 1 (t1) = antagonistic bacterial isolate + *F. graminearum* isolate 104 + Proline fungicide.
- Treatment 2 (t2) = antagonistic bacterial isolate + *F. graminearum*
- Treatment 3 (t3) = antagonistic bacterial isolate + Proline fungicide
- Treatment 4 (t4) = *F. graminearum* isolate 104+ Proline fungicide
- Control 1 = 10µl sterilized (autoclaved) distilled water (SDW)
- Control 2 = *F. graminearum* isolate 104

100 Wheat leaves of the same age below the flag leaf were harvested at growth stage 55 (Zadok et al. 1974)

The detached leaf assay was used as described by Imathiu et al. (2008).

Photo 20: bacterial isolate 132 detached leaf assay (t1-t4). Bacteria suspension was mixed with Proline® (0.5 mg/l; Bayer Crop Science)

For the leaves receiving a biocontrol treatment (treatment t1, t2 and t3), a 5µldrop of antagonistic bacteria (conc. (10^4 – 10^5cfu/ml) was placed on the center of the leaf surface. The fungicide (5 µl per leaf; conc.0.5 mg/l) was applied immediately after the application of the bacteria. After four days the harvested leaf segments were injured with a sterile needle four times at the center of the leaf, the spot where the fungicide, the bacteria and the fungal suspension have been applied to. Each leaf wounds received 5µl mycelial suspension of *F. graminearum* (isolate 104) for the treatments t1, t2 and t3.The treated leaves for each tested antagonistic bacteria were placed on humid filter paper in two plastic boxes and covered with a glass plate (Photo 20). The fungal growth of *F. graminearum* was measured after seven days incubation at room

temperature by determining lesion length (mm).The ten most antagonistic bacteria (Table5) including isolate 33 (morphological different) were tested at a concentration of $10^4 – 10^5$ cfu/ml..

3. Statistics

The correlation between pesticide treatment and number of colony forming units removed from winter wheat leaves was calculated by using the correlation function in MS Office Excel (Microsoft 2007). The calibration curves to determine the concentration of bacteria applied in the dual culture assay were calculated with the same program.

For further statistical analyses RStudio was used applying one-way ANOVA analysis followed by Tukey-HSD multiple comparison of means. For the dual culture assay the calculated inhibition of hyphal growth (mycelial area [cm^2]) for all three replicates will be tested if it the result was significantly caused by the different treatments. For the dual culture assay it will be assessed if “t1”, “t2”, “t4” and “control” are significantly different from each other. For “t1” it will be evaluated if its inhibition of *F. graminearum* is significantly different from the inhibition performance of “t2”, “t4” and control. For “t2” it is will be analyzed if it is significantly different from “t4” and control. The treatment “t3” only assesses if the antagonistic bacteria isolates survive the fungicide treatment.

A correlation analysis was carried out to determine for the combined treatment “t1” (biocontrol+chemical control) and for the single biocontrol treatment “t2” how strong and how the mycelia area size was influenced by the antagonistic performance of the bacteria isolate. Antagonistic performance was greater if the difference between the two radiuses was bigger, as the length of hyphae became shorter because fungal growth was inhibited by the antagonistic bacteria. The plate was divided in two halves, one half where the antagonistic bacteria were growing and inhibiting fungal growth (causing shorter hyphae = shorter radius) and the other half without antagonist growing in (Photo 14).

4. Results

4.1 Bacteria counting

Numbers of colonies were counted and the bacterial concentration cfu/ml was determined (Photo 21).

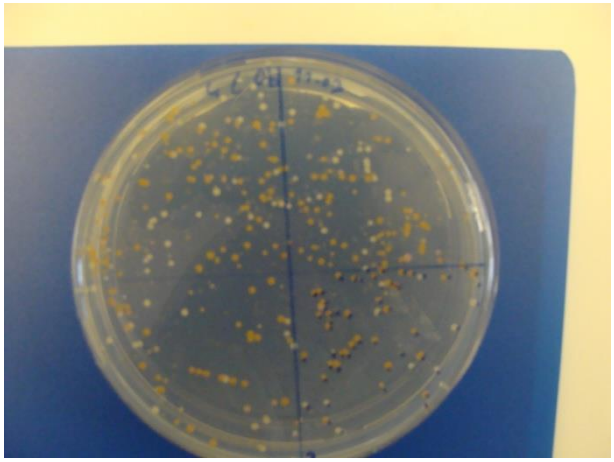


Photo 21: Example counting procedure bacterial colony forming units

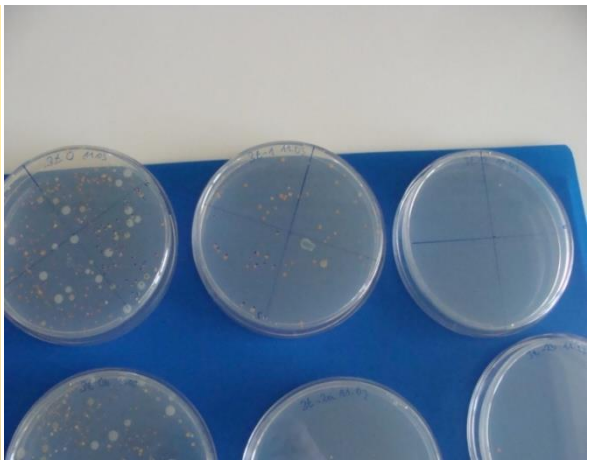


Photo 22: tenfold dilution series bacterial counting (two replicates per sample for validation of dilution method)

For some samples cfu/ml was determined after three days incubation and after five to six days for the more slow growing bacteria.

The results showed that for five fields, leaves sprayed with pesticides showed a lower amount of bacteria than non-treated leaves (e.g. field 11 and 12: Table 2). On the other hand for five other fields of the total 13 analyzed, wheat leaves receiving no pesticide treatment showed a higher amount of bacteria compared to leaves from the rest of the fields receiving pesticide treatment. For the remaining three fields it was found a similar amount of bacteria on the wheat leaves for both treatments. The bacterial concentration was only determined for the dilution “0” as this dilution was planned to be used in the dual culture assay later. The full table of results is provided in the Appendix (Table 6).

The correlation between treatment and bacterial abundance was calculated in Microsoft Excel to be 0.46.The summarized results of the bacterial counting are presented in Table 2.

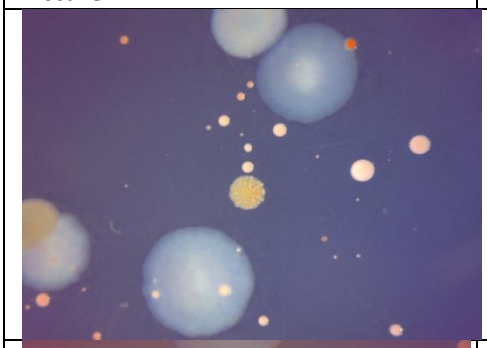
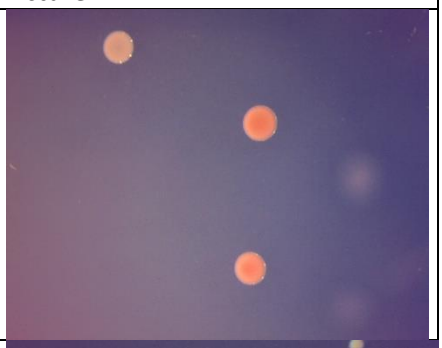
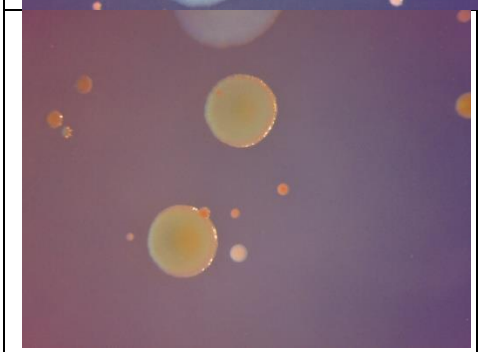
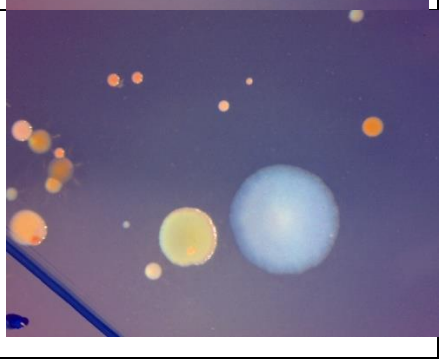
Table 2: Concentrations of bacteria found on 13 fields receiving spraying and no-spraying treatment on 26 plots cont= received no pesticide, t= received pesticides

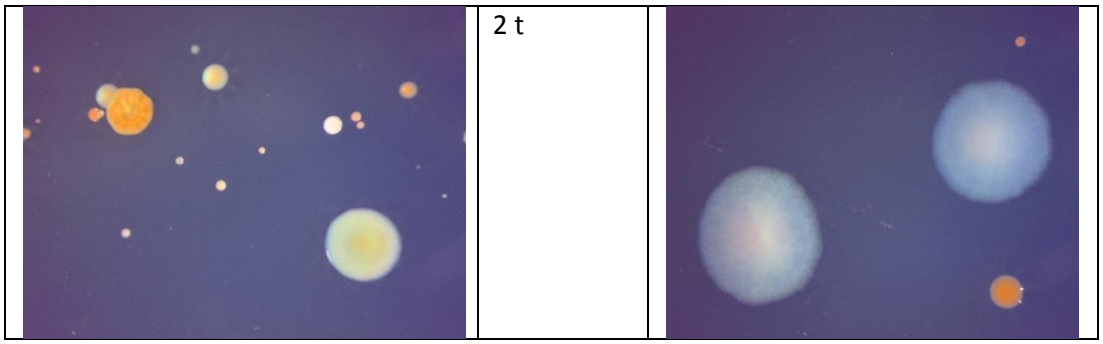
not sprayed plots	[cfu/ml]	sprayed plots	[cfu/ml]
1 cont	4342.95	1 t	tc
2 cont	3085.71	2 t	742.86
3 cont	5371.43	3 t	3257.14
4 cont	9333.33	4 t	4895.24
5 cont	6628.57	5 t	tc
6 cont	tc	6 t	2228.57
7 cont	1085.71	7 t	571.14
8 cont	tc	8 t	tc
9 cont	tc	9 t	tc
10 cont	357.14	10 t	571.43
11 cont	685.71	11 t	5457.14
12 cont	1942.86	12 t	2857.14
13 cont	tc	13 t	tc

tc= too numerous to count

4.2 Bacterial diversity

Figure1: Biodiversity of bacterial colonies removed from winter wheat leaves. Pictures were taken with a Leica Wild M3Z Stereo Microscope; numbers= number of field, cont/t = no pesticide/ pesticide treatment

Picture	Sample	Picture
	3 cont	
	2 cont	



The bacteria removed from winter wheat leaves were characterized by a great diversity of colors (white, green, yellow, orange, red, grey, milky, pink etc.) and shapes (e.g. round, spiky, slimy). Most of the antagonistic bacteria identified had a white, grayish, bluish color and a slimy texture and were fast growing (Figure1: 2t (picture on the right)). Bacterial isolate no. 33 was morphological different from the other antagonistic isolates as it was characterized by a greenish color and slimy texture (Photo 24).

4.3 Isolated bacteria

The isolated bacteria from the mixed cultures were obtained by the streaking method (Photo 23) and four isolates were “stored” together on one half strength NA plate by re-growing them (Photo 24).

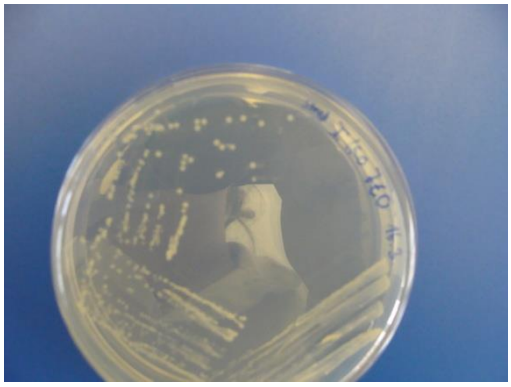


Photo 23: Isolation of bacteria species (streaking)

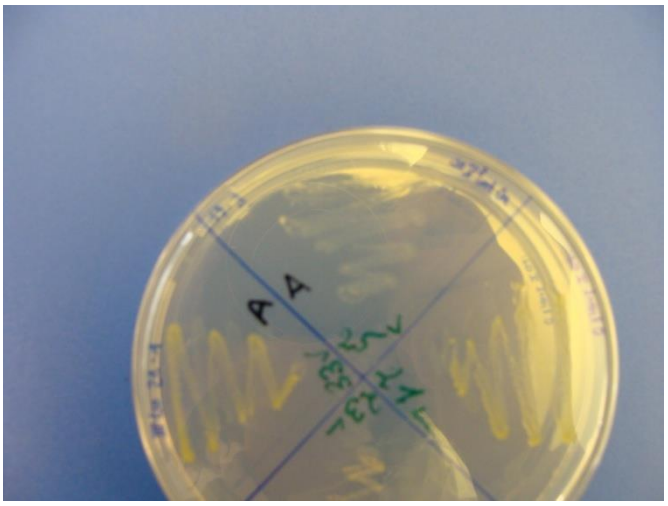


Photo 24:“Storage” of isolated bacteria species. Green numbers indicate storage number and “A” indicates found to be antagonistic against *F. graminearum* isolate 104. Here isolate 33 was found to be morphological different from isolate 25 by color.

In the Appendix Table 7 are listed all stored bacterial isolates, their storage number and if they showed antagonistic performance against *F. graminearum* isolate 104.

4.4 Antagonistic effects/identification of antagonistic bacteria

Table 3: Antagonistic bacteria isolates (total 29 isolates) against *F. graminearum* isolate 104; t= pesticide treatment, cont= no pesticide treatment

Sample: Field+treatment	Storage number
1 cont	2
2 cont	25
2 t	32
2 t	33
2 t	40
3 t	53
3 t	56
5 t	92
5 t	97
5 t	99
6 cont	103
6 cont	108
6 t	120
7 t	132
7 t	133
7 t	134
8 t	153
8 t	155
8 t	157
8 t	160
9 cont	164
9 t	171
9 t	173
9 t	174
9 t	175
9 t	176
9 t	177
9 t	178
9 t	180

For both treatments (t and cont) plots of the 13 fields have been found antagonistic bacteria. In total 29 bacteria communities showed antagonism against *F. graminearum* which looked, greenish and slimy (isolate 33), milky and slimy (all other antagonists except isolate 33) and were fast growing (three to four days).

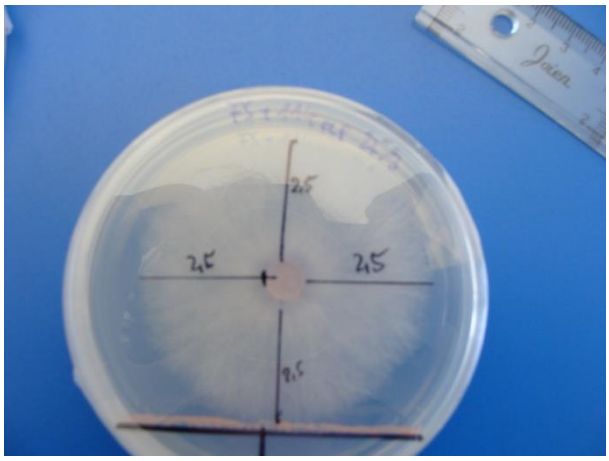


Photo 25: Non-antagonistic bacterial isolate (radius of mycelia mat is equal in all directions: 2.5 cm)

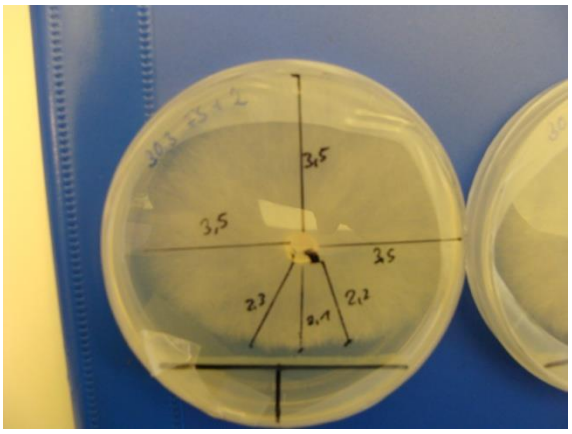


Photo 26: Antagonistic bacterial isolate (radius if mycelial mat in direction of bacteria culture on the line becomes less: three distances were randomly taken to calculate mean distance for ranking of the isolates)

Table5: Ranking of antagonistic bacterial isolates according to their antagonistic performance.10 most antagonistic bacteria isolates were selected (in bold letters)

Storage number	Mean length of hyphae [cm]
120	2
153	2.06
155	2.06
97	2.13
103	2.13
157	2.17
160	2.2
2	2.2
132	2.2
177	2.2
33 (morphological different to other isolates)	2.2
178	2.23
134	2.23
164	2.23
53	2.27
176	2.27
40	2.3
180	2.37
92	2.4
25	2.4
173	2.4
133	2.4
99	2.43
171	2.43
174	2.43

175	2.47
56	2.47
32	2.5
108	2.5

To calculate antagonistic performance it was taken the difference [cm] between the upper radius (the half of agar plate with no bacteria stroked on a line) and the mean lower radius of the mycelial mat (the half of agar plate where antagonistic bacteria were growing on a line) (Photo 26).

4.5 Test inhibition of fungal growth from macroconidia by 5 antagonists and antagonistic performance against *F. graminearum* isolate no. 40

Three of the five chosen antagonists (isolate 108, 180 and 134) showed an inhibitory effect on fungal growth from macroconidia (*F. graminearum* isolate no. 51) on half strength NA plates with two replicates. The bacterial isolates 97 and 164 did not show antagonistic effects after five days incubation.

The co-inoculation of five randomly chosen antagonists incubated together with the isolate 40 of *F. graminearum* failed. The isolate 40 was growing well on PDA but not on the half strength nutrient agar medium (half strength NA). Therefore, no antagonistic performance of the co-incubated bacteria could be observed.

4.6 Dual culture assay of F. graminearum and antagonistic bacteria on agar plates with and without the fungicide

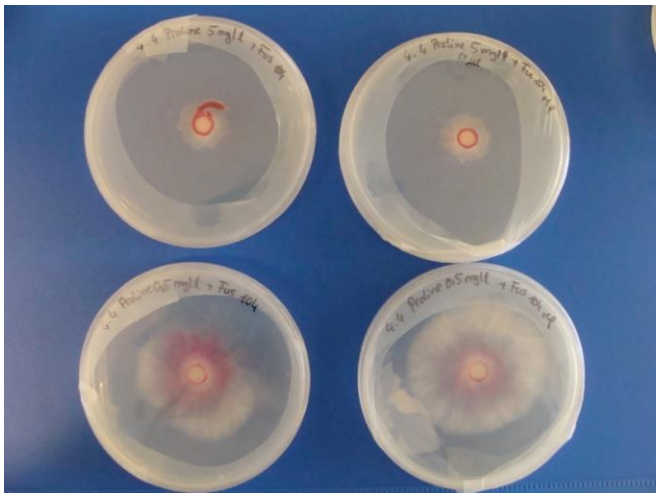


Photo 27:

F. graminearum isolate 104 after 5 days incubation with 5 mg/l Proline® (upper plates) and 0.5 mg/l Proline® (plates below): two replicates each

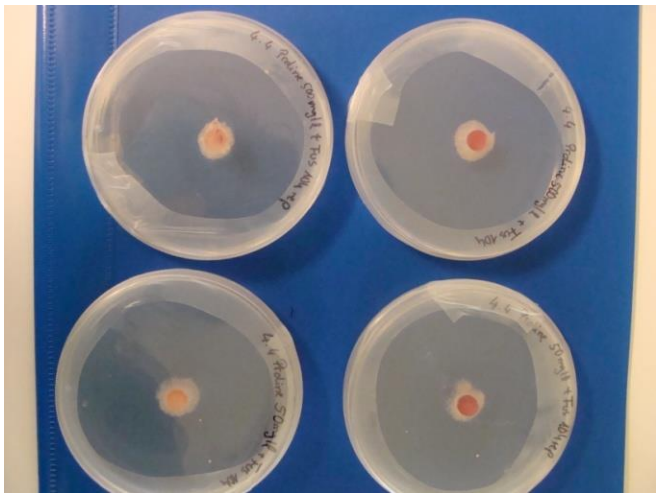


Photo 28:

F. graminearum isolate 104 after 5 days incubation with 500 mg/l Proline® (upper plates) and 50 mg/l Proline® (plates below): two replicates each

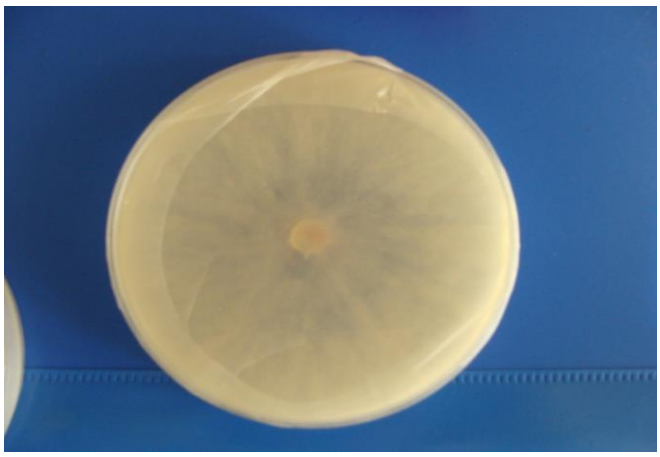


Photo 29:

Control: *F. graminearum* isolate 104 without Proline® after 5 days incubation

As shown in Photo 27-29the Proline® concentrations of 500mg/l (field dose), 50 mg/l and 5 mg/l showed an almost 100% inhibition of fungal (hyphal) growth of *F. graminearum* isolate 104 on half strength NA-plates after five days incubation compared to the control (no Proline added)(Photo 29). The applied concentration of 0.5 mg/l resulted at lowest inhibition of fungal growth(reduced growth rate) and therefore was chosen to be applied in the dual culture assay with *F. graminearum* and the antagonistic bacteria isolates. For this lowest concentration 0.5 mg/l the growth rate of hyphae was reduced, as the full hyphal cover of the agar surface was observed two days later than for the control treatment (observed for both replicates) as shown in photos 27, 29 and 30.

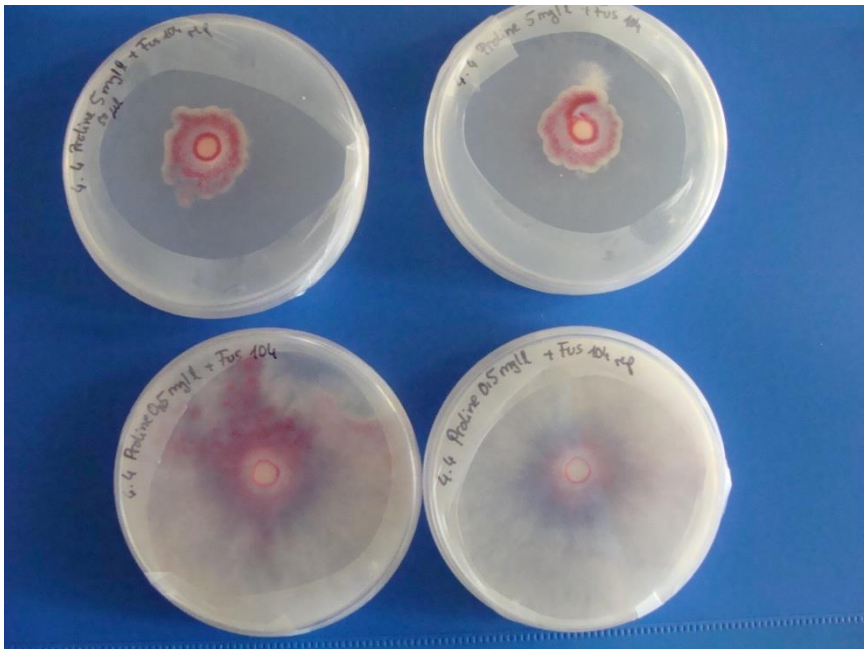


Photo 30:

First row: Proline® (5mg/l) + *F. graminearum* 104 after 7 days incubation (two replicates)

Second row: Proline® (0.5mg/l) + *F. graminearum* 104 after 7 days incubation (two replicates)

In addition to that it was observed that the hyphae growing on plates amended with the fungicide changed their color from white to pinkish, which did not happen for the control treatment.

4.7 Dual culture assay

The bacterial isolate 33 failed already after five days incubation to stop hyphal growth of *F. graminearum* isolate number 104 for treatment “t1” where fungicide and biocontrol agent applied in a combination but also in treatment “t2” where the bacterium were grown together with *Fusarium* without fungicide addition. Biocontrol was observed to have failed for this bacterial isolate as the hyphae were able to cross the line on the agar on which the antagonistic bacteria isolate have been distributed (Photo 33). The other 9 isolates tested (no. 160, 2, 103, 157, 120, 153, 155, 97 and 132) did stop fungal growth after seven days incubation, thus the hyphae were not crossing the line on which the bacteria isolates have been growing on the agar (observed for “t1” and “t2”) (Photo 32). For those bacterial isolates that succeeded in controlling fungal growth the *Fusarium* hyphae produced pink pigments if they got in contact with the antagonistic bacteria (observed for “t1” and “t2”) (Photo 31).

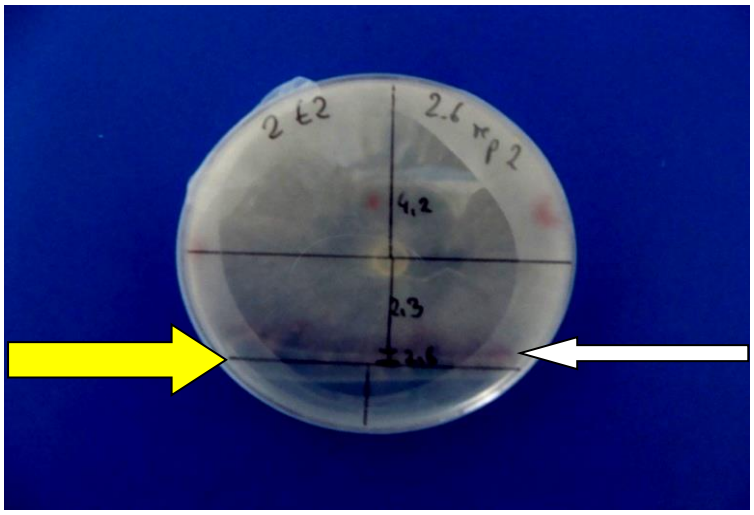


Photo 31: Pink pigment production of *F. graminearum* isolate 104 if getting contact to antagonistic bacteria (white arrow); after 7 days incubation; bacteria only were grown on the line indicated by the yellow arrow!

It was observed for the bacterial isolates marked in blue (Table 5), that if the fungal pathogen received a fungicide treatment, it as well produced pink pigments close to the agar plug and sometimes for the whole middle mycelia area (Photo 36) (observed for “t1” and “t4”).

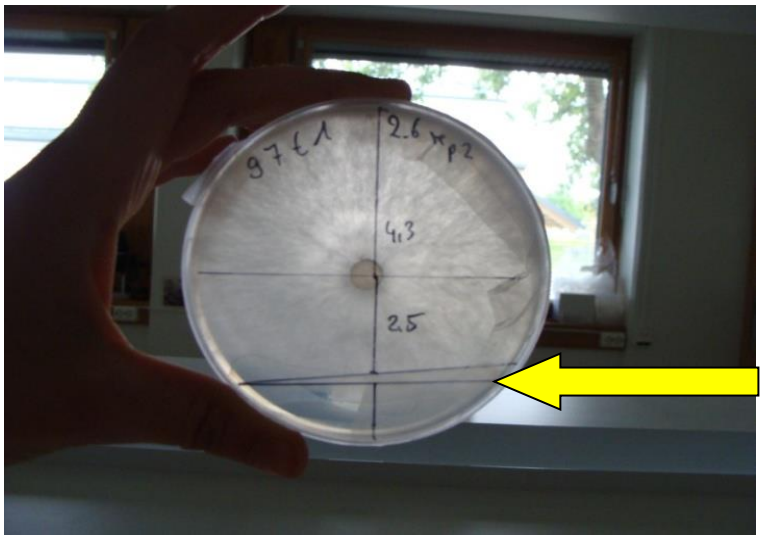


Photo 32: Example for successful biocontrol: hyphae did not cross the line on which antagonistic bacteria were growing (yellow arrow); after 7 days incubation

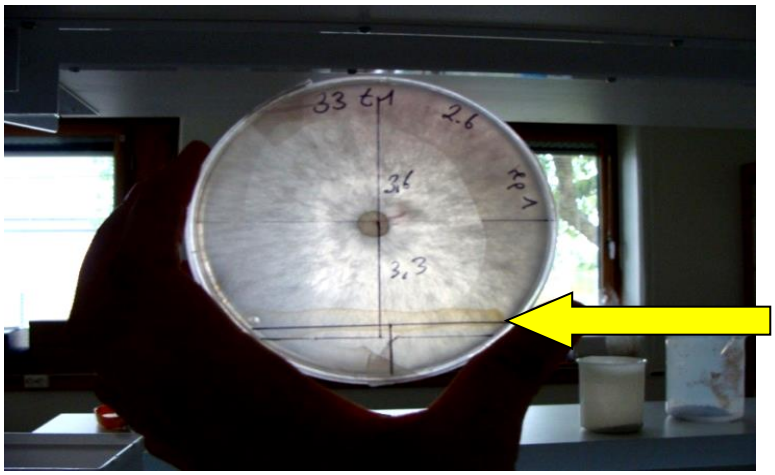


Photo 33: Example for not-successful biocontrol: hyphae crossed the line on which antagonistic bacteria were growing (yellow arrow); after 7 days incubation

Photo 34: Dual culture assay: Visual identification of fungicide failure treatment “t4”

For the treatment “t4” where *Fusarium* was growing with the single fungicide application, it was striking that after five days incubation it was observed that the fungicide sometimes did not work thus chemical control failed for some reason. These experiments are marked in red (Table 5). Fungicide failure was observed as the mycelia mat area size already after five days incubation became similar or equal to the control for treatment “t4”. Previous tests with the fungicide applied at a sub-lethal concentration (0.5 mg/l) after five days incubation should yield a smaller mycelia area compared to the control (Photo 27, 29). Another indicator for fungicide failure was that no pink pigments have been produced by the fungus (Photo 34). Fungicide failure also was observed for treatment “t1” for the bacteria isolates (153,155, 97 and 132). For bacteria isolate no. 120 fungicide failure only was observed for “t4” but not for “t1” (Table 5).

To assess the significance of treatment effect on mycelial area size this was only considered for those experiments where the fungicide worked at least for treatment “t1” (Table 5; bacteria isolates in the blue box). Before evaluating those experiments it was tested, by using one-way ANOVA analysis, if the fungicide failure caused a strong treatment assessment error in case of if all experiments of the dual culture assay would be considered, to assess if the treatments had a significant effect on mean mycelial area size [cm^2]. Therefore the ten different bacterial isolates tested were subdivided into two treatment groups: “yes”, if the fungicide worked (Table 5: bacteria isolates in blue box) and “no”, if fungicide did not work or biocontrol did not work (Table 5: bacteria isolates in red box). It was not found a significant difference between the mean mycelia area yielded by the experiments for which the fungicide worked (yielded smaller mean mycelia area) compared to the experiments for which the fungicide did not work (yielded bigger mycelia area; p-value 0.096) after fivedays incubation (Figure 2). After seven days incubation the treatment effect of fungicide error on mycelia area size became even less significant (p-value 0.394) (Figure 3).

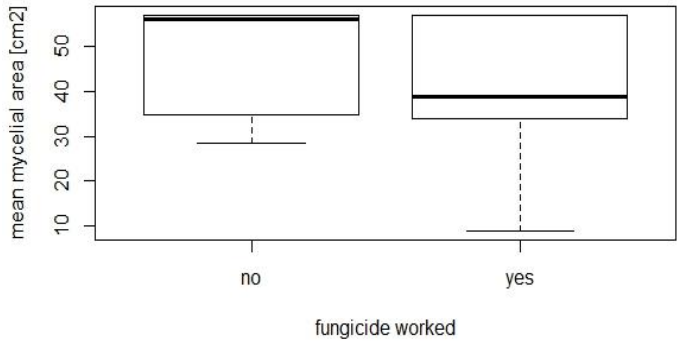


Figure 2: One-way ANOVA assessment of fungicide failure treatment effect on mycelia area size [cm^2] after 5 days incubation; p-value 0.096

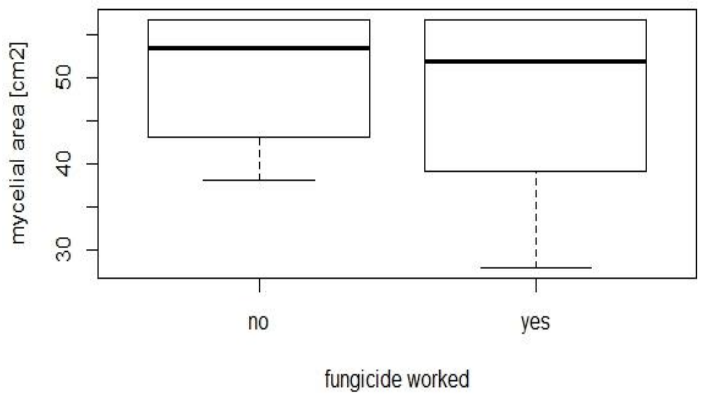


Figure 3: One-way ANOVA assessment of fungicide failure treatment effect on mycelia area size [cm^2] after 7 days incubation; p-value 0.394

Table 5: Dual culture assay: Mean mycelia area after 5 days incubation (single isolates) and after 5 and 7 days incubation (sum of all isolates). Multiple pair wise comparison of means (One-way ANOVA + Tukey HSD test (Results from RStudio)); *** = highly significant; ** = significant; * = not significant

Single isolates	Days of incubation	Control-t1	Control –t2	Control-t4	t2-t1	t4-t1	t4-t2	Fungicide worked	Biocontrol worked
160	5	***	***	**56.8 - 38.8; ** **	**	***	*		
2	5	***	***	56.8 - 39.5; ***	***	***	*		
103	5	***	***	56.8 - 40.7; **	*	***	***		
157	5	***	***	56.8 - 41.1; **	***	***	*		
120	5	***	***	56.8 - 56.8; ***	*	***	***	**no (t4) yes t1**	
153	5	***	***	56.8 - 56.8;*	*	***	***	no (t1;t4)	
155	5	***	***	56.8 - 56.8; *	*	***	***	no (t1;t4)	
97	5	***	***	56.8 - 56.8, ***	*	**	**	no (t1;t4)	
132	5	***	***	56.8 - 56.8; *	*	***	***	no (t4; t1)	
33	5	***	***	**56.8 - 56.8;***	***	***	*		**no**
all isolates	5	***	***	56.8 - 50.1; *	**	***	**		
	7	***	***	56.8 - 56.8 *	*	***	***		

In cases where the fungicide worked, treatment “t4” yielded a smaller mycelia area compared to the control (pair wise comparison of mean mycelia area (control – t4; Table 5). But the fungal hyphae continued growing and after seven days incubation “t4” yielded equal mycelia area size compared to the control. The single biocontrol treatment “t2” after five days incubation yielded a smaller mean mycelia area compared to single fungicide application “t4” and to the control (treatment effect was found to be not significant!)(Figure 5 and Appendix: Table 11). After 7 days incubation the mycelia area yielded by “t2” was still smaller than the mycelia area of “t4” and the control (treatment effect was found to be significant causing the difference of means; Figure 4, 6 and Appendix Table 10). Treatment “t1” after five and seven days incubation always yielded the smallest mycelial area compared to all other treatments (highly significant treatment effect compared to “t4”) (Figure 4,6 and Appendix Table 11). After five days incubation the combined treatment “t1” was significantly better than the single biocontrol treatment “t2” (Figure 4, 6 and Appendix

Table) which became highly significant result after seven days incubation (Figure 4 and 7 and Appendix Table 10).

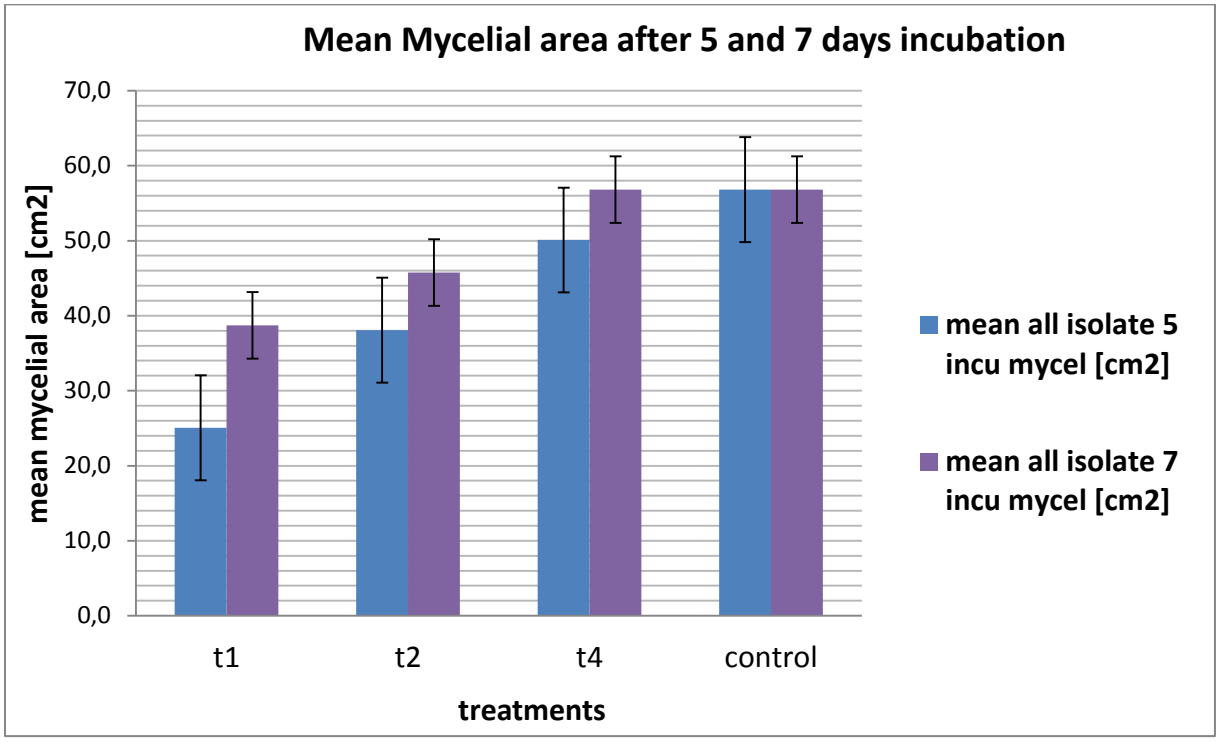


Figure 4: Mean mycelia area yielded by experiments where the fungicide worked for at least "t1": Combined treatment (biocontrol agent + fungicide) "t1", single biocontrol treatment "t2" and single fungicide application "t4" compared to the control (no treatment). Error bars indicate the standard errors of the experiments.

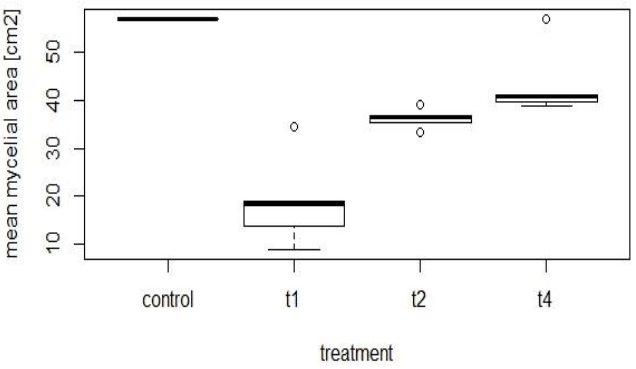


Figure 5: Mean mycelial area yielded by different control treatments after 5 days incubation; it was only considered the values derived from plates where the fungicide worked at least for "t1"! Corresponding results of Tukey HSD-multi comparison of means are available in the Appendix.

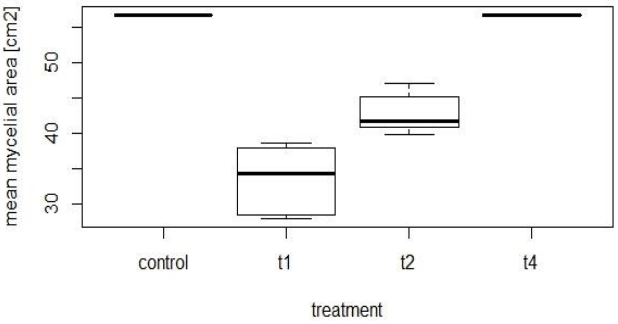


Figure 6: Mean mycelial area yielded by different control treatments after 7 days incubation; it was only considered the values derived from plates where the fungicide worked at least for "t1"!; Corresponding results of Tukey HSD-multi comparison of means are available in the Appendix.

Correlation between mycelia area size and antagonistic performance (mean radius difference)

For treatment "t1" and "t2" it was assessed the correlation between the mean mycelia area for all isolates (so including the experiments where fungicide failed). After five days incubation it was found a strong positive correlation between mean mycelia area size [cm^2] and the mean radius difference [cm]; correlation coefficient: 0.8136. A linear regression yielded R^2= 0.735 (Figure 7). After seven days incubation the correlation between mean mycelia area size [cm^2] and mean radius difference [cm] became negative. The linear regression yielded R^2= 0.574 (Figure 8).

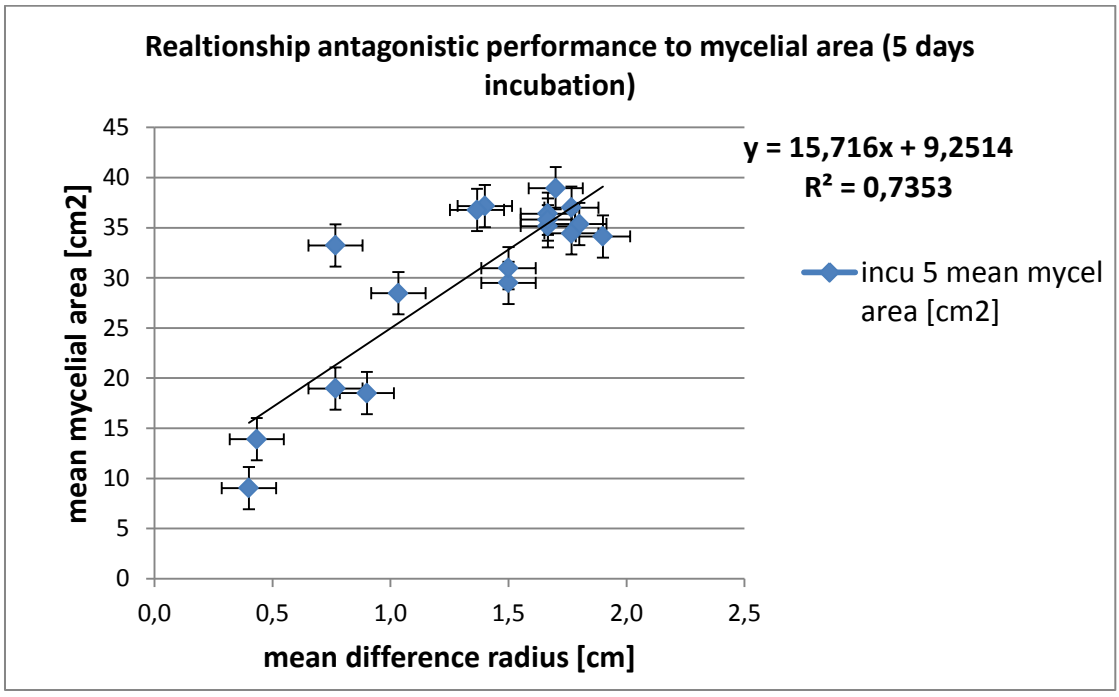


Figure 7: Linear regression mean mycelia area size dependent on antagonistic performance(mean radius difference) after 5 days incubation; determined standard errors

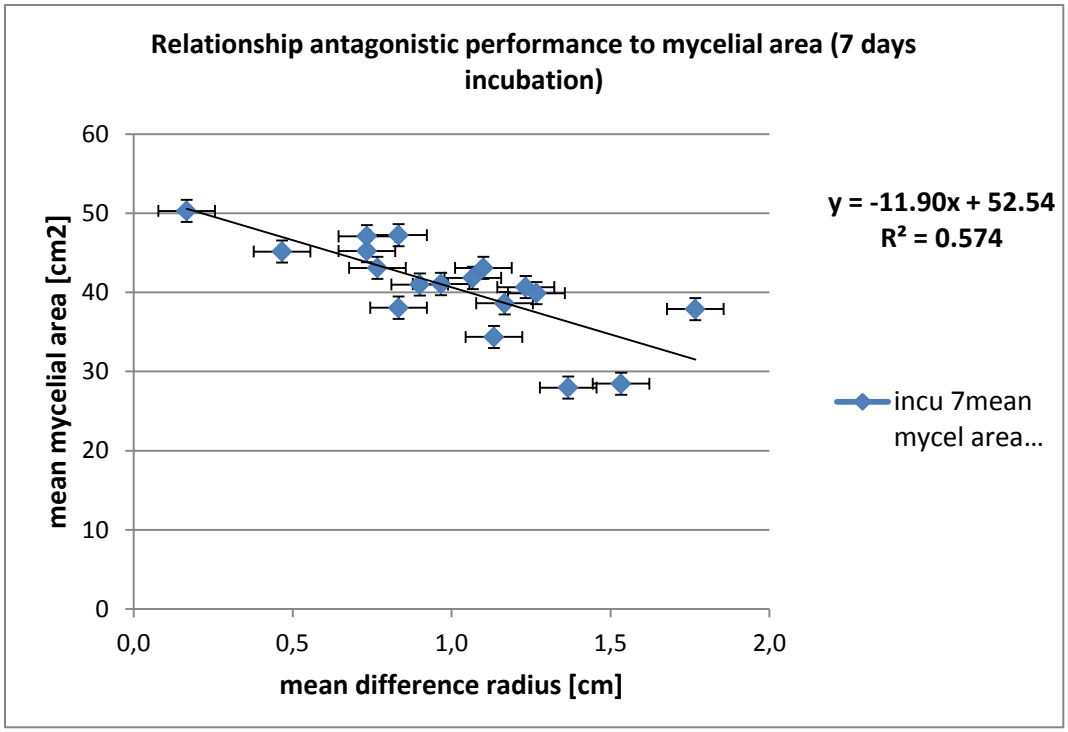


Figure 8: Linear regression mean mycelia area dependent on antagonistic performance (mean radius difference) after 7 days incubation; determined standard errors

4.8 Field dose testing

It was found that the ten bacteria isolates which have been applied in the dual culture assay are able to survive and grow if a field dose of Proline® has been applied (500mg/l).

4.9 Detached leaf assay

The detached leaf assay failed as it was impossible to determine lesion length. Failure was assumed to be because of experimental errors as the filter paper became too dry during the experiment. It was not enough time available to repeat the experiment.

5. Discussion

5.1 Bacteria abundance on wheat leaves consequently to pesticide treatment

The correlation between the pesticide or no pesticide treatment and the amount of colony forming units removed from winter wheat leaves harvested in 2011 was found to be low as the correlation factor was only 0.46. Therefore, from this data it could not be concluded that wheat leaves grown in fields receiving pesticide treatments necessarily host a lower quantity of bacteria than on leaves from fields receiving no pesticide treatment. The low correlation factor between pesticide application and

number of bacteria colonies removed from wheat leaves was expected because in this study for both field treatments (fungicide application/ no fungicide application) it was found the same amount of samples showing the higher abundance of bacteria removed from leaves receiving pesticides compared to leaves that did not receive pesticide treatment (Chapter 4.1). Thus, antagonistic bacteria could be found also if the crop had faced a pesticide treatment and in addition to that antagonistic bacterial species were mainly isolated from leaves which received pesticide spraying (Table 3).

From this I assumed that biocontrol agents removed from prothioconazole treated winter wheat leaves might be able to survive a treatment with Proline®, which also contains prothioconazole as active ingredient, which would reject the second hypothesis that bacterial antagonists might be killed by fungicide application. In this case fungicides apparently do not necessarily affect the bacterial antagonistic community on leaves. In the dual culture assay it was tested a sub-lethal dose and separately also a field dose of Proline® for its effect on antagonistic bacteria capability for survival. The bacteria survived both concentrations of the fungicide.

5.3 Macroconidia and F. graminearum no. 40 in a dual culture assay with selected antagonistic bacteria isolates

Three of the five tested antagonists inhibited the growth of fungal hyphae from macroconidia spores of *F. graminearum*. The macroconidia spores were obtained from another *F. graminearum* isolate (isolate number 51) than the isolate used to identify antagonistic bacteria (isolate no. 104).Therefore, antagonistic performance might be not only dependent on e.g. host resistance and *Fusarium* species resistance, but might also be Fusarium species specific. *F. graminearum* disperses and multiplies through an asexual (production of macroconidia) and sexual (production of ascospores) life cycle (Börner et al. 2009). During the most susceptible period of wheat for *Fusarium* infection (anthesis) Klix et al. (2007) detected a larger amount of ascospores than macroconidia above wheat heads, which raised the assumption that as primary inoculum ascospores might be more important than macroconidia. Thus, further research is needed to investigate how antagonistic performance of biocontrol agents varies in accordance to inhibit hyphal growth from different spores of *F. graminearum,* as this study did not focus on research questions investigating antagonistic interactions against different types of spores of *F. graminearum*.

In this study I found that different isolates of *F. graminearum* show different adaption and growth behavior to different substrates. The isolate 104 showed good fungal growth development on half strength NA-plates and on PDA. In contrast, the isolate 40 was observed to show inhibited fungal growth on half strength NA plates in comparison to isolate 104, but similar growth behavior on PDA. From this it could be concluded that the isolate 104 may be more competitive than isolate 40 as it adapts better to a less nutritious environment than isolate 40. The variation of survival and

aggressiveness of the different *F. graminearum* isolates could be explained by the phenotypic variation of *F. graminearum* (Talas 2011). During developing the methods it was found that if *F. graminearum* isolate 104 was re-grown more than five times on new half strength NA substrate that it failed to grow again. Therefore, after at least three times re-growing *Fusarium* on half-strength nutrient agar substrate the fungus needs to be re-cultured on PDA which offers better nutrition conditions than half-strength nutrient agar.

5.4 Concentration of pesticide applied in the dual culture assay on agar plates and spring wheat leaves

According to Müllenborn et al. 2007 Proline® the effective dose for Proline where 50% of the fungal growth of *F. graminearum* is inhibited (ED_{50}) is 0.4 ± 0.05 mg/l (*in vitro* experiments).

The concentration of 0.5 mg/l of prothioconazole applied on the agar plates is only slightly higher than ED_{50}(the concentration of an active substance of the fungicide to the target fungus and is much lower than the recommended concentration for field applications (0.7 g/l: given by 0.8 l Proline diluted in 300 l water per ha; Bayer Crop Science user manual).The effective dose (ED_{50}) is defined as the concentration of an active substance that reaches 50% of its maximum effect to a target organism. The higher the ED_{50} value the less sensible is the pathogen to the pesticide and consequently higher concentrations need to be applied to achieve effective control of the pathogen (Börner et al. 2009). The concentrations required and applied at field level are much higher than the concentrations applied for *in vitro* inoculations of *F. graminearum.* This finding was discussed by Reis et al. (2015) who determined *in vitro* for Proline 200 SC (250g/l prothioconazole) an even lower sensitivity of *F. graminearum* in order of pesticide application.

The field dose of Proline® (250g/l prothioconazole) recommended by the producer is 0.8 l/ha, which is the European standard recommended by Bayer Crop Science to provide sufficient control of *F. graminearum* in European Countries. However, according to Bayer Crop Science in Sweden only maximum 0.6l/ha are allowed to be applied on fields to control *Fusarium* because Bayer Crop Science could not justify higher doses in Sweden (Bayer Crop Science: employee information). For this study it was therefore decided to consider the field dose commonly applied in Sweden (0.6 l/ha), instead of the European standard.

In the dual culture assay and detached leaf assay the aim was to study what happens if not a field dose (500mg/l) is applied to control fungal growth of *F. graminearum,* in order to reduce the amount of pesticides applied in the field, by combining biological control agents (antagonistic bacteria) with chemical control agents (fungicides) to comply with Integrated Pest Management requirements by reducing amount of pesticides applied in the field. Therefore, the goal of this study was not to achieve most effective suppression of *F. graminearum* by chemical control

but to observe how biological and chemical control agents perform together in pathogen control.

It was decided not to apply field doses on agar plates in the dual culture assay as done by Audenaert et al. (2010) because it was discussed by Reis et al. (2015) and Klix et al. (2007) that the fungicide concentration applied in the field underlies further depletion and dilution processes during and after spraying (pesticides are very water soluble and sensible to UV-light and evaporation), which means that not the full initial concentration is in the end active in suppressing *Fusarium* spp. on wheat tissues. Both authors emphasize the finding that the pesticide concentrations used in the field (diluted in 200-600l water/ha) greatly exceed the EC_{50} level. In addition to that, no antagonistic effect caused by the bacteria can be observed if the lethal field dose (500mg/l) would be applied in the dual culture assay, because the fungicide would stop fungal growth before the hyphae can reach the bacteria cultures. Field dose was only applied for treatment t3 to assess if the bacteria are able to survive a concentration of 500mg/l of Proline®.

After five days incubation on agar plates the concentration of 0.5 mg L^{-1} of Proline® showed a clearly reduced the inhibitory effect on fungal growth of *F. graminearum* isolate 104 in comparison to all other tested concentrations which inhibited growth completely. Consequently, 0.5 mg/l Proline® was chosen as sub-lethal dose applied in the dual culture assay. The term “sub-lethal” was defined as an effective dose of the Proline® fungicide that lies between ED_{50} and ED_{90} for *F. graminearum*. A sub-lethal dose was intended to be applied because an additional *Fusarium* control agent (biocontrol agent) was co-incubated to compensate the reduced (suboptimal) chemical control of *F. graminearum*. The aim was to investigate if the chemical and biological control agent together are able to achieve an effective inhibition of fungal growth in comparison to if a lethal chemical dose of a fungicide is applied. The lethal dose here was defined as the dose of the fungicide applied in the field recommended by the producer (500 mg/l (Sweden) or 670 mg/L (European standard). It was observed that the isolate 104 of *F. graminearum* continued growing on the half-strength NA plates after five days incubation and covered the whole agar after seven days incubation. The control (fungal growth without fungicide application) showed a full scale agar cover by hyphae of *F. graminearum* isolate 104 after five days incubation. From that it was concluded that the application of suboptimal (sublethal) concentration of the fungicide might have caused a delay of hyphae growth (reduced growth rate) instead of stopping it.

5.5 Fungicide - biocontrol agent - cultivar interactions

Proline® is reported as to be very effective in inhibiting fungal growth (mycelia growth) of *Fusarium* species (Müllenborn et al. 2010). As described by Klix et al. (2007) its active substance prothioconazole inhibits an important enzyme of *Fusarium* (14α-demethylase) that is indispensable for the biosynthesis of ergosterol. Ergosterol is almost exclusively found in membranes of fungi and not in membranes of bacteria

(Mille-Lindblom et al. 2004). Therefore, bacterial survival after fungicide application can be explained by the fact that prothioconazole inhibits the biosynthesis of ergosterol, not present in bacteria.

According to Figure 4 the combined control treatment “t1” worked always better than single biocontrol “t2” and single chemical control “t4” after five and seven days incubation on half-strength NA plates. Treatment “t2” always worked better than treatment “t4”. Single chemical control “t4” (sub-lethal dose 0.5 mg/l applied) only after five days incubation yielded a smaller mean mycelia area [cm^2] compared to the control, but became equal to the control after seven days incubation. From this it was assumed that the sublethal dose of the fungicide Proline® only inhibited the growth rate of *F. graminearum* isolate 104 but did not stop fungal growth. The combined treatment “t1” and single biocontrol treatment “t2” completely stopped fungal growth on agar plates after seven days incubation. This could be observed as the hyphae did not cross the line on which the antagonistic bacteria have been growing but produced pink pigments instead along this line. Stopped fungal growth was assumed to be caused by a depletion of nutrients by the antagonistic bacteria, so that not enough nutrients were provided to the fungus to continue growing. Another possibility for the stopped fungal growth was assumed to be that the bacterial antagonists produced fungal growth inhibiting substances. For example according to Wachowska et al. (2012) antagonistic bacteria can control *F. graminearum* by producing inhibitory substances or for example the very antagonistic species Sphingomonas S11 elicited induced systemic response (ISR) of the host plant.

The bacterial isolate no. 33, which was the only isolate that was morphological different from the other isolates tested in the dual culture assay, surprisingly was the only isolate that failed to control and stop fungal growth of *F. graminearum* for both treatment “t1” and treatment “t2”, where isolate 33 was present. The question was if the bacterial isolate no. 33 failed to show antagonistic effects against *F. graminearum* because of the presence of the fungicide. This could not be concluded as biocontrol for this bacterium as well failed for treatment “t2” which had not received any fungicide application. Therefore, it could not be concluded that the fungicide application affected antagonistic performance of the bacterial isolate 33.

As explained earlier conclusions concerning which treatment controlled fungal growth of *F. graminearum* in the dual culture assay were only derived from replicates five out of ten bacterial isolates where the fungicide worked and where biocontrol was successful as well. After five days incubation single biocontrol treatment “t2” yielded a smaller mycelia area [cm^2] than the single chemical treatment “t4”, but the treatment effect was not significant (Figure 5) and “t4” yielded better control of fungal growth than the control but also here the treatment effect was found to be not significant. The finding that the fungicide Proline® (0.5 mg/l) effect failed in some cases was surprising as in previous tests (Photo 27) after five days incubation the mycelia area were registered be much smaller than the mycelia area of the control (Photo 29). It was assumed that the fungicide might have lost some of its efficacy as it was

standing two weeks after the testing, before it was applied in the dual culture assay. Another explanation could be that the fungicide was not homogenous or not sufficiently and evenly distributed sometimes. This might have been the reason why after five days incubation the treatment effect for “t1” in comparison to “t2” was significant but less significant than after seven days incubation. In case of the fungicide did not work, the mycelia area of “t1” became similar to the mycelia area of “t2” which caused a treatment assessment error that was reduced by excluding the plates for which the fungicide did not work. The biocontrol agents after seven days incubation were able to stop fungal growth even when the fungicide was applied.

All in all it was shown that biocontrol agents derived from wheat leaves and grown on half-strength nutrient agar, are able to compensate sublethal dose of of a fungicide targeting *F. graminearum,* as “t1” with antagonist always was the best treatment to control fungal growth no matter if the fungicide worked or not. Single biocontrol “t2” in the long run was found to be better in controlling fungal growth than single chemical control as it stops fungal growth instead of just reducing the growth rate. This finding is only valid for the particular combination of lab conditions, the *F. graminearum* isolate 104, the particular bacteria isolates tested a sub-lethal concentration of the fungicide Proline® (0.5 mg/l) applied, the particular fungicide application method and timing, antagonistic bacteria survival etc. There are many environmental factors and experimental method patterns that might have strongly influenced the outcome of the experiments. Why it is important to keep in mind that the findings of this study only have limited validity according to real-life conditions will be explained in the following paragraphs:

Timing of application and fungal resistance

Of high concern for the fungicide´s inhibitory performance against *F. graminearum* is the timing of application. Klix et al. (2007) reported that a the common timing in the field to control FHB was a fungicide application during anthesis (flowering) because this is the growth stage of wheat where it is most susceptible to infection by spores of *F. graminearum*. In this study the fungicide was applied to leaves, removed from wheat plants before anthesis started, because Edwards and Godley (2010) and the producer of the fungicide Proline® explained that an fungicide application before flowering provides additional FHB disease control in comparison to an application during flowering. Therefore, the leaves were sampled before anthesis to simulate optimal application timing of the fungicide. For the *in vitro* dual culture assay on agar plates the fungicide was applied at the same time as the biocontrol agent. The *F. graminearum* isolate 104 was incubated four days after both fungal growth inhibiting agents (chemical and biological) were added to the leaf. Hence, the bacteria were expected to have time to adapt to the substrate´s environment to increase its chances of survival (discussed in paragraph below). This could not be proven because the detached leaf assay failed.

In this study it was found that the antagonistic bacteria isolated from winter wheat leaves survived a concentration of 0.5 mg/l of the fungicide Proline® and even a field

dose of 500mg/l. For the application of biocontrol agents it is important to keep in mind microorganisms need time to establish in a new environment. Earlier studies has found , by testing different antagonistic microorganisms, that for Sphingomonas S1,a strong antagonist isolated from winter wheat leaves, failed in its antagonistic performance on wheat seedlings artificially infected with different isolates of *F. graminearum*(Wachowska et al. (2012). The fail of controlling *Fusarium* by the biocontrol agent was identified as to be caused by too late application timing. Wachowska et al. (2012) concluded that the bacteria needed at least 96 hours to adapt and grow in their new environment before they were able to suppress *F. graminearum* (Wachowska et al. (2012). This was the reason why it was decided to infect the wheat leave segments in the detached leaf assay four days after the chemical and biocontrol agents were applied.

In this study the concentration of the antagonistic bacteria suspension applied lied between 10^4 and 10^5 cells/ml. This was similar to the amount used by Wachowska et al. (2012) who used on their leaves 2x 10^5 cells ml^{-1}. The isolated antagonistic bacteria in this study have however not been taxonomically identified and it might be possible that their application requires species specific concentrations to achieve most effective control of *F. graminearum*.

Choice of cultivar

Another important role for the plant – fungicide – pathogen and biocontrol agent´s interactions plays the choice of the wheat cultivar used and its resistance against *F. graminearum* and the biocontrol agent´s survival performance in the host´s environment. Lenc (2011) identified the cultivar “Vinjett”, which was used in this study, in an organic farming system to be one of the most susceptible spring wheat cultivars regarding to *Fusarium* spp. In the study of Lenc (2011) *F. poae* was the dominant species that infected “Vinjett” and caused FHB but not *F. graminearum*. It remained unclear how susceptible “Vinjett” is to *F. graminearum* compared to other spring wheat cultivars. The *F. graminearum* resistance of a cultivar to can reduce the DON production by *Fusarium* significantly and therefore also can reduce the aggressiveness of the fungus (Mesterházy 2002). It might be possible that the bacteria isolated from winter wheat survive better on other wheat varieties and perform better in co-operation with a chemical control agent. This assumption is in agreement with Khan and Doohan (2009) who pointed out that the performance of a biocontrol agent to suppress a *Fusarium* is cultivar dependent.

The role of DON production affected by suboptimal fungicide application and the application of biocontrol agents

For the combined treatment “t1” and the single biocontrol treatment “t2” it was observed that the hyphae of *F. graminearum* isolate 104 produced pink pigments if they got in contact with the antagonistic bacteria. The production of pink pigments was not observed for the control (growing *F. graminearum* without any treatments). The production of pink pigments also was observed when the fungicide was applied

in the dual culture assay (t4 and t1). For the lethal concentrations of the fungicide (500mg/l, 50 mg/l and 5 mg/l), it was observed a particularly strong pink pigment production (Photo 27, 28 and 30). For the sub-lethal dose (0.5 mg/l) the pink pigment production concentrated on the middle of the agar plate (Photo 28, 30). After the fungus continued growing and covered the whole plate after seven days incubation, the hyphae did not produce further pink pigments (Photo 30). From this I assumed that the pink pigment of *F. graminearum* might be a kind of stress response of the fungus to fungicides or to deal with competing microorganisms.

Normally fungicides can also reduce levels of DON accumulation if it is applied at the right time to the plant and in the by the producer recommended concentration (Cromey et. al. 2001). But in this study it was aimed to investigate what happens if the fungicide is applied at a suboptimal dose. According to Audenaert et al. (2010) the application of sub-lethal concentrations of triazole fungicides (such as Proline®) triggers DON-production by *F. graminearum* caused by plant stress response to the fungicide. Sub-lethal concentrations were defined by Audenaert et al. (2010) as a suboptimal concentration of Proline® applied to inhibit conidia germination of *F. graminearum.* It was found that the fungicides induced an oxidative stress to the host plant which as a response elicits defense signals which triggered the fungal DON biosynthesis. This finding was found to be species specific as the increased DON production was detected for *F. graminearum* but not for *F. culmorum* (Audenaert et al. 2010).The increased DON production by suboptimal fungicide application has been proved also by other studies, but only for *in vitro* experiments. Under field conditions the evidence of different studies was conflicting (Edwards 2004). Audenaert et al. (2010) was testing *in vitro* a tenfold dilution series of the fungicide and came to the result that a dilution of 1/1000 of the field dose caused a higher DON production by the fungus and in a not significant inhibition of conidia germination 48h after treatment (Figure 9). The authors also found that suboptimal low concentrations of the Proline® fungicide resulted in non-significant inhibition of mycelia radial outgrow of *F. graminearum* which supports the findings in the present study.

This finding is also in agreement with Hrubošová-Hrmováet et al. (2011) who found that sub-optimal concentrations of fungicides increased DON production. There is a lack of knowledge how DON production might trigger the fungal growth of *F. graminearum* because there is lacking knowledge about at which development stages of the fungus the mycotoxins are produced in which amount and for what purpose. In this study it was assumed that maybe the DON production by *F. graminearum,* which makes this species so competitive against other *Fusarium* species, might be related to the production of pink pigments.

I assumed that maybe the DON production by *F. graminearum,* which makes this species so competitive against other *Fusarium* species, might be related to the production of pink pigments.

According to Börner et al. (2009) the production of mycotoxins is able to increase aggressiveness of a *Fusarium* species, but its influence on pathogenesis was determined to be not significant. Pathogenesis by Börner et al (2014) is defined as the genetically fixed ability of a pathogen to cause a disease and aggressiveness is defined as the ability of a pathogen to infect a plant, to use it as a source of nutrition and to multiply on it).It was assumed in this study that the increased DON production by *F. graminearum* caused by the sublethal fungicide treatment might have contributed in an unknown extent to mycelial growth ability of the fungus because mycotoxins are produced by *F. graminearum* in addition to enzymes to destroy cell walls (Börner et al. (2009).This assumption could not be proven in this study because DON production was not measured, but fungal growth (hyphae growth) at the early stages of infection. I concluded that mycotoxins might only be produced to increase the ability of the fungus to infect a host, but its production is not essential for initial infection, because the host can also be infected by *Fusarium* species that are not producing mycotoxins (Bai et al. 2002).Johansson et al. (2003) found that DON plays an important role in the infection process, resulting in many cases in a high correlation between level of infection and mycotoxin accumulation. According to Talas (2011) *F. graminearum* produces DON no matter if it is needed for aggressiveness or not but Bai et al. (2002) states that DON production is not crucial for the primary or secondary infection by *Fusarium*. DON is believed to be produced by *F. graminearum* to compete among other *Fusarium* species (Johansson et al. 2003). Hence, mycotoxins might mainly be produced for competitive reasons.

The question here is if the antagonistic bacteria applied in this study affect DON production as they inhibit fungal growth even if a sub-lethal ($<ED_{100}$) of fungicide is applied. This question could not be answered in the present study.

Bio- control agents

It is not much known about how pesticides might affect different biological control agents. Many studies about Integrated Pest Management concluded that maximum efficacy of biocontrol agents might be only achievable if the microorganisms reduce but not replace chemical control measures (Gilbert et al. 2013).To optimize Integrated Pest Management strategies it is necessary to work on a better understanding about the mechanisms of how antagonistic bacteria, fungi and yeast suppress different species and complexes of *Fusarium* spp.and to develop a better understanding how e.g. agricultural management practices, pesticide application strategies and climatic patterns might regulate biocontrol efficacy.

The question if antagonistic bacteria are able to survive if a fungicide additionally is applied is of high concern for optimizing integrated pest control. In this study it was found that the applied antagonistic bacteria species, which were removed from winter wheat leaves, survive if they were challenged with an applied sub-lethal concentration of 0.5 mg/l and a lethal field concentration (500mg/l) of Proline®. This fungicide works curative against *Fusarium* infections, thus can be applied at the same time as the artificial *Fusarium* infection on leaves (Börner et al. 2009). Most of

the antagonistic bacteria identified were removed from leaves which received pesticide treatment in the field. This finding also supports that antagonistic bacteria are not necessarily affected by the application of pesticides, especially fungicides.

In the present study antagonistic bacteria isolated from wheat leaves successfully reduced fungal growth of *F. graminearum* under suboptimal nutritional conditions (half-strength NA). Nutritious stress in the field is normally the case as well, but this study did not cover other environmental stress factors which highly might affect the performance and survival of the applied biocontrol agents. Especially the wheat leaves and spikes where the biocontrol agents would be applied to control the infection by *F. graminearum* before and during anthesis, the environmental conditions for bacteria are very challenging. The bacteria could be stressed by drought, high UV-radiation, nutritious stress and high temperatures, as the leaves and spikes are highly exposed to severe or stressful weather conditions (Gilbert et al. 2004).

To make biocontrol products marketable it is important to ensure the long-term survival and ability of the microorganisms to multiply in the field. The failure of survival and multiplication of biocontrol agents is currently the main problem for the application in the field. Gilbert et al. (2004) suggests different solutions to enhance the efficacy and survival ability of biocontrol agents in the field. The suggestions are for example the usage of growth stimulants mixed with the biocontrol agents or to find strains that show high resistance to environmental stress factors. The research on biocontrol agents is still really promising in order to reduce the amount of chemical control agents used and to prevent the emergence of fungicide-resistant strains, which is always a danger if a fungicide is applied over a long period of time to control *F. graminearum* (Gilbert et al. 2004). In addition to that, to explore opportunities of biocontrol of *F. graminearum* in wheat is necessary because currently taken control measures such as cultivation strategies, usage of resistant cultivars and usage of fungicides in the long run did not provide satisfying disease control (Müllenborn et al. 2007).

5.6 Good agricultural practice (Alternative control measures)

In addition to the application of biocontrol agents and chemical control strategies, good agricultural practice requires an integrated approach to control diseases caused by *Fusarium*. The EU legislation defines alternative measures to reduce the risk of mycotoxin contamination, *Fusarium* infection and spreading causing the outbreak of *Fusarium* diseases. These measures should consider research findings that increased understanding of infection pathways and the life cycle of the different *Fusarium* species (Edwards and Godley 2010). *F. graminearum* is well known as a soil and/or seed-born pathogen as it survives on crop debris (Klix et al. 2007). Therefore, it is recommended for crop rotations to avoid the use preceding crops that are susceptible to *Fusarium* infections such as corn, oat and wheat (Klix et al. 2007). Shallow burying of crop debris should trigger microbial decomposition of the inoculum source of *Fusarium* as antagonistic saprophytic microorganisms compete with the

pathogen for the nutrients provided by the crop debris. Most of the biocontrol agents are rather applied as seed treatment (Johansson 2003) or to the wheat heads (Jochum et al. 2006; Khan and Doohan 2009) instead of applying it to the wheat leaves. If the biocontrol agents were applied to the heads it was done normally under controlled environmental conditions in the greenhouse and not under real-life conditions in the field.

Choice and selective breeding of *Fusarium*-resistant wheat cultivars nowadays is believed to be the most effective way to reduce the risk of *Fusarium* infections and mycotoxin contamination of grains (Edwards and Godley 2010). But the problem with *Fusarium*-resistant wheat cultivars is that their number is very limited and that their resistance induces genetic features that often generate negative agronomic features e.g. lower yields (Edwards and Godley 2010).

5.7 Suggestions (Further research, improving experiment)

It was found that the antagonistic bacteria which were applied as biocontrol agents to the spring wheat leaves were capable of survival if 0.5 mg/l Proline® was applied on half-strength nutrient agar substrate. The survival of the antagonists was not tested for the detached leaf assay because of temporal restrictions for the experiment. Thus it could be interesting to test different doses of Proline® applied on wheat leaves *in vitro* as well as *in vivo* (in the field) for the capability of survival of the bacterial antagonists by using molecular techniques such as Real Time PCR. Using this detection method it would be necessary to taxonomically differentiate the bacterial species and to find out how their antagonistic performance against *F. graminearum* actually works e.g. if they produce inhibitory substances or/and inhibit fungal growth through competition for nutrients (see discussion biocontrol agents chapter 5.5). It would also be interesting to assess if and how antagonistic bacteria among each other interact and how that might affect their antagonistic performance against *F. graminearum*. They might work together if they are co-cultivated (testing mixtures of antagonistic bacteria). In addition to that testing well known *Fusarium* antagonists such as Bacillus subtilis, Pseudomonas species and Sphingomonas (Chapter 5.5) for their response to pesticide application could be another prospective experiment.

The taxonomically identification of the isolated bacteria is important for better understanding of their inhibitory performance against *F. graminearum* and to optimize their application in the field (ensure survival etc.). The bacterial isolates were stored in – 80 °C and are available for taxonomical differentiation. To identify the different bacteria the following methods can be applied: e.g. Phosphor Lipid Fatty Acid Analysis (PLFA) to determine to which bacterial group the isolated bacteria belong to (species specific biomarkers), DNA fingerprinting method AFLP (Janssen et al. 1996), 16S rRNA sequencing method (Janda and Abbot (2007) or by applying bacteria genome databases like e.g. “EnsemblBacteria.com”, “Microbial Genome Database for Comparative Analysis (MBGD)” or “Bacterial Isolate Genome Sequence Database (BIGSdb)” by applying species specific sequence tags.

Not only fungicides are applied to control *F. graminearum* but also herbicides can be a control the pathogen as weeds were identified to be one of the various sources of primary *Fusarium* inoculums for small grain cereals (Edwards 2004).It is therefore necessary to test not only fungicides but also herbicides for their effect on biocontrol agents for *F. graminearum* and how these chemical affect the saprophytic antagonistic microflora in the rhizosphere and phyllosphere. Further on, the role of DON production affecting the interactions between *F. graminearum,* the host plant and the bacterial antagonists was not assessed in this study but apparently DON production influences aggressiveness of the fungal species and is involved in infection pathways (see discussion role of DON production chapter 5.5). Further research is needed how DON production affects fungal growth and how it might affect the performance of biocontrol agents.

In this study fungal infection and the efficacy of chemical and biological control of *F. graminearum* was assessed by measuring lesion length and comparing the area of mycelial mats. A more precise technique to quantify the extent of fungal infection for the detached leaf assay would be determining the quantity of species specific fungal DNA by using Real Time PCR applying species specific primers. In addition to that it is reasonable to suggest spraying the biocontrol agent suspension on living plants mixed with the pesticide instead of using a detached leaf assay as it would represent better real-life working procedures in the field. In addition to that it was found that it is really challenging to remove the hyphae of *F. graminearum* from the solid nutrient agar. Hence, a better option would have been to cultivate the fungus in a liquid medium rather than on a solid medium (Schumann et al. 2013).

Regarding the dual culture assay on agar plates it is important to store the fungicide Proline® in a fridge to avoid multiplication of bacteria cultures inside the suspension. This was the main reason why the first trial of the dual culture assay failed because of unexpected bacterial contamination. The problem was solved by filtering the fungicide with a micro filter. A dose of 5mg/l of Proline® already effectively inhibited fungal growth of *F. graminearum* isolate 104, thus field doses do not need to be applied on half-strength NA substrate to achieve effective control of fungal growth which agreed on the findings of Reis et al. (2015). It would be interesting to test in the dual culture assay the behavior of different isolates of *F. graminearum* and mixtures of different isolates for their response to applied antagonists and chemical control agents.

Another suggestion to improve accuracy of the experiments is to increase the correctness of the calibration curves to determine the concentration of antagonistic bacteria in suspension by preparing a calibration curve for each single antagonistic species. For this a 10-fold dilution series containing at least 7 steps are needed to provide countable concentrations. In this study normally only the fifth dilution was countable and therefore the calibration curves had to be prepared by assuming similar absorption behavior of the taxonomically not differentiated antagonistic bacteria isolates at 280nm in the NanoDrop Spectrophotometer (Chapter 2.4). To

determine the concentration of the bacterial suspension it was calculated the mean concentration from three different calibration curves, because one of them did provide only a very low coefficient of determination but included all measured bacterial isolates (Appendix: Figure 10)

The last suggestion for further experiments *in vitro* is that environmental growth conditions could be changed for the dual culture assay on living plants (in the greenhouse) and agar plates because the life cycle and aggressiveness of *F. graminearum* is very dependent on temperature and moisture regimes (Johansson 2003).

6. Conclusion

In this study the selected bacterial antagonists survived a concentration of the fungicide Proline® an experimental dose of 0.5mg/l but also a field dose of 500 mg/l. This finding rejects the second hypothesis that antagonistic bacteria removed from winter wheat leaves and applied to half-strength nutrient agar die from a suboptimal dose (experimental dose) of Proline®. The isolate 104 of *F. graminearum* was able to grow on half-strength nutrient agar. Different *F. graminearum* isolates showed different capability to survive in the same environment. On half-strength nutrient agar the application of 0.5mg/l of the fungicide Proline® decreased the fungal growth rate as a full cover of the agar plate was delayed by two days in comparison to the control where the mycelia mat of the isolate 104 of *F. graminearum* covered the full agar surface already after five days incubation. Concentrations of the fungicide higher than 5mg/l completely inhibited the growth of hyphae from agar plug inoculants.

The dual culture assay showed that a combination of a sub-lethal dose of Proline® and antagonistic bacteria always provided a stronger inhibition of fungal growth of *F. graminearum*. All bacterial isolates (except isolate 33) stopped fungal growth during seven days incubation. The fungal growth rate was only slowed down after five days incubation when grown with the fungicide in a suboptimal dose, but was not stopped after seven days incubation. After seven days the combination of antagonistic bacteria and fungicide inhibited fungal growth better (highly significant) than the single biocontrol treatment. Therefore, a co-application of antagonistic bacteria with a fungicide was able to compensate the insufficient chemical control of fungal growth of *F. graminearum* in case if a suboptimal dose of the fungicide is applied. Bacterial antagonistic performance against *F. graminearum* was not affected by fungicide application and the antagonistic bacteria might be promising to be able to survive on wheat leaves and wheat heads as they were removed from wheat leaves and thus should be adapted to the corresponding environmental conditions. The co-application of biocontrol agents and fungicides according to the findings of the present study could be promising in order to reduce the amount of fungicides applied in the field to control *F. graminearum.*

7. Acknowledgements

I would like to thank Paula Persson from the department Crop Production Ecology at SLU for helping in developing experimental methods, identifying experimental errors and always giving me advice when I needed it. I also would like to thank Helena Bötker for her technical assistance and advice regarding experimental methods and result interpretation. I thank everybody who was interested to talk with me about my topic and giving me any help. I also would like to thank Sergey Sharameev for his help. Finally I would like to thank Paula Persson for giving me the opportunity to develop my own project independently. I learned a lot how to develop my own methods and how to deal with experimental and time schedule problems.

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Appendix

Table 6: Complete table of bacteria counting: To calculate the concentration of the bacteria suspension only the counts after 5/6 days incubation were considered. The term "cont" indicates no pesticide spraying; "t" indicates that the fields received pesticide treatment. Only one plate of the two replicates was counted.

plate	number of cfu after 3 days incubation	after 5/6 days incubation	bacteria concentration [cfu/ml] for 5 leaves
1 cont 0	272	508	4342.95
1cont -1	64	180	914.29
1 cont -2	8	44	114.29
1 cont 0a	308	572	4400
1 cont -1a	84	196	1200
1 cont -2a	12	20	171.43
1 cont 0b	332	548	4742.86
1 cont -1a	48	100	685.71
1 cont -2a	8	8	114.29
1t 0		tc	
1t -1		1040	14857.14
1t -2		96	1371.43
1t 0a		tc	tc
1t-1a		tc	tc
1t-2a		tc	tc
2 cont 0		216	3085.71
2 cont -1		36	514.29
2 cont -2		4	57.14
2t 0		52	742.86
plate	**number of cfu after 3 days incubation**	**after 5/6 days incubation**	**bacteria concentration [cfu/ml] for 5 leaves**
2t-1		8	114.29
2t-2		0	0
3cont 0		376	5371.43
3 cont -1		148	2114.29
3 cont -2		16	228.57
3t 0		228	3257.14
3t -1		56	800.00
3 t -2		4	57.14
4 cont 0		788	9333.33
4 cont -1		100	1428.57
4 cont -2		24	342.86
4 cont 0 a		692	9885.71
4 cont -1a		188	2685.71
4 cont -2a		32	457.14
4 cont 0b		480	6857.14
4 cont -1b		220	3142.86
4 cont -2b		28	400.00

4t 0		432	4895.24
4t -1		48	685.71
4t -2		8	114.29
4t 0a		368	5257.14
4t -1a		48	685.71
4t -2a		8	114.29
4t 0b		228	3257.14
4t -1b		24	342.86
4t -2b		4	57.14
5 cont 0		464	6628.57
5 cont -1		84	1200.00
5 cont -2		8	114.29
5t 0		tc	tc
5t -1		508	7257.14
5t -2		136	1942.86
6 cont 0		tc	tc
6 cont -1		476	6800
6 cont -2		36	514.29
6t 0		156	2228.57
6t -1		20	285.71
6t -2		4	57.14
7 cont 0		76	1085.71
7 cont -1		16	228.57
7 cont -2		4	57.14
7t 0		40	571.43
7 t -1		4	57.14
7t -2		0	0.00
8 cont 0		tc	tc
8 cont -1		332	4742.86
8 cont -2		128	1828.57
8t 0		tc	tc
8t -1		1040	14857.14
8t -2		96	1371.43
9cont 0	tc	tc	tc
9 cont -1	tc	tc	tc
9 cont -2	344	tc	4914.29
9t 0	tc	tc	tc
9t -1	tc	tc	tc
9t -2	tc	tc	tc
10 cont 0	21	25	357.14
10 cont -1	3	5	
10 cont -2	0	0	
10 t 0	40	40	571.43
10 t -1	2	2	
10 t -2	0	0	
11 cont 0	34	48	685.71
11 cont -1	5	7	
11 cont -2	0	0	
11 t 0	380	382	5457.14
11 t -1	120	121	
11 t -2	4	4	
12 cont 0	96	136	1942.86
12 cont -1	18	21	
12 cont -2	0	1	
12 t 0	136	200	2857.14
12 t -1	10	27	
12 t -2	1	2	
13 cont 0	tc	tc	tc
13 cont -1	tc	tc	
13 cont -2	376	400	
13 t 0	tc	tc	tc
13 t -1	256	280	
13 t -2	21	27	

Table 7: Storage numbers of isolated bacteria and their antagonistic effect against *F. graminearum* isolate 104. “a” indicates that bacteria isolate was determined to be antagonistic.

isolate	number storage	antagonistic effect
1 cont I	1	no
rep 1 cont II	2	a
rep 1 cont III	3	no
1 cont IV	4	no
1 cont V	5	no
1 cont VI	6	no
1 cont VII	7	no
1 cont VIII	8	no
1 cont IX	9	no
1 cont X	10	no
1t I	11	no
1t II	12	no
1 t III	13	missing
1 t IV	14	no
1 t V	15	no
1 t VI	16	no
1 t VII	17	no
1 t VIII	18	no
1 t IX	19	no
1 t X	20	no
2 cont I	21	no
rep 2 cont II	22	no
2 cont III	23	no
2 cont IV	24	missing
2 cont V	25	a
2 cont VI	26	no
2 cont VII	27	no
2 cont VIII	28	no
2 cont IX	29	no
2 cont X	30	no
2 t I	31	no
2 t II	32	a
2 t III	33	a
2 t IV	34	no
2 t V	35	no
2 t VI	36	no
2 t VII	37	no
2 t VIII	38	no
2 t IX	39	no
2 t X	40	a
3 cont I	41	no
3 cont II	42	no
3 cont III	43	no
3 cont IV	44	no
rep 3 cont V	45	no
3 cont VI	46	no
3 cont VII	47	no
3 cont VIII	48	no
3 cont IX	49	no
3 cont X	50	no
3 t I	51	no
3 t II	52	no
3 t III	53	a
3 t IV	54	no
3 t V	55	no
3 t VI	56	a
3 t VII	57	no
3 t VIII	58	no
3 t IX	59	no
3 t X	60	no
4 cont I	61	no
rep 4 cont II	62	no
rep 4 cont III	63	no
rep 4 cont IV	64	no
4 cont V	65	no
4 cont VI	66	no
4 cont VII	67	no
4 cont VIII	68	no
4 cont IX	69	no

4 cont X	70	no
4 t I	71	no
4 t II	72	no
4 t III	73	no
4 t IV	74	no
rep 4 t V	75	no
4 t VI	76	no
4 t VII	77	no
4 t VIII	78	no
4 t IX	79	no
4 t X	80	no
rep 5 cont I	81	no
rep 5 cont II	82	no
5 cont III	83	no
5 cont IV	84	no
5 cont V	85	no
5 cont VI	86	no
5 cont VII	87	no
5 cont VIII	88	no
5 cont IX	89	no
5 cont X	90	no
rep 5 t I	91	no
rep 5 t II	92	a
5 t III	93	no
5 t IV	94	no
5 t V	95	no
5 t VI	96	no
5 t VII	97	a
5 t VIII	98	no
5 t IX	99	a
5 t X	100	no
6 cont I	101	no
6 cont II	102	no
6 cont III	103	a
6 cont IV	104	no
6 cont V	105	no
6 cont VI	106	no
6 cont VII	107	no
6 cont VIII	108	a
6 cont IX	109	no
6 cont X	110	no
6 t I	111	no
6 t II	112	no
6 t III	113	no
6 t IV	114	no
6 t V	115	no
6 t VI	116	no
6 t VII	117	no
6 t VIII	118	no
6 t IX	119	no
6 t X	120	no
7 cont I	121	no
7 cont II	122	no
7 cont III	123	no
7 cont IV	124	no
7 cont V	125	no
7 cont VI	126	no
7 cont VII	127	no
7 cont VIII	128	no
7 cont IX	129	no
7 cont X	130	no
7 t I	131	no
7 t II	132	a
7 t III	133	a
7 t IV	134	a
7 t V	135	no
7 t VI	136	no
7 t VII	137	no
7 t VIII	138	no
7 t IX	139	no
7 t X	140	no
8 cont I	141	no
8 cont II	142	no
8 cont III	143	no
8 cont IV	144	no
8 cont V	145	no

8 cont VI	146	no
8 cont VII	147	no
8 cont VIII	148	no
8 cont IX	149	no
8 cont X	150	no
8 t I	151	no
8 t II	152	no
8 t III	153	a
8 t IV	154	no
8 t V	155	a
8 t VI	156	no
8 t VII	157	a
8 t VIII	158	no
8 t IX	159	no
8 t X	160	a
9 cont I	161	no
9 cont II	162	no
9 cont III	163	no
9 cont IV	164	a
9 cont V	165	no
9 cont VI	166	no
9 cont VII	167	no
9 cont VIII	168	no
9 cont IX	169	no
9 cont X	170	no
9 t I	171	a
9 t II	172	no
9 t III	173	a
9 t IV	174	a
9 t V	175	a
9 t VI	176	a
9 t VII	177	a
9 t VIII	178	a
9 t IX	179	no
9 t X	180	a
10 cont I	181	no
10 cont II	182	no
10 cont III	183	no
10 cont IV	184	no
10 cont V	185	no
10 cont VI	186	no
10 cont VII	187	no
10 cont VIII	188	no
10 cont IX	189	no
10 cont X	190	no
10 t I	191	no
10 t II	192	no
10 t III	193	no
10 t IV	194	no
10 t V	195	no
10 t VI	196	no
10 t VII	197	no
10 t VIII	198	no
10 t IX	199	no
10 t X	200	no
11 cont I	201	no
11 cont II	202	no
11 cont III	203	no
11 cont IV	204	no
11 cont V	205	no
11 cont VI	206	no
11 cont VII	207	no
11 cont VIII	208	no
11 cont IX	209	no
11 cont X	210	no
11 t I	211	no
11 t II	212	no
11 t III	213	no
11 t IV	214	no
11 t V	215	no
11 t VI	216	no
11 t VII	217	no
11 t VIII	218	no
11 t IX	219	no
11 t X	220	no
12 cont I	221	no

12 cont II	222	no
12 cont III	223	no
12 cont IV	224	no
12 cont V	225	no
12 cont VI	226	no
12 cont VII	227	no
12 cont VIII	228	no
12 cont IX	229	no
12 cont X	230	no
12 t I	231	no
12 t II	232	no
12 t III	233	no
12 t IV	234	no
12 t V	235	no
12 t VI	236	no
12 t VII	237	no
12 t VIII	238	no
12 t IX	239	no
12 t X	240	no
13 cont I	241	no
13 cont II	242	no
13 cont III	243	no
13 cont IV	244	no
13 cont V	245	no
13 cont VI	246	no
13 cont VII	247	no
13 cont VIII	248	no
13 cont IX	249	no
13 cont X	250	no
13 t I	251	no
13 t II	252	no
13 t III	253	no
13 t IV	254	no
13 t V	255	no
13 t VI	256	no
13 t VII	257	no
13 t VIII	258	no
13 t IX	259	no
13 t X	260	no

Calibration curves applied to determine bacteria concentration applied in the dual culture assay

Figure 10: Calibration curve 1 for the antagonistic bacteria isolates 177, 155, 53, 103, 134, 2 (Mean values). R^2 = coefficient of determination. For the lower dilutions the number of colony forming units was too numerous to count. Therefore I only had only one mean value for each isolate. Y= concentration, X=absorbance.

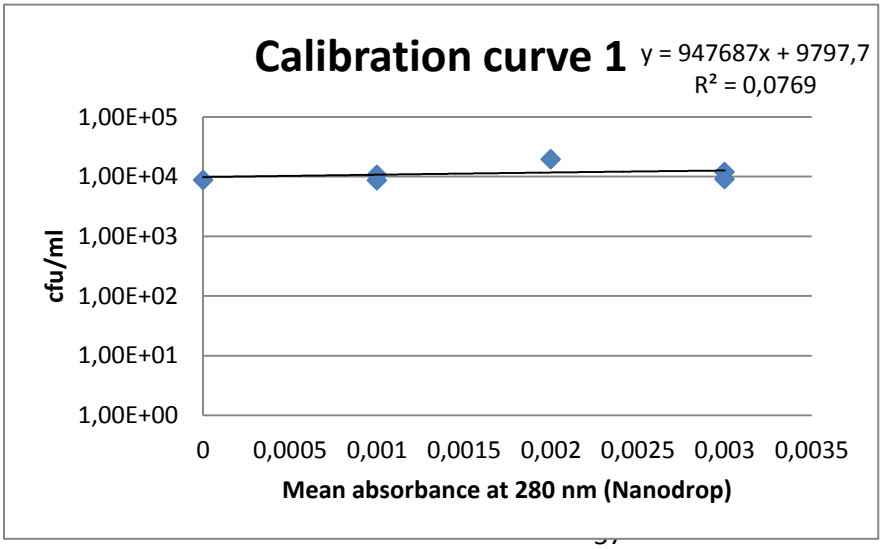

Table9: NanoDrop mean absorbance at 280 nm of seven randomly chosen antagonists and the corresponding bacterial concentration [cfu/ml].

Bacteria isolate	Mean absorbance at 280 nm	concentration [cfu/ml]
177	0	8.73E+03
155	0.001	1.06E+04
53	0.003	1.18E+04
103	0.002	1.94E+04
134	0.001	8.63E+03
2	0.003	9.06E+03

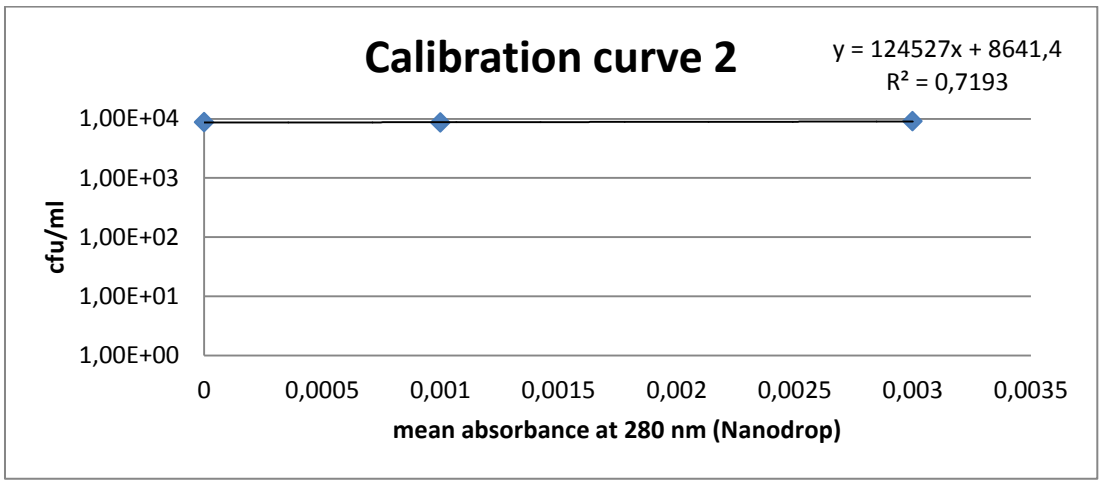

Figure 11: Calibration curve 2 : bacteria isolates 103, 134, 2.

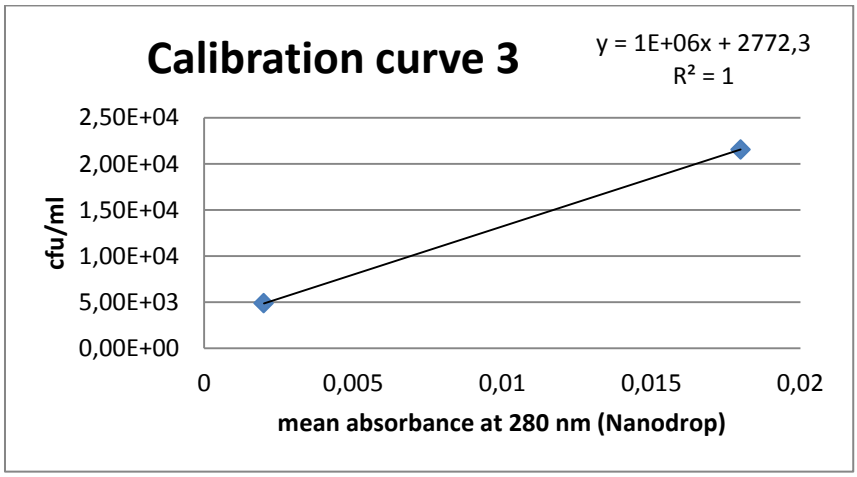

Figure 12: Calibration curve 3: bacteria isolate 33

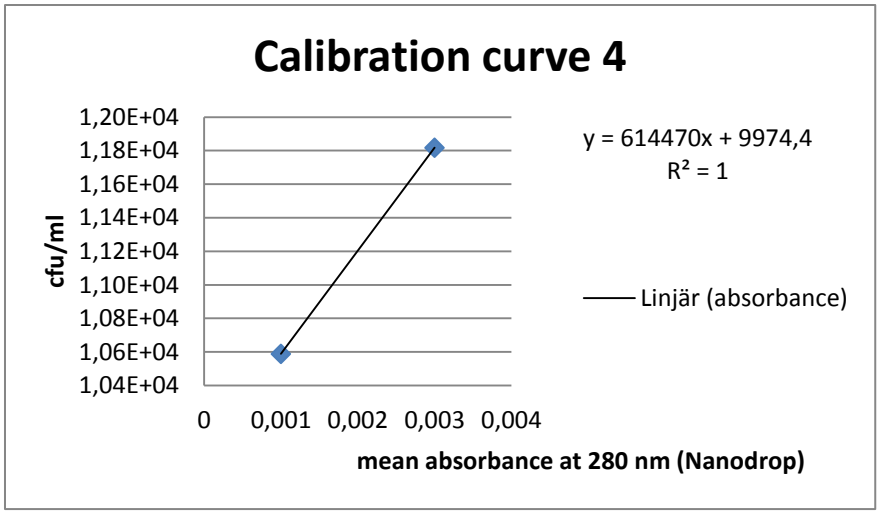

Figure 13: Calibration curve 4: bacteria isolates 155, 53

Table 9: Bacteria concentration derived from calibration curves for 10 tested bacteria isolates. For bacterial isolate 33 it was prepared a separate calibration curve (curve 3) as it was morphologically different from the other isolates. For the other isolates it was calculated a mean concentration derived from the calibration curves 1, 2 and 4.

isolate	600 nm Abs.	280 nm absorb.	mean absorb. at 280 nm	curve 3 [cfu/ml]	curve 1 [cfu/ml]	curve 2 [cfu/ml]	curve 4 [cfu/ml]	Mean conc [cfu/ml]
33	0.096	0.476	0.5465	5.49E+05				
33	0.117	0.617						
153	0.175	0.909	0.908		9.58E+04	1.99E+04	6.58E+04	**6.05E+04**
153	0.171	0.907						
155	0.145	0.762	0.7535		8.12E+04	1.80E+04	5.63E+04	**5.18E+04**
155	0.148	0.745						
132	0.153	0.843	0.8485		9.02E+04	1.92E+04	6.21E+04	**5.72E+04**
132	0.16	0.854						
103	0.163	0.841	0.811		8.67E+04	1.87E+04	5.98E+04	**5.51E+04**
103	0.152	0.781						
97	0.141	0.679	0.7005		7.62E+04	1.74E+04	5.30E+04	**4.89E+04**
97	0.145	0.722						
2	0.149	0.78	0.812		8.67E+04	1.88E+04	5.99E+04	**5.51E+04**
2	0.171	0.844						
160	0.113	0.646	0.6365					
160	0.111	0.627			7.01E+04	1.66E+04	4.91E+04	**4.53E+04**
157	0.13	0.709	0.6775					
157	0.114	0.646			7.40E+04	1.71E+04	5.16E+04	**4.76E+04**
120	0.109	0.606	0.6265					
120	0.117	0.647			6.92E+04	1.64E+04	4.85E+04	**4.47E+04**

Table 10: Results One-way ANOVA for the experiments where the fungicide worked at least for treatment "t1"; Multiple Comparison of mean **mycelial area after 7 days incubation** of the different treatments; t1 = combined treatment (antagonists + fungicide), t2 = single biocontrol treatment (antagonists without fungicide), t4 = single chemical treatment (fungicide without antagonists), control = no treatment

Anova 7 days incubation fungicide worked						
	Df	**Sum Sq**	**Mean Sq**	**F value**	**Pr (>F)**	**significance**
treatment	3	1948	649.3	74.77	1.25E-09	***
residuals	16	139	8.7			
Tukey HSD Multiple comparison of means for balanced data						
treatment pairs	**p adj.**	**significance**			**p-value**	
t1 - control	0	***		**Shapiro-test:**	0.03154	
t2 - control	0.0000083	***		**Kruskal - test:**	0.0003729	
t4 - control	1	*		**Significance code**	**P- value**	
t2 - t1	0.0005394	***		***	p < 0.001	
t4 - t1	0.0000615	***		**	P < 0.001	
t4 - t2	0.0000083	***		*	P < 0.01	Not sign.

Table 11: Results One-way ANOVA for the experiments where the fungicide worked at least for treatment "t1"; Multiple Comparison of mean **mycelial area after 5 days incubation** of the different treatments; t1 = combined treatment (antagonists + fungicide), t2 = single biocontrol treatment (antagonists without fungicide), t4 = single chemical treatment (fungicide without antagonists), control = no treatment

One-way Anova 5 days incubation fungicide worked						
	Df	**Sum Sq**	**Mean Sq**	**F value**	**Pr (>F)**	**significance**
treatment	3	3728	1242.8	32.64	4.73E-07	***
residuals	16	609	38.1			
Tukey HSD Multiple comparison of means for balanced data						
treatment pairs	**p adj.**	**significance**			**p-value**	
t1 - control	0.0000002	***		**Shapiro-test:**	0.0007116	
t2 - control	0.0003824	***		**Kruskal - test:**	0.0006821	
t4 - control	0.016003	*		**Significance code**	**P- value**	
t2 - t1	0.0022621	**		***	p < 0.001	
t4 - t1	0.00007116	***		**	P < 0.001	
t4 - t2	0.2852773	*		*	P < 0.01	Not sign.

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