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Combined Use of Antagonistic Microorganisms against Fusarium Head Blight on Wheat

Master Thesis

Submitted by

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In Partial Fulfilment of Requirements
for the Degree of

Diplom-Ingenieur

Vienna, February 2013

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Abstract

Fusarium Head Blight (FHB) is a serious disease of small grain crops such as wheat and barley. FHB is mainly due to infections by the fungi *Fusarium graminearum* (teleomorph: *Gibberella zeae*) and *F. culmorum*. Apart from causing severe yield losses, these fungi can also produce mycotoxins such as deoxynivalenol and zearalenone, preventing contaminated crops to be further used as food or feed. Control of FHB can be achieved through cultural practices (tillage, crop rotation), use of fungicides, resistant cultivars or biological control agents (BCAs). In the present work, three wheat cultivars were inoculated with *G. zeae* using the kernel spawn method to infect wheat ears in a natural way. Three different BCAs, each of which acts through a distinct control mechanism (SAR-inducing, antibiosis, competition for resources) were then sprayed onto the wheat plants at different developmental stages and in different combinations. To find out which BCA combination acted best and to which extent FHB symptoms were reduced, the disease incidence DI (%), disease severity DS (%) and percentage of Fusarium damaged kernels FDK were scored and the disease intensity DINT (%) computed. Wheat cultivars exerted the strongest effects on disease development. Neither of the BCA treatments nor the fungicide Folicur® were able to reduce DI (%) or FDK. When applied alone, BCA P183 reduced the area under the disease pressure curve (AUDPC) DS (%) by 25% ($P<0.05$) compared to the control, similar to the fungicide (30% reduction, $P<0.05$). BCA combinations also decreased the AUDPC DS (%). Analysis of the AUDPC DINT (%) showed similar results. There is some evidence that cultivars responded differently to the BCA treatments. As even the fungicide did not control FHB to a high degree, it is assumed that more conclusive results can be obtained under less pronounced disease pressure, which seems to have been exceptionally high.

Zusammenfassung

Die Ährenfusariose ist eine schwerwiegende Krankheit an Weizen, Gerste und anderem Getreide. Erreger der Krankheit sind Pilze der Gattung *Fusarium*, hauptsächlich *Fusarium graminearum* (teleomorph: *Gibberella zeae*) und *F. culmorum*. Neben Ertragseinbußen ist vor allem die Kontamination des Getreides mit für Mensch und Tier giftigen Mykotoxinen von Bedeutung. Mittels geeigneter ackerbaulicher Maßnahmen, Fungizideinsatz, Sortenwahl oder dem Einsatz antagonistischer Mikroorganismen (AM) kann die Krankheit bekämpft werden. In dieser Arbeit wurden drei Weizensorten mit *G. zeae* auf naturnahe Weise infiziert und drei AMs mit jeweils unterschiedlichem, antagonistischem Wirkmechanismus in verschiedenen Kombinationen und zu verschiedenen Zeitpunkten auf die Pflanzen appliziert. Der Bekämpfungserfolg wurde mittels visueller Bonitur der Befallshäufigkeit (BH%), der Befallsschwere (BS%), des Anteils Fusarium geschädigter Körner (FDK) und der Berechnung der Befallsintensität (BI%) festgestellt. Die Weizensorten hatten den größten Einfluss auf alle Parameter. Weder die AM-Behandlungen noch das Fungizid Folicur® vermochten BH% oder FDK zu reduzieren. In Einzelapplikation verringerte der AM P183 die Area Under Disease Pressure Curve (AUDPC) BS% um 25% (P<0.05) relativ zur Kontrolle, und war vergleichbar mit dem Fungizid (30%, P<0.05). AM Kombinationen verringerten die AUDPC BS% ebenfalls. Die Auswertung der AUDPC BI% zeigte ähnliche Resultate. Es wurden ebenfalls Hinweise auf eine Weizensorte-AM-Behandlung-Interaktion gefunden. Da selbst das Fungizid keinen zufriedenstellenden Bekämpfungserfolg hervorbrachte, wird davon ausgegangen, dass der Krankheitsdruck außerordentlich hoch war und unter anderen Bedingungen schlüssigere Resultate ausgewertet werden könnten.

ACKNOWLEDGEMENTS

I thank Ao. Univ. Prof. Dipl. Ing. Dr. nat. tech. Marc Lemmens for providing me with the opportunity to perform this master thesis, for his excellent supervision and guidance,

I also thank Andrea, Lisa, Imer and Matthias for their kind help and good spirits.

I am deeply grateful to my family who made my studies possible and encouraged me all the way.

And to my friends, who turned the last five years into an invaluable experience.

List of Abbreviations

Abbreviation	Explanation
*	*P*-value ≤0,05
**	*P*-value ≤0,01
***	*P*-value ≤0,001
+	*P*-value >0,1
ANOVA	analysis of variance
AUDPC	area under disease pressure curve
BBCH code	a number identifying the phenological development stage of a plant
BCA	biological control agent
cf.	compare
CIMMYT	International Maize and Wheat Improvement Centre
daa	days after anthesis
DF	degrees of freedom
DI	disease incidence
DINT	disease intensity
DON	deoxynivalenol
DS	disease severity
e.g.	for example
EC	European Commission
ELISA	enzyme-linked immunosorbent assay
et al.	and others
EU	European Union
F.	*Fusarium*
FDK	Fusarium damaged kernels
FHB	Fusarium Head Blight
fig.	figure
FRAC	fungicide resistance action committee
G.	*Gibberella*
i.e.	that is
LSD	least significant difference
MS	mean squares
n.s.	*P*-value ≤0,1
NF	neighbouring field

PCR	polymerase chain reaction
QTL	quantitative trait loci
r	Pearson correlation coefficient
ROS	reactive oxygen species
rpm	revolutions per minute
rt	room temperature
SAR	systemic acquired resistance
SNA	synthetic nutrient-poor agar
UAV	unmanned aerial vehicle
W	windbreak
ZEA	zearalenone

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1 Introduction and Purpose of the Present Work

Fusarium Head Blight (FHB), also known as Fusarium Ear Blight or scab, is a serious disease of cereal crops mostly caused by the fungus *Fusarium graminearum* (teleomorph: *Gibberella zeae*). Due to its devastating effects on crops and worldwide occurrence (McMullen et al., 1997) it is of major importance to agriculture and has therefore been the subject of numerous studies.

There are several ways of tackling FHB, one of which consists in using biological control agents (BCAs) able to prevent or reduce disease development in susceptible plants. Among others, the Institute of Biotechnology in Plant Production of the Department for Agrobiotechnology IFA-Tulln has focused its research on FHB control using such BCAs. Several promising BCAs have been isolated and tested in glasshouse and field experiments.

The present work builds upon results obtained by Thüringer (2011) who separately applied different BCAs on three wheat cultivars artificially inoculated with *G. zeae.* The three BCAs with the best control efficiency were then selected for a similar but more complex use in this study: the wheat cultivars, also inoculated with *G. zeae*, were treated at different developmental stages with different BCA combinations. Control effects were visually assessed by scoring disease symptoms.

With the applied experimental design we expected to gain further knowledge of:

- ***a)*** *the FHB control efficiency of BCA combinations compared to single BCAs*
- ***b)*** *the importance of the plant's developmental stage at the time of BCA application*
- ***c)*** *the mode of action exerted by each BCA*
- ***d)*** *differences in control efficiency between wheat cultivars*

2 Literature Review

This chapter provides an overview of the different aspects of the FHB disease and illustrates why FHB has been and still remains subject of worldwide research. Emphasis is put on *F. graminearum* and common wheat (*Triticum aestivum*), as this is the most studied FHB pathogen-host system – ones that has been employed in this work.

2.1 The Causal Agents

The Fusarium Head Blight disease is caused by several fungi of the genus *Fusarium*. Which species is most prominent in provoking the disease in a specific region depends on the region's climate and can therefore vary from year to year (Gale, 2003). FHB in the USA, Canada, China, southern and eastern Europe is known to be mostly caused by *F. graminearum*, whereas *F. culmorum* prevails in northern, western and central Europe (Gale, 2003; Wagacha & Muthomi, 2007). Other important species can be *F. avenaceum*, *F. poae, F. equiseti* or *F. sulphureum* (Bai et al., 2003; Shaner, 2003).

Teleomorphs are not known for all *Fusarium* species. *Gibberella zeae*, teleomorph of *F. graminearum*, is classified as follows (Goswami & Kistler, 2004):

> Superkingdom Eukaryota; Kingdom Fungi; Phylum Ascomycota; Subphylum Pezizomycotina; Class Sordariomycetidae; Subclass Hypocreomycetidae; Order Hypocreales; Family Nectriaceae; Genus *Gibberella*

FHB (or "scab") can occur on wheat, barley, oat, rice and several other monocot plants (Goswami & Kistler, 2004; Shaner, 2003; Tekle et al., 2012). Root rot, seedling blight and foot rot, as well as the stalk and ear rot disease on maize are also caused by FHB pathogens (Leplat et al., 2012; Mesterházy et al., 2012; Walter et al., 2010).

Figure 1 : Two macroconidia of *F. graminearum* (figure from Leplat et al., 2012).

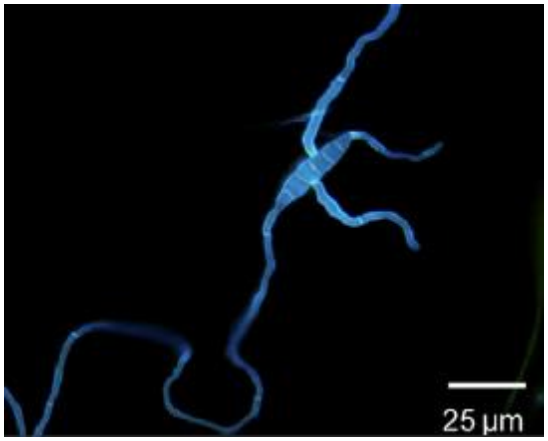

Figure 2 : Macroconidia of *F. culmorum* with four germ tubes (figure from Wagacha et al., 2012).

Detection and identification of *Fusarium* species was traditionally based on macro- and micro-morphological features, which is laborious and requires training (Demeke et al., 2005). Macroconidia (examples see fig. 1-2) are the most important cultural characters in the identification of *Fusarium* species (Leslie & Summerell, 2006). Molecular tools such as the polymerase chain reaction can be employed to complement or substitute traditional identification methods (Spanic et al., 2010b).

2.2 Biology, Pathology and Impact

FHB causing fungi overwinter saprophytically on crop debris. Depending on the *Fusarium* species, different fungal units ensure survival, e.g. *F. graminearum* endures in form of mycelia, ascospores (sexual spores of *G. zeae*), macroconidia or chlamydospores (Bai and Shaner, 1994).

The disease cycle of *F. graminearum* is summarized in figure 3. FHB is regarded as a monocyclic disease (Bai and Shaner, 1994) with infected crop residues being the principal source of inoculum (Perez et al., 2008). In the case of *F. graminearum*, mycelium on crop debris produces sticky ascospores or macroconidia (Leplat et al., 2012). By means of wind, rain, insects or rain splash, respectively, these then reach and infect the wheat ears (Goswami & Kistler, 2004; Shaner, 2003).

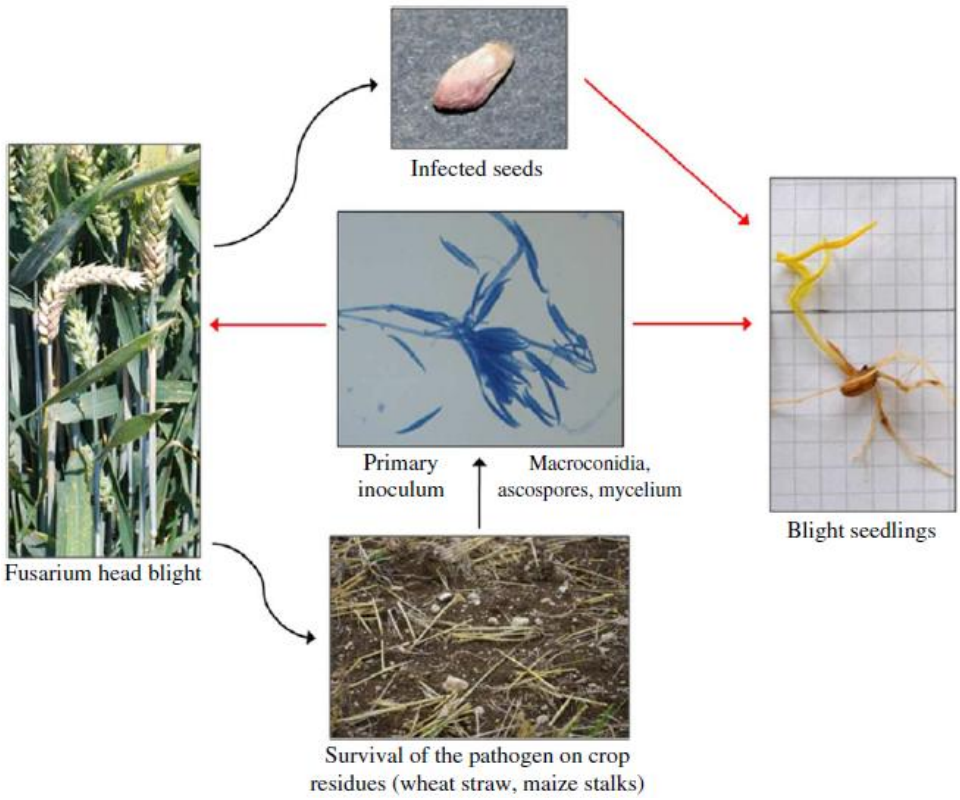

Figure 3 : Disease cycle of *F. graminearum*. Infected seeds and crop residues provide habitats for the fungus (black arrows) and are sources for FHB or seedling blight later in the season (figure from Leplat et al., 2012).

Leplat et al. (2012) reviewed the ecological requirements for *F. graminearum* persistence on crop debris. They concluded that, although the fungus is able to resist extreme conditions, *F. graminearum* growth and development of the teleomorph are heavily influenced by temperature, water, light and oxygen availability and that thanks to its enzymatic outfit, the fungus is able to use crop residues as nutrient source. Quantity and quality of these residues are also important survival factors (Leplat et al., 2012).

Survival deeper in the soil has been documented for some *Fusarium* species but does not seem of importance for FHB development (Shaner, 2003). Recently Wagacha et al. (2012) found that conidia develop on lower senescing leaves of wheat plants during a growth period, which may be another source of inoculum for FHB. There is no evidence that infected seeds contribute to later head blight of developed plants (Wagacha & Muthomi, 2007), however they could lead to seedling blight (Leplat et al., 2012) or allow the pathogens to spread into new areas (Shaner, 2003). Schmale et al. (2012) used UAVs to collect fungal dispersal units 40-350 meters above ground and suggested that long-distance transport in higher altitudes

may cause *Fusarium* population shifts or allow *Fusarium* pathogens to colonize new regions.

The FHB infection process starts when conidia or ascospores are deposited on the host ear (Goswami & Kistler, 2004). The incubation period in the field lasts for four to five days (Bai & Shaner, 1994). Susceptible infection stages are anthesis (BBCH code 61) to soft dough (BBCH code 85) (Bushnell et al., 2003). Shaner (2003) states that susceptibility at given developmental stages may differ between plant cultivars. Temperature and moisture are the most important infection parameters. In general, warm and wet conditions promote infection. At 25°C (temperature optimum) only 16 hours of moist weather are necessary for successful infection - depending on plant cultivar and development stage (Bai & Shaner, 1994). High susceptibility at anthesis is probably due to the fact that pathogens use extruded anthers as preliminary nutrient source. These contain high amounts of betaine and choline that seem to stimulate fungal growth (Tekle et al., 2012) and thus provide a base for further infection, although this has been contested by Engle et al. (2004).

The infection process of *F. graminearum* has been reviewed in detail by Bushnell et al. (2003): fungal spores either enter florets directly when these briefly open at dehiscence or germinate on the outer surfaces of florets and glumes (fig. 4). In the latter case, hyphae use stomata and underlying chlorenchyma, wounds, crevices between the palea and lemma - which widen during grain filling - , or the thin-walled epidermal cells at the base of wheat glumes as entry points. Subcuticular growth on outer surfaces of lemmas and glumes can also occur and may serve as a mechanism for further spread. Host tissue is then invaded by infection hyphae (Wanjiru et al., 2002). Confirming the above, Rittentour & Harris (2010) found that *F. graminearum* developed “subcuticular hyphae” and “bulbous infection hyphae” during infection of detached wheat glumes. Recently Boenisch & Schäfer (2011) were able to demonstrate the formation of foot structures, infection cushions and compound and lobate appressoria by *F. graminearum* on infected spikelets, further elucidating the fungus’ infection pathway. Also, it seems that FHB pathogens apply host specific and substantially different infection strategies (Kazan et al., 2012), e.g. *F. graminearum* showed a distinct gene expression pattern when infecting wheat and barley (Lysøe et al., 2011), illustrating the complexity of the FHB disease.

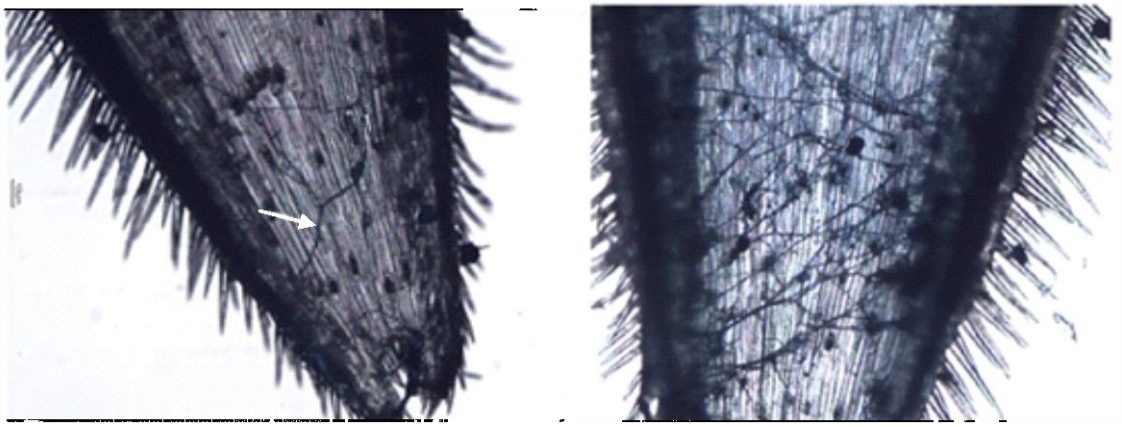

Figure 4 : Hyphae of *F. graminearum* on the surface of an oat palea one day after inoculation (left, arrow) and hyphal network on an oat palea four days after inoculation (right). Staining agent: lactophenol (figure from Tekle et al., 2012, modified).

Once access to the inside of a floret is gained, the ovary and other flowering organs are readily infested (Bushnell et al., 2003). Thus, the fungus has infected the organs that later form the grain.

Spread from floret to floret in the same spikelet and from spikelet to spikelet happens through the rachis and rachilla (see Brown et al. (2010) for details) or, under wet conditions, over the exterior spikelet surfaces (Bushnell et al., 2003). It is thought that, at the beginning of floret infection, the pathogen-plant relationship is of biotrophic nature and later switches to necrotrophic, allowing a more intense colonization of host tissue (Wagacha & Kistler, 2004). Brown et al. (2010), however, found no evidence for necrotrophy during *F. graminearum* pathogenesis and say that its lifestyle is somehow different from any of the classical definitions (biotrophy, hemibiotrophy and necrotrophy, as defined in Agrios (2005)).

For wheat and barley, macroscopic symptoms normally become visible on the earliest flowering florets, often in the middle of the ear (Bushnell et al., 2003). Dark brown, water-soaked spots then appear on the surfaces of florets and glumes (fig. 5A) (Bai & Shaner, 1994). Awns may deform and curve downwards (Goswami & Kistler, 2004) and entire florets become bleached (“blighted”). Under wet conditions, orange-pinkish mycelium and spores or black perithecia can develop on infected spikelets or kernels (fig. 5B-C), and apical portions of ears can turn white (premature ripening) due to clocking of the rachis by the pathogen (fig. 5B), which then results in formation of small kernels (Bushnell et al., 2003). Goswami & Kistler (2004) noted that FHB infections of barley might be harder to detect in the field than in the case of wheat. Heavily infected kernels may shrivel and

become covered with white mycelium (“tombstone kernels”) (fig. 5D) and sometimes infected spikelets do not develop grain at all (Bushnell et al., 2003). Similar symptoms can be seen on infected oats (for details see Tekle et al. (2012)).

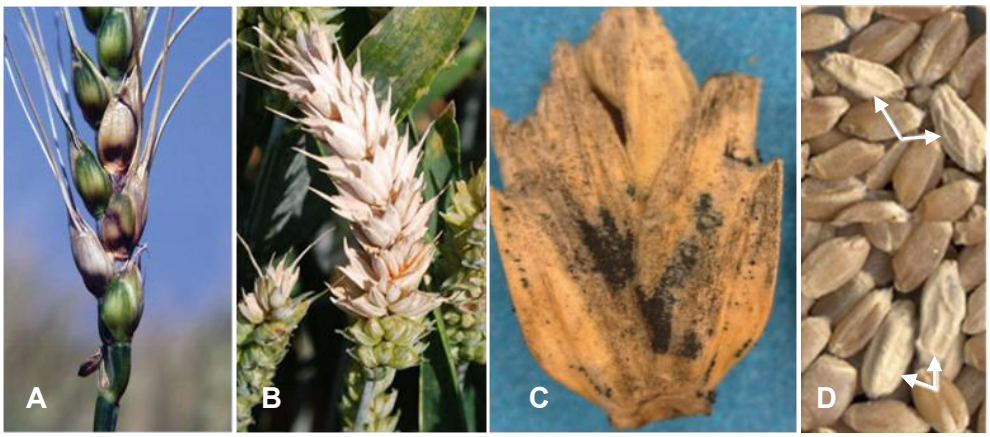

Figure 5 : FHB symptoms: water-soaked spots and blighted spikelets (A), orange-pinkish spores on a partially blighted ear (B), perithecia on a wheat spikelet (C) and shrivelled tombstone kernels among healthy kernels (D, arrows) (A, B, C: modified, from Goswami & Kistler, 2004; Leplat et al., 2012; Xue et al., 2009, respectively; D: modified, from www.agriculture.alberta.ca, property of the Government of Alberta).

As for other plant pathogens, cell wall-degrading enzymes are required to penetrate, infest and degrade host tissue. In the case of FHB enzymes such as cellulases, xylanases and pectinases are suspected to allow intense and rapid host tissue colonization (Wanjiru et al., 2002). Variation in virulence between isolates could be due to differences in the production of these enzymes, as has been suggested by Kikot et al. (2009) for the pectic enzyme group.

FHB fusaria are also known to produce a vast array of species specific mycotoxins (“*toxic substances [...] capable of causing illnesses [...] and death to animals and humans that consume* (them) *[...]*” (Agrios, 2005)). Different toxins may be produced by strains (“chemotypes”) of the same *Fusarium* species. Among others, this has also been reported for *F. graminearum* (de Kuppler et al., 2011). Since mycotoxins are resistant to food processing and high temperatures (de Kuppler et al., 2011), grain contaminated with such substances is unfit for further use as food or feed (McMullen et al., 1997). Trichothecenes, fumonisins, zearalenones, beauvericin, butenolide, enniatins, equisetin and fusarins are examples of mycotoxins produced by *Fusarium* species (Desjardins & Proctor, 2007), while FHB infected cereal grains mostly contain trichothecenes, zearalenones or fumonisins (D’Mello et al., 1999). The trichothecene deoxynivalenol (DON) and zearalenone (ZEA) are the most important toxins of *F. graminearum* (Bai & Shaner, 1994). DON is a vomitoxin that interferes

with protein synthesis (Desjardins & Proctor, 2007) and hence causes emesis and feed refusal when fed to animals, whereas ZEA metabolites have oestrogenic effects, provoking reproductive disorders (D'Mello et al., 1999). DON and other trichothecenes are synthesised in specialised infection structures (Boenisch & Schäfer, 2011) and are not essential for initial plant infection (Foroud et al., 2012; Horevaj et al, 2012) but seem to be important virulence factors (Kazan et al., 2012). Also, it was found that they are produced only after successful plant infection and allow fungal entry into the rachis for further disease spread (Foroud & Eudes, 2009). An increase in mycotoxin content may even occur postharvest if grain is inappropriately stored (Pirgozliev et al., 2003). The toxins can also be leached out from infected plants by free water, possibly causing aquatic environmental pollution (Gautam & Dill-Macky, 2012).

The fact that the genomes of *F. graminearum* and *F. verticillioides* have been sequenced (Desjardins & Proctor, 2007) and extensive transcriptome, proteome as well as metabolome information of *F. graminearum* and infected cereal hosts are available (Kazan et al., 2012; Walter et al., 2010) further demonstrates that FHB is a disease of major concern. FHB can have devastating effects on cereal production. Epidemics have caused food shortages, economic losses, food quality problems and in some regions drastically reduced cereal proportions in crop rotations (McMullen, 2003). If conditions are favourable, FHB can completely destroy a crop within a few weeks (McMullen et al., 1997). According to the CIMMYT, it is one of the major factors limiting wheat production in many parts of the world (Goswami & Kistler, 2004). Economic losses result from yield reductions and quality discounts which are due to damaged kernels, low test weight or toxin contents (Johnson et al., 2003). Baking quality is reduced and infected barley is unsuitable for malt production (Pirgozliev et al., 2003). Indirect losses are caused by reduced seed germination, seedling blight and poor stand of infected plants (Bai & Shaner, 1994). In the European Union, maximum levels for several *Fusarium* mycotoxins have been set and thus prohibit the use of over threshold contaminated foodstuffs as food ingredient (EC, 2006). Also, bans on mixing contaminated with uncontaminated grains and chemical detoxification have been issued by the EU, further restricting the usage of affected crops.

2.3 Host Defence Mechanisms

In order to better understand FHB disease development, cereal resistance mechanisms and host responses are also being investigated. According to Foroud & Eudes (2009), resistance to FHB has been divided into five types:

(1) resistance to initial infection
(2) resistance to spread of infection within a spike
(3) resistance to kernel infection
(4) tolerance against FHB and trichothecenes
(5) resistance to trichothecene accumulation
- a: by chemical modification of trichothecenes
- b: by inhibition of trichothecene synthesis

Defining such resistance types provides useful working hypotheses for tackling FHB (Bushnell et al., 2003), especially in the field of resistance breeding. With the exception of type 1 and 2 there is no agreement on what numbers designate what resistance type (Bushnell et al., 2003). Resistance components are inherited independently but are often linked in many genotypes (Mesterházy, 2003). It is agreed that FHB resistance is polygenic and that resistance genes can be accumulated in cultivars (Khatibi et al., 2012; Wagacha & Muthomi, 2007). So far immunity to the disease has not been observed (Foroud & Eudes, 2009).

Apart from waxy head tissue surfaces reducing water availability to the pathogens, type 1 resistance mostly seems to be of morphological and physiological nature such as plant height, timing and duration of anthesis, absence or presence of awns or spikelet density (Walter et al., 2010). Foroud et al. (2012) also propose involvement of a systemic response in uninfected tissues that prevents or minimizes secondary infections. In oat, high type 2 resistance is provided by the long pedicels (Tekle et al., 2012). Similarly, disease spreading to neighbouring spikelets in barley is impeded by the rachis node and rachilla (Khatibi et al., 2012). In wheat rachillae are shorter, type 2 resistance is therefore less pronounced and more likely a form of local resistance (Foroud et al, 2012). Type 4 resistance is present if crop yield and quality are maintained despite FHB presence (Foroud & Eudes, 2009). Boutigny et al. (2008) have reviewed resistance type 5: trichothecene detoxification (type 5a) happens by means of chemical modification or degradation, mainly through glycosylation. In transgenic plants, acetylation and de-epoxidation reactions have been shown to

allow detoxification. Endogenous plant compounds may inhibit mycotoxin synthesis, giving rise to type 5b resistance.

It has been shown that *F. graminearum* and *F. culmorum* are able to infect *Arabidopsis* (Urban et al., 2002). This pathosystem currently serves as an instrument for further examining plant signalling pathways of FHB resistance (Kazan et al., 2012). The molecular crosstalk between FHB fusaria and their host and host defence mechanisms are intensively studied and have been reviewed in detail by Walter et al. (2010): in summary, plant defence starts with pathogen detection. Cereal hosts possess a range of proteins able to recognize the pathogens. Pathogen recognition is then followed by secretion of substances that inhibit fungal degradative enzymes or directly attack fungal cells, e.g. with chitinases or glucanases. To limit pathogen spread, plant cell walls are reinforced through thickening, lignification and enrichment with phenolic or antifungal proteins. Effects of DON induced oxidative bursts are countered by DON metabolization, DON export or ROS detoxification, thus avoiding cell death.

2.4 Disease Control

The final goal of all FHB related research is of course to prevent disease outbreaks or at least limit them to a tolerable level. Proper agricultural practices, application of fungicides, use of resistant cereal cultivars and biological control are means to tackle FHB (Pirgozliev et al., 2003). The best way of sustainably managing FHB, however, would be an integrated approach (Wagacha & Muthomi, 2007).

Agricultural practices

Considering the FHB disease cycle, appropriate land preparation and crop rotations allow decreasing primary FHB inoculum. Inversion tillage buries fusaria that are overwintering on plant debris, promotes crop residue decomposition and controls weeds that might serve as FHB hosts (Leplat et al., 2012). Thus, pathogen survival is reduced and newly planted cereals are less likely to become infected. A well designed crop rotation comprising non FHB hosts has similar effects. It has been shown that the risk of an FHB outbreak is a lot higher if the preceding crop is susceptible to the disease This is especially true for maize as it produces high amounts of crop residue (Leplat et al., 2012). Blandino et al. (2012) showed that

appropriate tillage and crop rotations, together with fungicide application and the right plant cultivar, have synergistic effects on FHB suppression as well as toxin contamination and should be employed in integrated pest management systems in regions with high FHB risk. However, Yuen & Schonewise (2007) mention that, due to economic reasons, such FHB limiting practices are not likely to be adopted. Prices for maize are rising and plowing would reduce the benefits of low-till practices.

The effect of herbicides on FHB development has not been extensively examined but Fernandez et al. (2009) mention that usage of glyphosate might be associated with higher FHB levels in fields. Given the trends of reduced soil tillage and sustainable pesticide use, Leplat et al. (2012) argue that the role of weeds, some of which also harbour the FHB disease, will become more and more important in the future. Also, Heier et al. (2005) report that immoderate use of nitrogen-fertilizers can increase mycotoxin contamination even if environmental conditions are unfavourable for *Fusarium* spp., further putting emphasis on the importance of appropriate agricultural practices for FHB control.

Chemical control

In contrast to diseases like rusts or powdery mildew, there are no fungicides able to control FHB to a very high degree (Ito et al., 2012; Mesterházy, 2003). Thus, solely relying on fungicides for FHB control is risky. Integrating cultivar resistance with fungicides however can be an effective control strategy (Wegulo et al., 2011; Willyerd et al., 2012). Mesterházy et al. (2012) came to similar conclusions. Additionally they point out the importance of the right nozzle type used for fungicide spraying. Poor coverage of cereal heads may have contributed to the unsatisfying results obtained in many fungicide trials. A higher number of fungicide applications, probably increasing the pathogens' stress, may promote mycotoxin contamination of infected cereals, as was the case in an experiment performed by Giraud et al. (2011). They found that triple fungicide application led to higher grain DON content than in the case of two or single applications. Yoshida et al. (2012a) investigated the effect of timing of fungicide application on FHB and mycotoxin contamination and found that FHB is best controlled when spraying at anthesis, while an application at the late milk stage allows reducing toxin contents in matured grain.

At present, only three broad spectrum fungicides containing metconazol or tebuconazol as active ingredients are registered for FHB control in Austria. Both ingredients have the same FRAC code. Another fungicide containing spiroxamine and tebuconazol may be used to decrease mycotoxin contents. This should be borne in mind when considering resistance management.

Resistance breeding

At the moment, the use of resistant plant cultivars seems to be the most promising and sustainable FHB management option (Wagacha & Muthomi, 2007). As already noted, inheritance of resistance to FHB is of quantitative nature. This requires different breeding strategies than for gene-for-gene resistances. FHB resistance breeding therefore relies on quantitative trait loci (QTL) mapping and marker-assisted selection, reviewed in detail by Buerstmayr et al. (2009). Thanks to its excellent type 2 resistance, the Chinese spring wheat cultivar Sumai 3 is one of the most resistant hexaploid wheat cultivars and has been used as a crossing partner in many breeding programs (Bai & Shaner, 2004; Basnet et al., 2012). Several resistance QTL have been found in hexaploid wheat cultivars (Buerstmayr et al., 2009) while in barley and tetraploid durum wheat resistance sources are scarce (Bai & Shaner, 2004; Huhn et al., 2012). Foroud & Eudes (2009) point out that, due to the limited number of resistance sources for breeding, an “arms-race scenario” between pathogen and host as observed in gene-for-gene resistances might appear, eventually leading to the selection of highly virulent pathogen strains able to overcome host defences. Transferring FHB resistance genes from alien species to wheat or other cereals (Cai et al., 2005) might be a way to prevent this.

Resistance mechanisms other than type 2 may also become important selection traits in the future. While investigating the bioactivity of volatile organic compounds against *F. avenaceum* and *F. graminearum*, Cruz et al. (2012) found that some of these compounds possess potential in FHB control and thus could prove useful in breeding programmes. Similarly, selecting for resistance-associated cereal host metabolites may allow speeding up selection processes (Kazan et al. 2012).

Additionally, genetic engineering may help in the fight against FHB (Dahleen et al., 2001). Mycotoxin contamination may especially be reduced if engineered crops are enabled to metabolize the toxins, remove them from cells by efflux or inhibit their

synthesis (Foroud & Eudes, 2009). Di et al. (2010) successfully produced transgenic wheat expressing a modified ribosomal protein, the target site of DON, and Ferrari et al. (2011) managed to incorporate polygalacturonase-inhibiting proteins of *Phaseolus vulgaris* into wheat plants. Both transformations resulted in increased FHB resistance.

Biological control

Fighting FHB with biological control agents (BCAs) may become an important component in the management of this disease (da Luz et al., 2003; Khan et al., 2001) and is environmentally friendly compared to chemical treatments (Kazan et al., 2012). Organic farmers would especially benefit from a highly effective BCA.

In general there are several modes of action by which BCAs may affect pathogens. These are a) competition for food and space, b) direct parasitism, c) direct toxic effects by secretion of antibiotic substances, d) indirect toxic effects by volatile substances released by the metabolic activities of the antagonist and e) indirect effects by activating host plant defences (Agrios, 2005; Kazan et al., 2012). The risk of selecting for resistant FHB pathogen populations when applying BCAs for disease control thus seems to be rather low (Yuen & Schoneweis, 2007).

Several bacteria, yeasts and filamentous fungi are able to attack FHB pathogens and reduce disease damages (da Luz et al., 2003; He et al., 2009; Jochum et al., 2006; Khan & Doohan, 2009b; Matarese et al, 2012; Palazzini et al., 2007). In most cases, BCAs used in FHB control studies were isolated from wheat anthers, kernels, crop debris or agricultural soils, thus being adapted to the environmental conditions of FHB target sites and increasing the chances of effective FHB suppression (Yoshida et al., 2012b). Possible BCA uses could be spray applications at wheat anthesis, seed applications or crop residue treatments (Gilbert & Fernando, 2004). For example, fungi of the genus *Trichoderma* have been shown to reduce FHB inoculum on crop residues (Gilbert & Fernando, 2004; Matarese et al., 2012) and *F. graminearum* survival can be limited by enhancing crop debris decomposition processes (Leplat et al., 2012).

Targeted activation of host plant defences before plant diseases develop may also serve as biological crop protection strategy in FHB management. Khan & Doohan (2009b) reduced FHB damage by inducing wheat plant resistance through application of the biochemical chitosan. Virulent or avirulent pathogen

isolates and non-pathogen organisms are also able to activate the plant defence machinery (Choi & Hwang, 2011). One form of inducible plant resistance is systemic acquired resistance (SAR): aerial plant tissues exposed to a SAR inducer, e.g. a pathogen or BCA, produce systemic signals alerting all other plant organs of the inducer's presence and thus activating defence mechanisms in the whole plant (Shah, 2009; Vlot et al., 2008). Expression of SAR can occur already 24 hours after induction (Agrios, 2005) and confers enhanced and long lasting resistance against multiple plant pathogens (Shah, 2009; Vlot et al., 2008).
However, the discrepancy between BCA performances under environmentally controlled and field conditions is frequently observed and often prevents BCAs from being used as a commercial biocontrol product (Pirgozliev et al., 2003). Also, it has been reported that in some cases the effectiveness of a BCA may be host cultivar specific (Khan et al., 2004), which further complicates large scale application.

FHB control is not only limited to the field. Multiple postharvest measures are available. Next to limiting the outgrowth of fungi and mycotoxin production by adjusting water activity and temperature during storage of infested grain, several chemical and physical treatments may also be an option (Audenaert et al., 2012). Ito et al. (2012) managed to isolate a bacterial strain that is able to decrease DON contents in harvested, naturally contaminated wheat and barley grain. Also, Girotti et al. (2012) recently developed a method for early FHB and trichothecene detection in field collected samples which might help prevent mycotoxin contamination in the first place.

3 Materials and Methods

3.1 Experimental Design

The three commercial wheat cultivars Capo, Kronjet and Trappe were used in this work. All three are “little to moderately” susceptible to FHB (AGES, 2012). To infect them in a natural way with FHB, the plants were inoculated with *G. zeae* using the kernel spawn method. Thirteen different treatment variants were then applied in five replications to each cultivar in 1 m² plots in a completely randomised block design (fig. 6). Thus all in all 13×5×3 = 195 plots were treated.

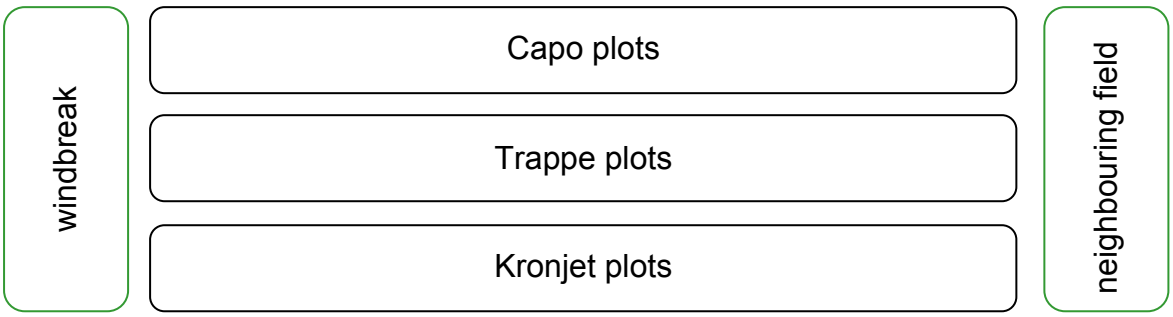

Figure 6: Sketch of the experimental setup.

In order to minimize edge effects, the external borders of the cultivar blocks on the windbreak- and field-side were skirted with pathogen inoculated but untreated buffer plots. The two remaining outer edges of the Capo and Kronjet blocks were neighboured by untreated and uninoculated wheat strips. Furthermore, an automated misting device generating high air humidity was installed. This ensured best possible homogeneous FHB infection conditions in the cultivar plots.

The thirteen treatment variants applied to tackle FHB are listed in table 1. Control plots did not receive any treatment at all. Names of the BCAs IFA350 and P183 are coded due to potential patent applications. IFA350 was used as a systemic acquired resistance (SAR) inducer, while P183 was suspected to exert antibiotic effects on FHB agents. Botector® is a commercial plant protection product used in viticulture to prevent colonization of the grape surface by *Botrytis cinerea*, a fungus able to cause severe yield losses in vineyards. Botector® is a mixture of two strains of *Aureobasidium pullulans*, a yeast-like fungus competing with pathogens for food and space. Folicur® is a commercial systemic broad-spectrum fungicide able to reduce

FHB on wheat. Its active ingredient is tebuconazole which targets the C14-demethylase in sterol biosynthesis (FRAC code 3).

Table 1: Treatment variants employed. "Mix": a mixture of two BCAs has been applied. "–": BCAs have been applied on consecutive dates.

Treatment Number	Treatment Composition
1	Control
2	Folicur®
3	Botector®
4	IFA350
5	P183
6	IFA350 – P183
7	IFA350 – Botector®
8	Botector® – P183
9	P183 – Botector®
10	Mix (Botector® + P183)
11	IFA350 – P183 – Botector®
12	IFA350 – Botector® – P183
13	IFA350 – Mix (Botector® + P183)

Control and Folicur® plots were meant to serve as references to estimate the FHB control efficiency of the different BCA variants. To find answers to the questions posed at the beginning of this work and because of wheat plants being most susceptible to FHB infection at anthesis (BBCH code 61), a more complex treatment application scheme was employed, as illustrated in figure 7.

IFA350 was always deployed five days prior to anthesis onto the leaves of the respective wheat cultivar (treatments 4, 6, 7, 11-13). All other substances were sprayed onto the ears. Folicur® and P183 in treatments 5, 6, 9, 11, Botector® in treatments 3, 7, 8, 12 and "Mix" in treatments 10, 13 were applied two days prior to anthesis. In treatments 8, 12 and 9, 11, P183 and Botector® were deployed two days after anthesis, respectively.

It was assumed that five days should suffice to allow SAR to develop in the wheat plants treated with IFA350. The other two BCAs were suspected to interact directly with the FHB agents, either through competition for space and nutrients (Botector®) or antibiosis (P183). To ensure that they coincided in time and space with the FHB pathogen, they were applied onto the ears, shortly before or after anthesis, depending on the treatment variant.

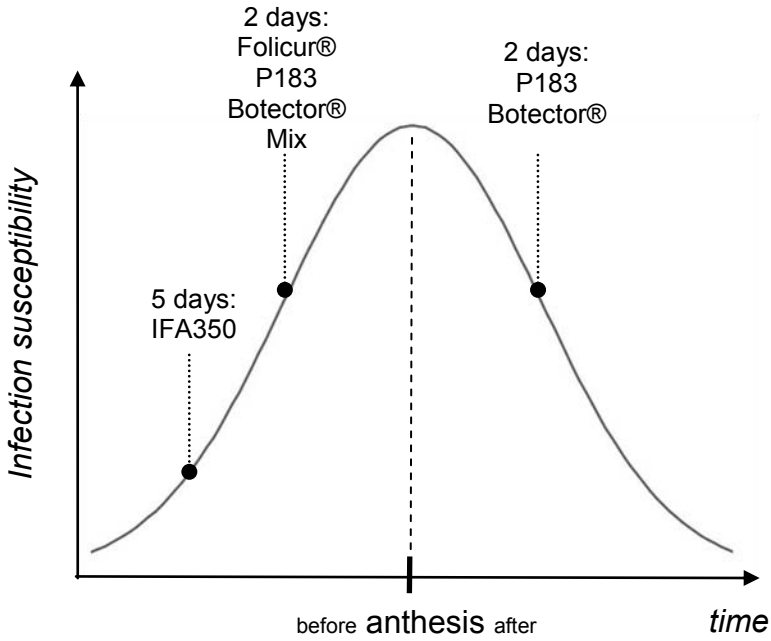

Figure 7 : Treatment application scheme. IFA350, Folicur® and Mix were always deployed before anthesis, P183 and Botector® before or after, depending on the treatment variant (see text).

FHB symptoms were then visually scored at defined time intervals. Scored were the disease incidence and disease severity as well as the percentage of Fusarium damaged kernels.

3.2 Cultivation measures

The winter wheat Capo was sown on 4/11/2011, summer cultivars Trappe and Kronjet on 16/3/2012. Sowing densities were 18 g / m². Fertilizers were applied in all three cultivars on 3/4/2012 (NPK 16:6:18+5S, 300 kg / ha) and on 15/5/2012 (CAN 27% N, 200 kg / ha). Herbicides were used as follows:

Cultivar	Date	Product Name and Spray Rate
Capo	30/4/2012	Andiamo® Maxx 1,5 l / ha
	10/5/2012	Puma® Extra 1 l / ha
Tappe, Kronjet	8/5/2012	Andiamo® Maxx 1,25 l / ha
	15/5/2012	Puma® Extra 1 l / ha

There was no other use of chemicals. Preceding crop was maize.

3.3 Pathogen and BCA Inoculation

On 24/4/2012, four to six weeks before the wheat plants were expected to reach anthesis, FHB inoculum was deployed using the kernel spawn method: 50 g / m² of *G. zeae* infected, overnight water soaked maize kernels were scattered among the wheat plants on the ground (fig. 8). The kernels originated from maize plants that had artificially been inoculated with *G. zeae* isolates IFA65, IFA71 and IFA73 in 2011 ten days after 50% silking and harvested at the end of that season.

Figure 8 : Infected kernels spread among the wheat plants (left). After one month most kernels were covered with dark *G. zeae* perithecia (right, arrows).

Table 2 : Inoculation dates of the different treatment variant components for the different wheat cultivars. Capo reached anthesis on 29/5/2012, Kronjet on 6/6/2012 and Trappe on 12/6/2012.

Capo	21/5/2012	25/5/2012	2/6/2012
Kronjet	30/5/2012	4/6/2012	8/6/2012
Trappe	31/5/2012	8/6/2012	15/6/2012
Treatment			
1	/	/	/
2	/	Folicur®	/
3	/	Botector®	/
4	IFA350	/	/
5	/	P183	/
6	IFA350	P183	/
7	IFA350	Botector®	/
8	/	Botector®	P183
9	/	P183	Botector®
10	/	Mix	/
11	IFA350	P183	Botector®
12	IFA305	Botector®	P183
13	IFA305	Mix	/

The different treatment variants were applied to the three wheat cultivars on different dates (table 2), depending on when these reached anthesis. Due to the fact that plant development was difficult to predict, the exact inoculation timetable of the treatment components as described above (fig. 7) could not be followed (e.g. for Capo IFA350 was applied eight days prior to anthesis, and not five as was planned). Using hand sprayers, 200 ml of the respective treatment components were deployed per plot and date. For Folicur® 0,15 ml / 200 ml, for Botector® 0,4 g / 200 ml were applied. Tap water served as solvent. Concentration of IFA350 was 0,069 g dry matter per 200 ml. For P183 it was 0,075 g / 200 ml in the Capo and 0,084 g / 200 ml in the Kronjet and Trappe treatments. The IFA305 and P183 solutions had been produced as follows:

Stock cultures of P183 (on 10% glycerine, frozen) and IFA350 (on earth, refrigerated) were used to grow cultures on SNA-plates (incubated for 10 days at room temperature). The plates were then checked for contaminations, 5×5 mm² discs cut out, added into separate 8 l yeast media (24 g yeast extract, 24 g malt extract, 40 g peptone, 80 g glucose, 160 g agar, pH 6,2, filled up with ddH_2O, autoclaved for 20 min at 121°C) containing flasks and cultivated using the bubble breeding method (Spanic et al., 2010a) for three days at room temperature (fig. 9), then refrigerated (4°C) for storage.

Figure 9 : Cultivation of IFA350 and P183 in yeast media containing flasks. Sterile air was blown into the flasks to promote BCA growth (bubble breeding method).

Shortly before each inoculation, the cultures were homogenized with a blender and diluted with tap water (1:5 for IFA350, 1:2 for P183). The treatment variant component “Mix” contained equal amounts (100 + 100 ml) of IFA350 and P183.

For the determination of IFA350 and P183 dry matter concentration, twelve Falcon tubes (four for IFA350, four for P183 in Capo and four for P183 in Kronjet/Trappe treatments) were dried overnight at 45°C, their dry weight measured and filled with 50 ml of the respective homogenized and undiluted BCA-yeast-medium solution. These were then centrifuged (4000 rpm, 8 min, rt), the supernatants removed, 25 ml osmosis water per tube added, vortexed until the pellets dissolved, again centrifuged,

the supernatant removed and finally dried at 45°C to constant weight. Desiccators were used to prevent air humidity from influencing the weighing.

Figure 10 : Misting device among the wheat plots. The windbreak can be seen in the back.

As noted above, an automated misting device was employed to provide optimal infection conditions for the FHB pathogen (fig. 10). From 21/5/2012 to 14/6/2012 misting occurred on every second day from 15:00 to 12:00 every 20 minutes (during the day) or every hour (at night) for 10 seconds if weather conditions were dry (regulated by an automated humidity sensor). On average approximately 5 mm water were applied per day.

3.4 Scoring

3.4.1 Disease Incidence and Disease Severity

Definitions:

> Disease incidence: *number of plant units sampled that are diseased expressed as a percentage of the total number of units assessed*
>
> Disease severity: *number of diseased spikelets per number of total spikelets in diseased ears, expressed as percentage*

Using hand held tally counters disease incidence (DI) and disease severity (DS) were scored in each plot fourteen, eighteen, twenty-two and twenty-six days after anthesis of the respective wheat cultivar. Selection of plants for disease assessment in each plot occurred at random.

An ear with at least one infected spikelet was assessed as diseased and thus increased the DI. The total number of infected spikelets per infected ear was scored as DS. DI and DS of scored wheat plants of the same plot were summed up, so that each plot received one DI and one DS value per scoring date. The number of wheat plants assessed depended on the disease incidence: if incidence in a plot was below 50% (half of the plants without FHB symptoms) fifty ears per plot were examined; if incidence was above 50% it were only twenty-five.

DI and DS plot values were then extrapolated as if 100 plants had been investigated (i.e. multiplied by either 2 or 4). These extrapolated DI plot values equal the percentage of diseased plants per hundred ears investigated DI (%).

As by definition DS also has to be expressed as percentage, the average number of spikelets per ear was estimated by counting the spikelets of fifty randomly selected plants. This was done separately for each wheat cultivar. Multiplying the so obtained average with the extrapolated DI (= DI (%)) gives the total number of spikelets in infected ears. DS (%) as defined above was then calculated according to the following formula:

$$\mathrm{DS}\ (\%) = \frac{\text{extrapolated DS value}}{\text{extrapolated DI} \times \text{number of spikelets per ear}} \times 100$$

3.4.2 Fusarium Damaged Kernels

The parameter Fusarium damaged kernels (FDK) describes the amount of shrivelled, deformed or discoloured kernels in a given sample. In this work FDK was expressed as percentage.

Wheat cultivars were harvested with a combine harvester (Delta Plot combine, Wintersteiger AG, Austria) on 17/7/2012 (Capo) and on 30/7/2012 (Kronjet and Trappe). The cleaning fan speed was reduced to 75% of normal speed to make sure that shrivelled kernels were harvested as well. This required additional cleaning of the kernels using a laboratory thresher (LD350, Wintersteiger AG, Austria) which was performed after hot air drying (42 °C) of the kernels.

Capo FDK was then assessed by comparing approximately one hundred kernels of each plot to FDK standards (fig.11). Standards each contained exactly one hundred Capo kernels of which 5, 10, 15, 20, 30, 40, 50, 66, 75 or 90 percent were diseased.

Figure 11 : Capo FDK standards with 20% (left) and 90% (right) diseased kernels.

For Kronjet and Trappe, healthy and damaged kernels were very similar in appearance. Therefore a different FDK scoring method had to be employed: approximately one hundred kernels of each plot (the amount was compared to a reference amount of exactly one hundred kernels) were separately put onto a black surface and roughly divided into groups of ten kernels. FDK was then assessed in these groups and added up to give the FDK value for the respective plot. For Kronjet and Trappe this method allowed better discrimination between healthy and diseased kernels than the method used for Capo.

3.5 Data Processing and Statistical Analysis

In addition to FDK, DI (%) and DS (%) the disease intensity (DINT) was also analysed. DINT is a general term for the amount of disease expressed in percent. Here, it combines the information provided by DI and DS. For each scoring date and plot, DINT was calculated using the respective DI and DS values according to the formula:

$$\mathrm{DINT}\ (\%) = \mathrm{DI}\ (\%) \times \mathrm{DS}\ (\%)$$

To sum up the results obtained for each plot on the different scoring dates the area under disease pressure curve (AUDPC) was calculated for DI (%), DS (%) and DINT (%):

$$\mathrm{AUDPC} = \sum_{i=1}^{n} \left\{ \frac{y_i + y_{i-1}}{2} \times (x_i - x_{i-1}) \right\}$$

where y_i is the value of DI (%), DS (%) or DINT (%) of the i^{th} observation, x_i the number of days after anthesis on the i^{th} observation, and n is the total number of observations (modified from Buerstmayr et al., 2000).

Statistical analysis of the different scoring parameters was done with the GLM procedure of SAS® Version 9.2 for Windows (North Carolina, USA). Performed were analyses of variance (ANOVA) and Fisher's LSD tests. α in Fisher's LSD tests was 0,05; ANOVA mean squares were of type 3. Pearson correlation coefficients and their P-values were computed with the CORR procedure. Data normality was checked for using the UNIVARIATE procedure and not normally distributed data was transformed using the square root transformation $\sqrt{x + 0{,}5}$. The model used in the ANOVA was:

$$Y_{ijt} = \mu + C_i + T_j + R_t + e_{ijt}$$

in which μ stands for the grand mean, C for the wheat cultivar (i = 1...3), T for treatment variant (j = 1...13), R for replication (t = 1...5) and e for random error.

4 Results

If not stated otherwise, results shown are mean values of the five treatment replications of all three wheat cultivars (mean of 5×3 values). Parameter values of the different scoring dates are designated by the respective number of days after anthesis (daa) added to the parameter, e.g. DS $(\%)_{18}$ stands for DS (%) values obtained at the second scoring performed 18 daa. Where appropriate, values were rounded to three decimal places. ***; **; *; n.s. and + stand for *P*-values ≤0,001; ≤0,01; ≤0,05; ≤0,1 or >0,1, respectively.

Disease Incidence DI (%)

Assessments of DI (%) and DS (%) started 14 daa. Mean DI (%) values for all treatments were above 90% 22 daa and reached almost 100% at the last scoring date, as illustrated in figure 12.

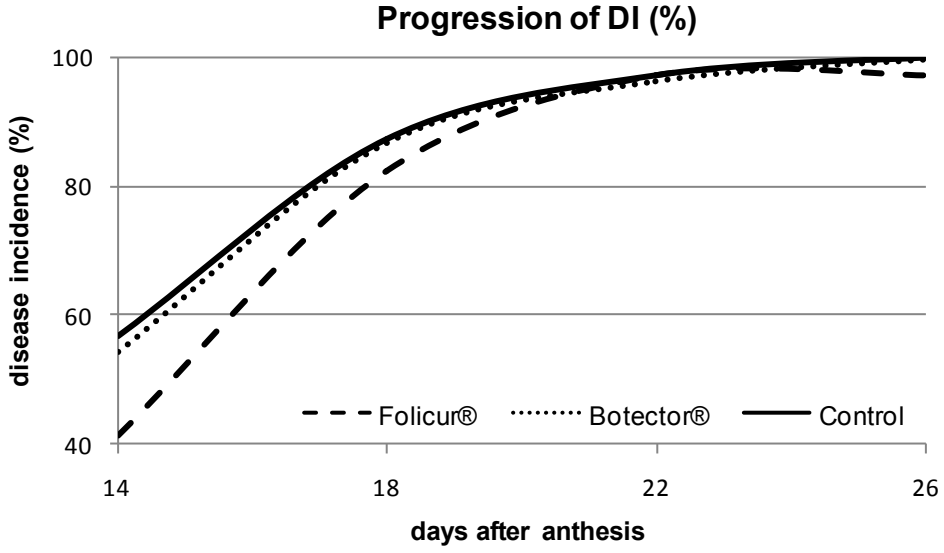

Figure 12 : Progression of DI (%) of one of the best (Folicur®), average (Botector®) and worst (Control) treatment variants.

Neither cultivars nor treatments had a statistically significant effect on the AUDPC DI (%) which summarizes all the DI (%) data (table 3, transformed data).

Table 3 : ANOVA results of the parameter AUDPC DI (%) (transformed data). DF: degrees of freedom; MS: mean squares

Source	DF	MS
		$AUDPC_{DI(\%)}$
Cultivar	2	7,381+
Treatment	12	6,69 n.s.
Replication	4	6,424+
Error	176	4,028

Disease Severity DS (%)

ANOVA results of all DS (%) parameters are shown in table 4. In all cases, wheat cultivars and treatments had statistically significant effects on the scores, in contrast to treatment replications.

Table 4 : ANOVA results of the different DS (%) parameters. DF: degrees of freedom; MS: mean squares.

Source	DF	MS				
		$DS(\%)_{14}$	$DS(\%)_{18}$	$DS(\%)_{22}$	$DS(\%)_{26}$	$AUDPC_{DS(\%)}$
Cultivar	2	3798,112***	7166,189***	8698,366***	13161,559***	2090586,996***
Treatment	12	51,796***	114,178***	210,003***	228,836**	34310,299***
Replication	4	21,112+	40,137+	82,469+	52,191+	11082,334+
Error	176	15,912	27,127	52,2	88,231	7082,053

Average numbers of spikelets per ear of each cultivar, needed for the calculation of DS (%), were 20,04 for Capo, 17,36 for Kronjet and 17,48 for Trappe.

The Control treatment had the highest DS (%) at all scoring days, reaching 58% 26 daa (fig. 13). Best treatment was the Folicur® treatment with 46% 26 daa (12% DS (%) reduction).

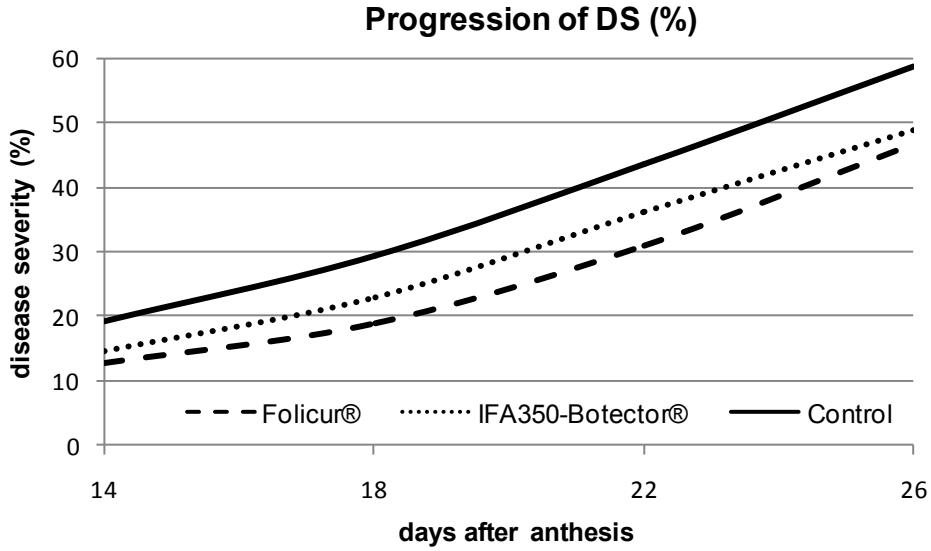

Figure 13 : Progression of DS (%) of treatment variants with best (Folicur®), moderate (IFA305-Botector®) and worst (Control) effects on DS (%) reduction.

DS (%) ranking of treatments did not change from 22 daa to 26 daa. Given that the AUDPC DS (%) includes this data, the ranking in this parameter is almost the same as the one of 22 daa / 26 daa and is shown in figure 14.

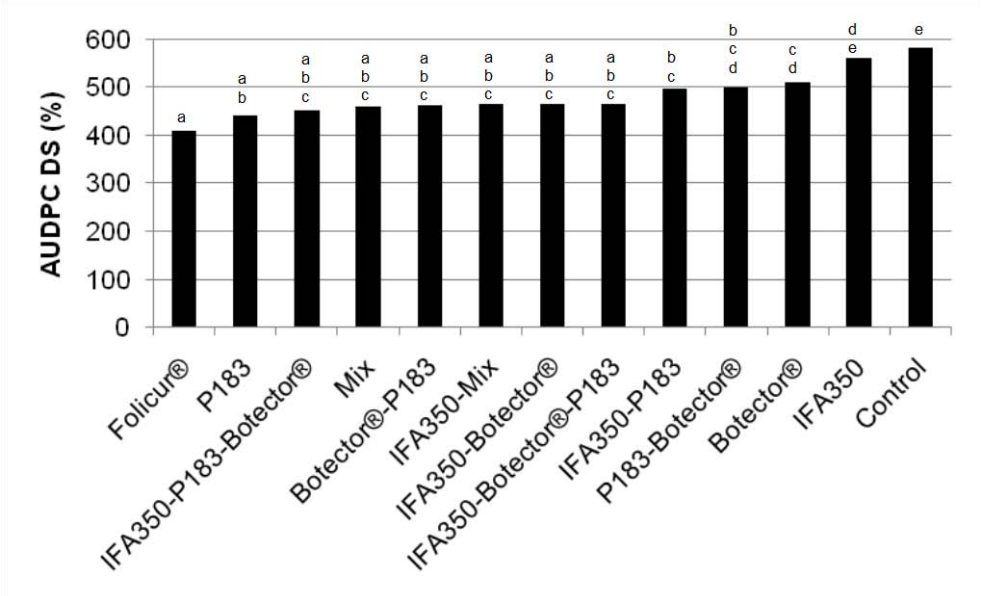

Figure 14 : AUDPC DS (%) values of all treatment variants. Treatments sharing the same letter(s) do not significantly differ from each other (Fisher's LSD test, α = 0,05, LSD = 60,645).

The Folicur® treatment produced the lowest AUDPC DS (%) value with a reduction of 30% compared to the control, although not significantly different from the next seven best treatments. The most efficient BCA treatment was P183 with a reduction of 25%. Only one treatment had a value comparable to the control's.

Cultivar specific AUDPC DS (%) scores of the control treatments were 391,39 for Capo, 578,99 for Kronjet, and 779,58 for Trappe. Thus, taking all treatment per cultivar data together, Capo proved to be the best cultivar in DS (%) reduction with a mean AUDPC DS (%) of 296,74 (24% reduction compared to the AUDPC DS (%) of the control treatment) followed by Kronjet (495,23; 15% reduction) and then Trappe (654,71; 16% reduction). Fisher's LSD test showed these three mean AUDPC DS (%) values to significantly differ from each other (LSD = 29,133).

Disease Intensity DINT (%)

Similar as for disease severity, ANOVA also showed statistically significant effects of cultivars and treatments for all DINT (%) parameters and none for the replications - except for DINT $(\%)_{14}$ (table 5).

Table 5 : ANOVA results of the different DINT (%) parameters. DF: degrees of freedom; MS: mean squares.

Source	DF	MS				
		$DINT(\%)_{14}$	$DINT(\%)_{18}$	$DINT(\%)_{22}$	$DINT(\%)_{26}$	$AUDPC_{DINT(\%)}$
Cultivar	2	752,245***	7659,288***	8793,496***	13091,241***	1377475,096***
Treatment	12	30,601***	138,691***	209,592***	254,642**	31218,623***
Replication	4	21,881*	46,075+	80,555+	62,129+	11500,632+
Error	176	7,264	30,205	55,393	92,11	6231,131

Eleven treatment variants produced AUDPC DINT (%) values significantly different from the control (fig. 15). Folicur® performed best (34% reduction), along with three BCA treatments. Again P183 was the best BCA treatment with a 27% reduction, but not statistically significantly different from most other BCA treatments.

Cultivar specific AUDPC DINT (%) scores of the control treatments were 340,3 for Capo, 488,16 for Kronjet, and 636,82 for Trappe. Capo had the lowest mean AUDPC DINT (%) value (244,15; 36% reduction compared to the AUDPC DINT (%) of the control treatment), followed by Kronjet (421,6; 34% reduction) and Trappe (532,77; 34% reduction). Again, Fisher's LSD test showed that the mean AUDPC DINT (%) values significantly differed from each other (LSD = 56,885).

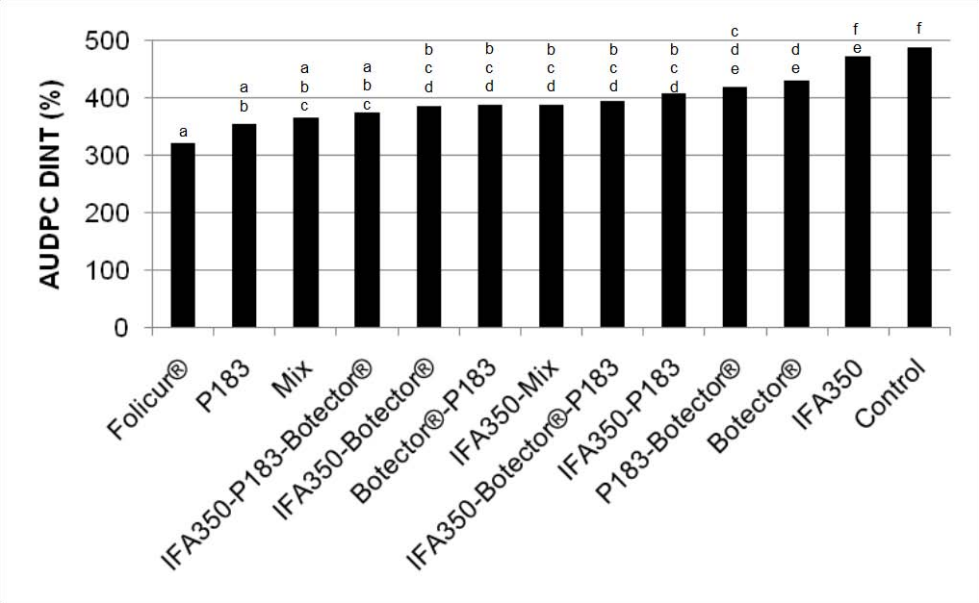

Figure 15 : AUDPC DINT (%) values of all treatment variants. Treatments sharing the same letter(s) do not significantly differ from each other (Fisher's LSD test, α = 0,05, LSD = 60,645).

Fusarium Damaged Kernels FDK

For the parameter FDK, ANOVA showed no statistically significant effect of treatments or replications, in contrast to cultivars (table 6, transformed data). However, one should consider that for Capo a different scoring method was used than for Kronjet and Trappe (as discussed on page 37). *Note: due to improper sample handling, one of the five replications of the P183 treatment of Kronjet could not be integrated into the evaluation.*

Table 6 : ANOVA results of the parameter FDK (transformed data). DF: degrees of freedom; MS: mean squares.

Source	DF	MS
		FDK
Cultivar	2	17.51***
Treatment	12	1.186 n.s.
Replication	4	0.808+
Error	175	0.697

To provide an impression of the proportion of Fusarium damaged kernels, FDK mean scores of some treatments are shown in table 7. Percentage of damaged kernels ranged from 16,8% (P183) to 25,4% (Control).

Table 7 : FDK mean values and standard deviations of six treatments (untransformed, rounded to one decimal place).

P183	IFA350 – Mix	Botector®
16,8±9,1%	20,3±8,8%	24±13,3%
Folicur®	Mix	Control
17±3,2%	21,3±8,1%	25,4±13,6%

Correlations

For each wheat cultivar, disease incidence and disease severity data of all treatments for the scoring date 18 daa were correlated (table 8). In the case of cultivars Capo and Kronjet, Pearson correlation coefficients were low and not statistically significant. Only in the case of Trappe, DI $(\%)_{18}$ and DS $(\%)_{18}$ were correlated to a moderate degree.

Table 8 : Pearson correlation coefficients (r) and respective *P*-values for cultivar specific DI $(\%)_{18}$ to DS $(\%)_{18}$ correlations.

Capo	Kronjet	Trappe
r = 0,367	r = 0,44	r = 0,684
P = 0,217	*P* = 0,132	*P* = 0,01

Correlation coefficients of FDK to AUDPC DINT (%) turned out to be moderately high and statistically significant only for Capo (table 9).

Table 9 : Pearson correlation coefficients (r) and respective *P*-values for cultivar specific FDK to AUDPC DINT (%) correlations.

Capo	Kronjet	Trappe
r = 0,713	r = 0,322	r = 0,377
P = 0,006	*P* = 0,283	*P* = 0,204

Using the cultivar specific treatment AUDPC DINT (%) scores, differential effects of treatments on cultivars were also investigated with correlations (fig. 16). Correlation coefficients were all statistically significant but not very high, varying from 0,566 to 0,617.

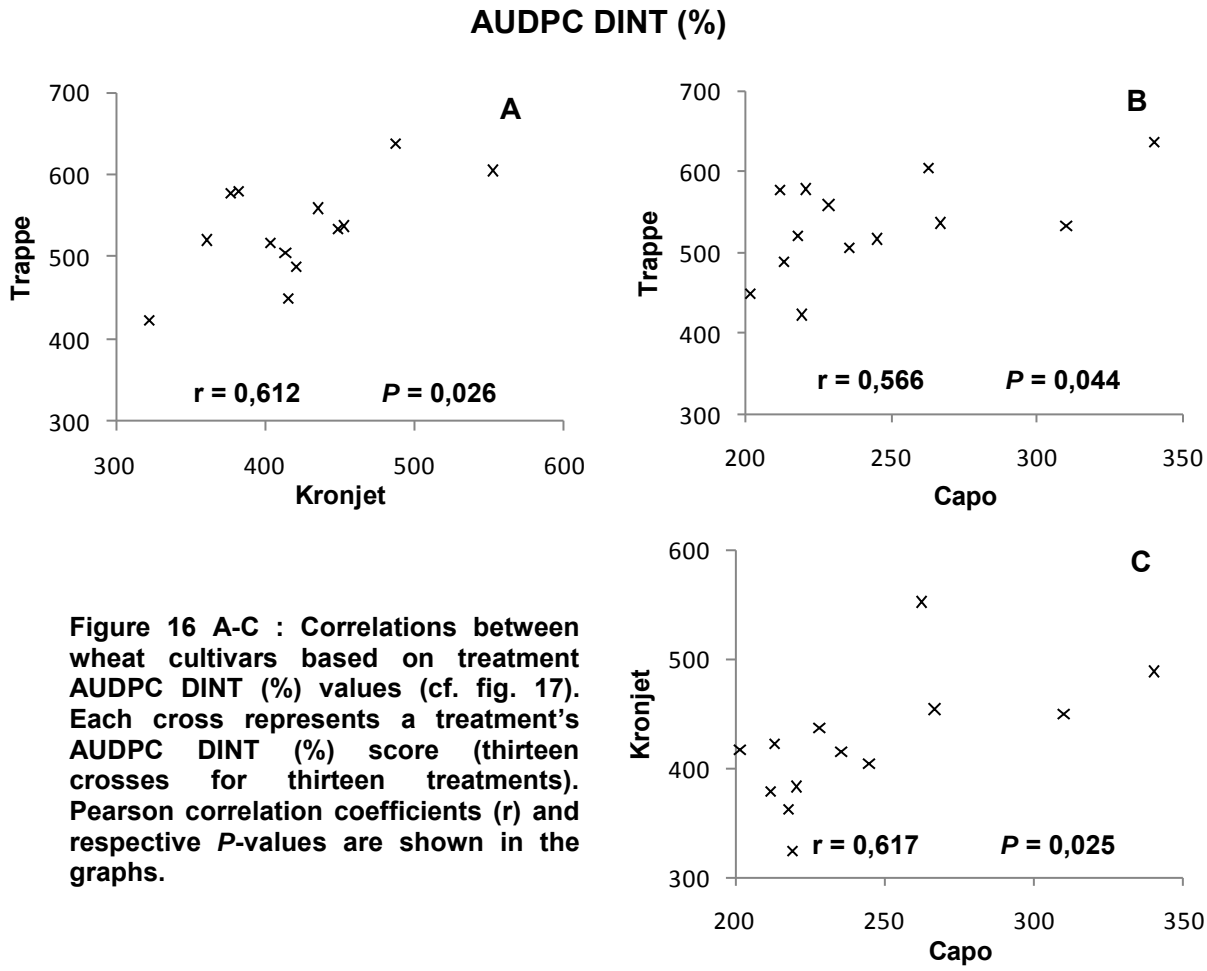

Figure 16 A-C : Correlations between wheat cultivars based on treatment AUDPC DINT (%) values (cf. fig. 17). Each cross represents a treatment's AUDPC DINT (%) score (thirteen crosses for thirteen treatments). Pearson correlation coefficients (r) and respective *P*-values are shown in the graphs.

In figure 17, a treatment ranking based on the AUDPC DINT (%) is shown for Trappe, and its treatment AUDPC DINT (%) scores can be compared to those of the other cultivars. The treatment ranking observed for Trappe differs from both Capo's and Kronjet's, e.g. treatment 4 (IFA350) for Kronjet performed even worse than the control (treatment 1), while it was the second worst for Trappe and proved to possess a rather average disease suppression effect in the case of Capo.

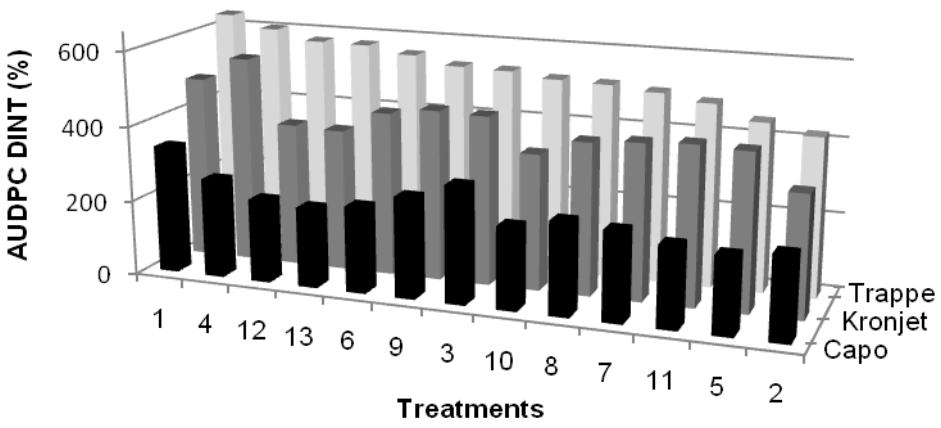

Figure 17 : Comparison of treatment effects on parameter AUDPC DINT (%) for the three wheat cultivars. Numbers designate the different treatments (cf. table 1)

5 Discussion

Tackling the devastating, multiple crops affecting Fusarium head blight disease (FHB) is hard. Several control measures are available but often none allow for satisfying disease control when applied alone. Using effective biological control agents (BCAs) may become an additional way for FHB suppression to be employed in integrated or organic pest management. In this work, the control efficiencies of three different BCAs applied in different combinations at different dates onto three artificially FHB inoculated wheat cultivars were investigated in a field trial. FHB control success of the different BCA treatments was measured by visually assessing the disease incidence DI (%), disease severity DS (%) and percentage of Fusarium damaged kernels FDK. FDK assessment occurred post-harvest, while DI (%) and DS (%) were scored 14, 18, 22 and 26 days after anthesis (daa). DI (%) and DS (%) data were used to calculate the disease intensity DINT (%), a general term for the amount of disease present. To combine the information obtained on the different scoring dates and describe treatment control performances during the whole experimental period, the area under the disease pressure curve (AUDPC) was calculated for DI (%), DS (%) and DINT (%). Using Pearson correlations coefficients, the relations between DI (%) and DS (%) as well as between FDK and AUDPC DINT (%) were examined. In the same way, a possible cultivar specific response to the different BCA treatments was investigated.

Disease Incidence DI (%)

ANOVA of AUDPC DI (%) showed no significant effect of cultivars on disease incidence and only a trend for treatments (table 3). Starting 22 daa, DI (%) values of all treatment variants were very high (> 90%) and attained almost 100% 26 daa. Therefore it can be said that none of the treatments offered complete protection against FHB. However, a common DI (%) value of nearly 100% does not necessarily mean that the treatments had no differential effect at all on disease incidence. For example 14 and 18 daa, the Folicur® treatment produced lower DI (%) values than the control (fig. 12; the statistical significance of the DI (%) reduction was not tested). Theoretically, two distinct treatment variants may lead to different disease incidences on an earlier scoring date (fig. 18, time point A) but in the end reach an equally high incidence level (fig. 18, time point B).

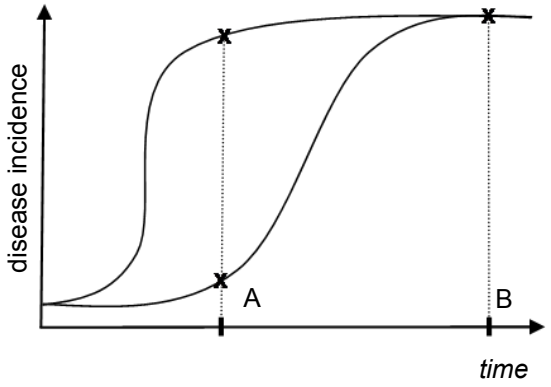

Figure 18 : Theoretically possible disease incidence progressions of plants treated with two different treatments.

It could be that a disease pressure threshold, e.g. a certain amount of infective FHB ascospores, has to be reached to overwhelm plant defences or the protections offered by treatments. Different treatments might thus be effective only until their respective threshold is attained. Disease pressure may increase in time if environmental conditions are favourable, which would perfectly explain the observations made in this work: the Folicur® treatment and others as well may have had thresholds higher than the control's and were exceeded at a later date, causing the differences in DI (%) values 14 and 18 daa, but finally leading to a common 100% incidence. This also illustrates the usefulness of multiple scoring dates and the calculation of the AUDPC: if disease pressure is exceptionally high and one looks only at data of a very late scoring date, one would not identify treatments that might prove effective in FHB control under less pronounced disease pressure. This should especially be borne in mind when screening for new BCAs or examining the efficiency of any other disease control measure.

Thüringer (2011) tested BCA performances on FHB control using an experimental design almost identical to the one employed here (i.e. same wheat cultivars, amount of FHB infected maize kernels and Folicur® concentration). However, he reported a DI (%) of only 35-40% for the control and 10-15% for the Folicur® treatment (~70% reduction). Therefore, it seems likely that very high disease pressure, possibly caused by environmental conditions favourable to FHB development, was at the origin of the unsuccessful control of FHB disease incidence by any of the treatment variants applied in this work. Jochum et al. (2006) also mention inconsistent FHB control success with fungicides and Mesterházy et al. (2011) state that fungicide use may not allow sufficient control during a strong FHB epidemic. This could of course also be true for BCA based treatments.

Disease Severity DS (%)

Treatments as well as cultivars had statistically significant effects on the disease severity at all scoring dates (table 4). In experiments performed by Khan et al. (2004) BCA applications reduced FHB disease severity by up to 60%. Other research groups also managed to significantly reduce FHB severity by means of BCA treatments (Khan & Doohan, 2009b). In the present work, the best BCA treatments reduced FHB severity by only 12% (26 daa) or 30% (AUDPC DS (%)), but performed as well as the fungicide treatment, as shown by Fisher's LSD test. In Thüringer's work (Thüringer, 2011), Folicur® and the best BCAs reduced AUDPC DS (%) values by 83% and 51-58%, respectively. Surprisingly, he found that IFA350 and Botector® treatments were comparable to the P183 treatment. Results here show, however, that only the P183 treatment was comparable to the fungicide treatment while IFA350 and Botector® ranked among the worst BCA treatments (fig. 14). Thüringer's Botector® treatment was five times more concentrated, which probably explains the better performance of that BCA product in his experiments. In any case, the huge difference in control efficiency between the fungicide treatments (30% vs. 83%) again indicates that disease pressure in the work at hand was exceptionally high and probably explains the observed high disease severity. Still, the DS (%) data shows that, in contrast to the disease incidence, there was a control effect lasting throughout the experimental season (fig. 13), that some BCA treatment variants exerted the same control efficiency as the fungicide and that only one treatment had no effect on disease severity suppression (fig. 14).

Comparison of the cultivar specific AUDPC DS (%) scores showed that disease severity was less pronounced on Capo than on Kronjet and Trappe, although all three cultivars are rated equally susceptible to FHB (AGES, 2012). The better performance of Capo could be due to a treatment×cultivar interaction which is discussed on page 39. Another explanation might be that the cultivars reached anthesis, stage of highest FHB susceptibility, on different dates and perhaps infection conditions (temperature, amount of infective ascospores) were least favourable at the time Capo was flowering. Thus, some sort of disease×environment interaction could also have been of importance. By setting up in-field spore traps, the amount of ascospores could be assessed. Looking for environmental variables that might account for the differences between cultivar disease scores seems rather hard, as Kriss et al. (2012) found that the relationship between disease symptoms and

environmental factors is very complex and not consistent. It could also be that the FHB susceptibility rating of the three cultivars is not appropriate, at least not for the environment and season this experiment was performed in. The cultivar specific AUDPC DS (%) values of the control treatments were quite different (391,39 for Capo; 578,99 for Kronjet; 779,58 for Trappe), which indicates that the cultivars are not, in contrast to their rating by AGES (2012), equally susceptible to FHB. Differences in FHB susceptibility would then possibly have contributed to the scoring result differences between the three cultivars. If this were true, however, one would expect the fungicide to be more effective when applied onto resistant cultivars than onto susceptible ones (Šíp et al., 2010). This was clearly not the case, as the Folicur® treatment lowered AUDPC DS (%) values for all three cultivars to the same degree by approximately 30% (data not shown).

Disease severity scores are often used to describe the degree of spread of the FHB pathogen from a primarily infected spikelet to an uninfected one through the rachis and rachillae. It should be noted that secondary FHB infections, i.e. additional infections of healthy spikelets by ascospores or conidia in an already diseased ear, cannot macroscopically be distinguished from spikelet infections caused by pathogen spread within the ear. Secondary infections are therefore included in visually assessed disease severity scores. Under high disease pressure with a lot of fungal inoculum able to produce large amounts of spores, it is imaginable that a large proportion of the observed disease severity is actually not due to disease spread within the host tissue but originates from multiple secondary infections. This might be particularly important in FHB resistance breeding programs where the disease severity is used to assess type 2 resistances of cultivars.

Disease Intensity DINT (%)

Three BCA treatments performed as well as the fungicide (fig. 15). Apart from that, analysis of AUDPC DINT (%) data showed almost the same treatment ranking and LSD grouping as for AUDPC DS (%) data (compare figures 14 and 15). The disease intensity is the product of disease severity and disease incidence. 22 daa and 26 daa DI (%) treatment values were very similar for all treatments and therefore differences between treatment AUDPC DINT (%) values are most likely due to the underlying DS (%) scores. 14 and 18 daa DI (%) scores probably account for the minor differences between treatment rankings of AUDPC DINT (%) and AUDPC DS (%). Hence, in this work, one could think on omitting the calculation of the AUDPC DINT (%) as its information on treatment specific FHB control efficiency is very similar to the information provided by the AUDPC DS (%). On the other hand, the AUDPC DINT (%) analysis further confirms that none of the treatments were able to reduce disease incidences, which might be of some importance given that DI (%) values were not analysed in detail.

The disease index is another parameter used in FHB disease assessments. It is calculated in the same way as DINT (%) was here (cf. Groth & Ozmon, 1999), except that index calculations are usually performed solely for the last scoring date. Computing the disease intensity therefore is not superfluous in general, it only proves to be of little use in this work. In large resistance breeding programs and experiments of bigger scale, however, a lot more data might be generated and thorough examination of each breeding line and / or treatment might not possible, due to limited resources. In such cases the disease intensity of only one late scoring date (= disease index) would allow for rapid selection of the best breeding lines and / or treatments and would then be very useful.

Replications had a significant effect on DINT (%) 14 daa (table 5). This is surprising, given that replications were not significant for any other parameters, and cannot be explained.

As in the case of AUDPC DS (%), comparison of the cultivar specific AUDPC DINT (%) values showed that disease intensity was least pronounced on cultivar Capo, followed by Kronjet and then Trappe. Possible reasons for this are discussed in the disease severity section above (page 34) and correlations section below (page 39).

Fusarium Damaged Kernels FDK

Similarly as for the parameters AUDPC DS (%) (fig. 14) and AUDPC DINT (%) (fig. 15), FDK values were lowest for treatments P183 and Folicur® (table 7). However, differences between treatments were not statistically significant (table 6) and treatments possessed only a tendentious effect on FDK. This is surprising, knowing that in Thüringer's experiment (Thüringer, 2011) P183 and Folicur® were also the best treatment variants and considerably reduced FDK values. The ability of BCAs and fungicides to decrease FDK symptoms has been confirmed by others. In experiments performed by Xue et al. (2009), Folicur® was able to reduce FDK to almost 0%. As already said, the fungicide had no statistically significant effect on FDK in the present work, which, as for the disease incidence, could be explained by a possibly very high disease pressure (see page 32).

Wheat cultivars had a statistically significant effect on FDK (table 6). This result is to be doubted, however. Due to the similar appearance of healthy and infected kernels of the two spring wheat cultivars Kronjet and Trappe, a different FDK scoring method than for the winter wheat cultivar Capo had to be employed (see page 22 for details). In general, Capo FDK scores were higher than for Kronjet and Trappe, while scores of the latter two were very similar (data not shown). This was probably caused by the usage of the two different scoring methods. Therefore the ANOVA result should be interpreted only with caution. Also, Capo had the lowest AUDPC DINT (%) values for all treatments (fig. 17) and it seems unlikely that the cultivar with the lowest disease intensity had the highest FDK percentage. To avoid problems with the visual scoring of FDK, other methods may be employed. Suchowilska & Wiwart (2006) propose the use of several parameters of kernel colour and shape in digital image analysis to assess the FDK percentage. Wegulo & Dowell (2008) compared near-infrared to visual FDK assessment and concluded that the automated near-infrared scoring system assesses the FDK percentage to a better degree and could replace visual scoring. Rudd et al. (2001) mention that, although other diseases and some environmental conditions may affect the results, kernel evaluations have proven to be useful FHB scoring tools and should not be omitted.

Quantitative real time PCR or quantitative ergosterol measurements can also be used to measure fungal biomasses in kernel samples and estimate their degree of infection. This, however, would differ from genuine FDK scoring, as not all damaged

kernels have to be infected. For example, uninfected kernels can shrivel, and so be damaged, when ear rachises are clogged by FHB pathogens.

Another very - if not the most - important aspect of the FHB disease was not investigated at all in this work: the mycotoxin contamination of grain. A BCA used by Xue et al. (2009) reduced the DON kernel content, an ability that the BCAs employed in the present work might also possess, and this should be taken into account in future studies. Furthermore, because there is the possibility that an interaction between FHB pathogens and other (BCA-) fungi results in elevated mycotoxin contaminations (Magan et al., 2003), a potential BCA must be able to decrease both FHB symptoms and mycotoxin content (Palazzini et al., 2007). In addition, toxin contents after BCA treatments could be compared to those of fungicide treatments, which can also stimulate mycotoxin production (Khan & Doohan, 2009a; Ramirez et al., 2004). Knowledge of the FDK percentage of a given sample suffices to estimate its mycotoxin content only approximately (Suchowilska & Wiwart, 2006), as infected, shrivelled as well as healthy looking kernels can, but do not have to be contaminated (Sinha & Savard, 1997). Mycotoxin detections should therefore be performed, e.g. using ELISA kits or high performance liquid chromatography coupled with mass spectrometers.

Correlations

Disease scoring is very time consuming, especially for the parameter disease severity as every single wheat spikelet has to be checked for disease symptoms. If disease severity were highly correlated with the disease incidence, which can be assessed much faster, it would suffice to score the disease incidence and derive the disease severity thereof. The correlation between DI $(\%)_{18}$ and DS $(\%)_{18}$ of all treatment variants was checked for the three wheat cultivars (table 8). In the case of Capo and Kronjet, no correlation was found. For Trappe it was only moderate. Preferably, the correlation of DI (%) and DS (%) of the last scoring date should have been investigated to reveal a possible relationship between the two parameters. However, it was not possible to do so because, as already noted, DI (%) values starting 22 daa reached almost 100%. DI (%) scores could thus not increase any further in contrast to DS (%) scores, making a correlation analysis futile. In years with less pronounced disease pressure, where the disease incidence does not attain

100%, it might be appropriate to reduce disease scoring to the parameter DI (%); but then again one would have to predict the disease pressure in the first place. Based on the present findings, omitting the scoring of disease severity cannot be recommended.

A correlation between AUDPC DINT (%) and the percentage of Fusarium damaged kernels was also examined (table 9). A moderately high and statistically significant correlation was found for cultivar Capo, but not for Kronjet or Trappe. It stands to reason that this was due to the different FDK scoring method that had to be employed for Kronjet and Trappe (see page 22). Because there is no clear correlation between the two parameters, it does not seem that the FDK scoring can replace field observations, or vice versa. Instead, the method for FDK scoring should be improved, as already discussed on the previous pages. With an improved scoring method, better correlations between FDK and preharvest disease symptoms might become apparent.

It is imaginable that not all cultivars reacted in the same way or proportion to a specific BCA treatment. This has already been reported by other authors (e.g. Jochum et al., 2006; Khan et al., 2004). A speculative explanation for this phenomenon could for example be cultivar specific ear surface structures that generate microclimates of different suitability for BCA growth, or some cultivars might provide better nutrient sources for the colonizing BCAs. However, due to the experimental layout of the field trial (completely randomised block design), the significance of a possible treatment×cultivar interaction could not be statistically evaluated. By correlating the cultivar AUDPC DINT (%) scores to each other (fig. 16 A-C), the presence of a treatment×cultivar can at least be discussed. If there were no such interaction, the correlations would have to be almost perfect ($r \approx 1$). With correlation coefficients around 0,6 however, this is clearly not the case. In figure 17 treatments are ranked for Trappe according to their decreasing AUDPC DINT (%) scores. Capo and Kronjet show different treatment rankings, further substantiating the presence of an interaction. From a practical point of view, this type of interaction is not desired because it would probably limit the number of wheat cultivars that could be treated with a specific BCA product for FHB control. To allow for a more profound statistical evaluation of the (alleged) treatment×cultivar interaction and find out whether it is stable over seasons, the experiment should be repeated and its layout changed, e.g. to a split-block design (cf. Little & Hills, 1978).

Miscellaneous

During the disease scoring, it was noticed that, for all three wheat cultivars, plots on the “windbreak” (W) side (cf. fig. 6) were most diseased and that symptoms decreased in direction of the “neighbouring field” (NF) side. Thus it seems that there was an increasing disease pressure gradient from NF to W. Possible explanations for this could be that a) as the W side laid downwind, wind blew airborne ascospores into that direction, or b) the windbreak reduced wind speeds and hours of direct sunlight on the W side, making the microclimate there moister (e.g. longer humidity due to slower drying of dew) than on the NF side and thus improving infection conditions.

If the treatment plots are distributed at random inside each cultivar block, as was the case in this work, it is possible that all five replication plots of a treatment variant are concentrated in one part of the block, e.g. just next to the windbreak, which would then influence the scoring results of that treatment variant. To avoid such influences of the disease pressure gradient, each cultivar block could be divided into five sub-blocks. Each sub-block would then contain one replication plot of every treatment variant with plots being distributed at random in the sub-blocks. Figure 19 illustrates such an experimental layout for six imaginary treatment variants. Preferably, the experimental design chosen should also allow for the calculation of the treatment×cultivar interaction (cf. page 39).

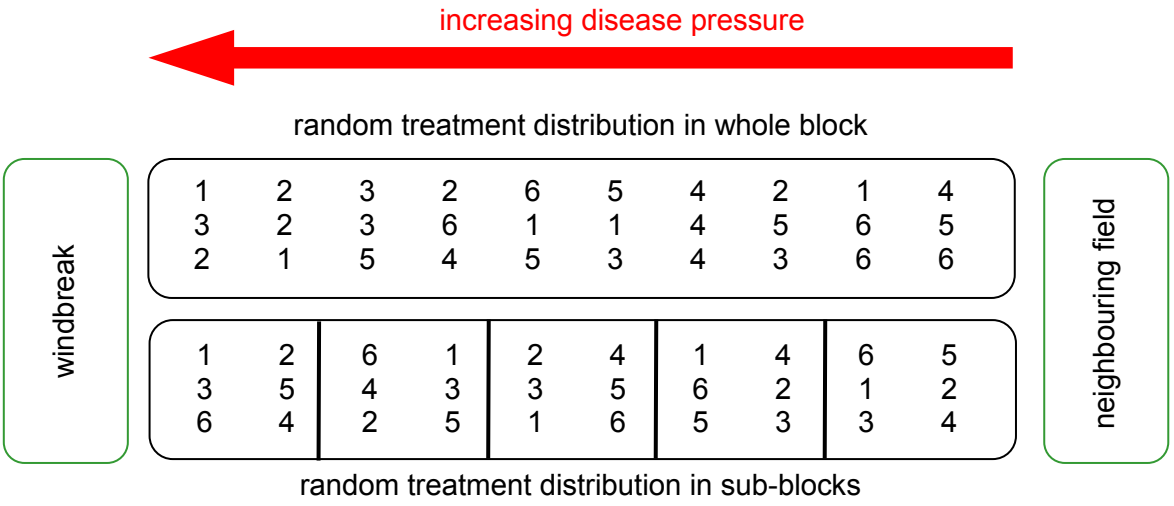

Figure 19 : Proposed improvement of the experimental layout that would take into account the observed disease pressure gradient and reduce its possible impact on scoring results. Each number stands for an imaginary treatment variant.

Deploying the BCAs and fungicide with hand sprayers, as performed in this work, ensures good coverage of the wheat heads (Mesterházy et al., 2003). For fungicides, Mesterházy et al. (2011) state that FHB control improvements can further be achieved by using the right nozzle type for application. This could of course also be true for BCA treatments. Optimizing BCA production processes and formulations might also increase the disease control success (Xue et al., 2009). To assure that the use of a potential BCA product is profitable over a larger geographic region, its FHB suppression effectiveness would eventually have to be tested at different locations and environments. However, in such an early stage of BCA product development as in the present case, this does not seem necessary yet.

To guarantee that IFA350 did not directly influence FHB pathogens and acted solely as plant defence activator, it was sprayed onto the wheat leaves and not the heads. Although IFA350 did not reduce overall AUDPC DS (%) and AUDPC DINT (%) scores (figs. 14 and 15), for Capo AUDPC DINT (%) scores were reduced (treatment 4 in fig. 17, the statistical significance of the reduction was not tested). This finding suggests that IFA350 indeed activates plant defences, but maybe not (sufficiently) on all cultivars. According to its producer, the commercial product Botector® *"contains highly efficient microorganisms"* that act *"through natural competition for space and nutrients"* and *"does not attack the metabolism of fungal pathogens"*. The exact mode of action exerted by P183 remains unknown. It is improbable that P183 and Botector® interacted in a negative way when present on the same wheat head, as growth inhibitions were not observed in cultures of P183- Botector® mixtures (Lemmens, personal communication). Whatever their influence on each other, results show that combinations of these two BCAs did not differ from most of the other BCA treatments in terms of FHB control.

As stated in the beginning, this work had four main foci of interest, which can now be addressed as follows:

a) *the FHB control efficiency of BCA combinations compared to single BCAs*

Absolute AUDPC DS (%) and AUDPC DINT (%) scores showed that P183 was the best BCA treatment when single-applied, in contrast to single applications of IFA350 or Botector® which had the lowest disease control effect. However, differences between these single BCA treatment variants and most BCA combinations were not high enough to be statistically significant.

b) *the importance of the plant's developmental stage at the time of BCA application*

No definite conclusion concerning the importance of pre- and post-anthesis application of the BCAs can be drawn.

c) *the mode of action exerted by each BCA*

There is some evidence that the BCA IFA350 induced plant defences. Information on the mode of action of BCA P183 was not obtained.

d) *differences in control efficiency between wheat cultivars*

Results indicate that wheat cultivars responded differently to at least some of the BCA treatments. The experimental layout of the trial did not allow testing the statistical significance of these differences.

Repeating the experiment with the stated improvements under less pronounced disease pressure would probably provide more conclusive results concerning differences between BCA combinations, presumed treatment×cultivar interactions and the importance of BCA application timing.

6 List of Tables

7 List of Figures

8 References

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