Norwegian University of Life Sciences

Preface

This project has been founded by the Research Council of Norway and has been carried out at Bioforsk Plant Health. I am grateful for the help all the people there have given me, and I will miss the including and inspiring working environment.

I would like to thank my supervisors at Bioforsk, Sonja Klemsdal and Hege Divon for accepting me as their student. Sonja; Thank you for your patience and good advice, and for always taking the time to answer questions. Hege; Thank you for all your advice and good spirit. Your positive and encouraging approach has been a great help.

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Finally, thanks to my family for their infinite support through my studies.

Ås, July 2012

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Abstract

Different *Fusarium* species are the cause of *Fusarium* head blight in wheat and have been regarded as one of the most devastating crop pathogens in the world. Some *Fusarium* can produce mycotoxins that can contaminate grain and be harmful when ingested by humans and animals. Different species of *Fusarium* can occur on the same wheat ear, where they possibly can have an effect on each other. In this study, three different *Fusarium* species (*Fusarium avenaceum*, *F. culmorum* and *F. graminearum*) were inoculated on wheat, alone or in pairwise combinations. DNA from each of the species were isolated from infected wheat and quantified to see if growth was influenced by interaction in young wheat and wheat at the ripening stage. Expression levels of the two mycotoxin related genes *Esyn* (enniatin production in *F. avenaceum*) and *Tri1* (deoxynivalenol production in *F. graminearum*) were investigated by real-time quantitative PCR in young wheat. The amount of the mycotoxins enniatins and deoxynivalenol were measured by gas chromatography–mass spectrometry in wheat at the ripening stage. Signs of interaction between *F. avenaceum* and *F. graminearum* were demonstrated, both in respect to DNA quantity and mycotoxin production. The upregulated *Tri1* in *F. graminearum* in the initial stages is in agreement with previous knowledge that DON is necessary for *F. graminearum* establishment in wheat.

To further investigate the possible interaction between *F. graminearum* and *F. avenaceum*, an *in vitro* culture plate study was done. A *F. graminearum* mutant constitutively expressing red fluorescence protein and expressing green fluorescence protein (GFP) when inducing *Tri5*, a gene involved in the toxin pathway, was used. The *F. graminearum* mutant was together with *F. avenaceum* co-inoculated on plates and investigated in confocal laser scanning microscopy (CLSM) every third hour. No obvious signs of interaction were seen, possibly because of the relatively short time span. *F. graminearum* did however grow much faster than *F. avenaceum*, revealing its more aggressive nature.

F. langsethiae is a relatively newly discovered species, often found in barley and oat, but seldom in wheat. Why this specie is rarely found in wheat is still unknown. Its interaction with wheat is also poorly understood. Two inoculation experiments on wheat were done: 1) In a detached leaf assay (DLA) conidial suspensions of a *F. langsethiae* mutant constitutively expressing GFP (*F. langsethiae* GFP) were inoculated on wheat leaves. 2) A greenhouse experiment where wheat heads were inoculated with conidial suspension of *F. langsethiae* at anthesis. Infection in leaves from DLA were later measured and investigated with CLSM. Hyphal assemblies around stomata

of the plant were seen repeatedly, indicating that stomata could be the entering point for *F. langsethiae* infection on wheat leaves. Scanning electron microscopy of the florets from the greenhouse experiment showed that *F. langsethiae* was able to infect the outer layers of the floret. When growing directly on the caryopsis, however, hyphae where lysed, possibly by defence mechanisms from the host.

Sammendrag

Ulike *Fusarium* arter forårsaker aksfusariose i hvete og har blitt kjent som en av verdens mest ødeleggende patogener i kornproduksjon. Noen *Fusarium* arter kan produsere mykotoksiner som kan kontaminere korn og være helseskadelig når det blir spist av dyr og mennesker. Ulike arter *Fusarium* kan opptre i grupper på samme hveteaks, hvor de muligens kan ha en påvirkning på hverandre. I denne studien ble tre ulike arter *Fusarium* inokulert på hvete, *Fusarium avenaceum*, *F. culmorum* og *F. graminearum*, alene eller i parvise kombinasjoner. DNA ble isolert fra hveten og kvantifisert fra hver av artene for å se om veksten var påvirket av interaksjon mellom artene både i umoden og moden hvete. Ekspresjonsnivå av de mykotoksinrelaterte genene *Esyn* (enniatinproduksjon i *F. avenaceum*) og *Tri1* (deoxynivalenol produksjon i *F. graminearum*) ble undersøkt ved realtime kvantitativ PCR i ung hvete. I moden hvete ble mengde mykotoksiner målt ved hjelp av gasskromotografi-massespektrometri. Tegn på interaksjon mellom *F. avenaceum* og *F. graminearum* ble demonstrert, både i DNA-mengde og mykotoksinproduksjon. Oppregulering av *Tri1* i *F. graminearum* i startstadiet av infeksjonen indikerer at DON er nødvendig for *F. graminearum* etablering i hvete.

For ytterligere å undersøke interaksjonen mellom *F. graminearum* og *F. avenaceum* ble et *in vitro* platekulturforsøk utført: De to artene ble ko-inokulert på plater og undersøkt i konfokalt laser scanning mikroskop (KLSM) hver tredje time. En *F. graminearum* mutant ble benyttet, denne uttrykte konstitutivt rødt fluorescens protein og grønt fluorescens protein (GFP) når et gen involvert i toksinsyntese, *Tri5*, ble indusert. Ingen åpenbare tegn på interaksjon ble observert, muligens på grunn av den relativt korte inkubasjonstiden i forsøket. *F. graminearum* viste seg imidlertid mer aggressiv enn *F. avenaceum*, med hensyn til dens mye raskere vekst.

F. langsethiae er en relativt nyoppdaget art og er ofte funnet i bygg og havre, noe mer sjelden i hvete. Grunnen til dens sjeldnere kolonisering av hvete er fortsatt ukjent. Interaksjonen til denne arten med hvete er også dårlig forstått. To inokulasjonseksperimenter på hvete ble utført: 1) I en

analyse gjort på avkuttede blad (DLA) ble conidie løsninger av en *F. langsethiae* mutant som konstitutivt uttrykker GFP brukt til å inokulere hveteblader. 2) I et veksthus eksperiment hvor hveteaks ble inokulert med *F. langsethiae* conidieløsninger ved hveteblomstring. Infeksjon i blader fra DLA ble senere undersøkt med CLSM. Hyfeansamlinger rundt spalteåpningene ble observert gjentatte ganger, noe som indikerer at stomata kan være inngangspunktet for *F. langsethiae* på hveteblader. Scanning elektron mikroskopi av hvetekorn fra veksthusforsøket viste at *F. langsethiae* var i stand til å infisere de ytre lagene i kornet. Ved vekst direkte på kornet, imidlertid, var hyfene lysert, muligens på grunn av forsvarsmekanismer fra verten.

Abbreviations

15-ac-DON	15-acetyldeoxynivalenol
3-ac-DON	3-acetyldeoxynivalenol
Avr	Avirulence
BF	Bright field
Bp	Base pair(s)
BTUB	β-tubulin protein
cDNA	Complimentary deoxyribonucleic acid
CLSM	Confocal laser scanning microscopy
cox	Cytochrome c oxidase gene
CPD	Critical Point Drying
C_q	Quantification cycle
DLA	Detached Leaf Assay
DNA	Deoxyribonucleic acid
DON	Deoxynivalenol
dpi	Days post inoculation
FA	*F. avenaceum*
FC	*F. culmorum*
FG	*F. graminearum*
FHB	*Fusarium* head blight
FU	Fluorescence
GC-MS	Gas chromatography-mass spectrometry
GFP	Green fluorescence protein
HKG	Housekeeping gene(s)
Hpi	Hours post inoculation
kb	Kilo base pair(s)
MBA	Mung bean agar
miRNA	Micro ribonucleic acid
mRNA	Messenger ribonucleic acid
Nd	Not determined
NIV	Nivalenol
PCR	Polymerase chain reaction
qPCR	quantitative polymerase chain reaction
R	Virulence gene
RNA	Ribonucleic acid
rRNA	Ribosomal ribonucleic acid
RT	Room temperature
RT-qPCR	Reverse-transcriptase quantitative polymerase chain reaction
SDS	Sequence detection software
SDW	Sterile distilled water
SEM	Scanning electron microscopy
SNA	Synthetic nutrient agar
SNB	Synthetic nutrient broth
SOD	Superoxide dismutase
UBC	Ubiquitin conjugating enzyme
WA	Water agar

Contents

Chapter 1 Introduction

1.1 *Fusarium* life cycle and morphology

The *Fusarium* genus is placed in phylum ascomycota, and contains a large number of different species. *Fusarium* species are able to colonize a remarkable number of ecological niches in most geographical areas worldwide. This may be explained by the high degree of variation between the species, in respect to morphological, cultural, and physiological characteristics (Nelson et al. 1994).

The morphology of *Fusarium* is extensively reviewed by Nelson and co-workers (1994). *Fusarium* can produce three types of spores; macroconidia, microconidia and chlamydospores. Macroconidia (Figure 1) are produced in a specialized structure called sporodochium. The sporodochium is produced on phialides which are hyphal structures with only one (monophialide) or several (polyphialide) openings or pores. Conidia are extruded through these openings. Species forming macroconidia are for example *Fusarium graminearum*, *F. culmorum* and *F. avenaceum*.

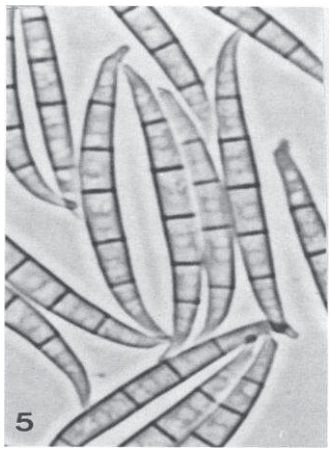

Figure 1. Cano shaped macroconidia of *F. graminearum.* The general macroconidia form in *Fusarium* spp. Magnification x 950. (Nelson et al. 1994)

Another spore type, microconidia, is only produced in aerial mycelium, upward or outward from the substrate, either from mono- or polyphialides or in chains. *F. langsethiae* is an example of a fungus that produces only microconidia.

In the winter when no suitable host is available, some *Fusarium* species produces chlamydospores. The chlamydospore is a thick walled resting spore filled with a lipid like material. They are produced singly, in clumps or in chains by for example *F. oxysporum*.

Spores can serve as a nutrient store; containing large amounts of nutrients like glycerol and trehalose which are mobilized when germinating (Thevelein 1984).

The life cycle of *Fusarium* is most extensively studied in *F. graminearum* (Figure 2) as reviewed by Trail (2009), and is in general the assumed life cycle of these fungi. In contrast to *F. graminearum* most *Fusarium* species are lacking a sexual step in their lifecycle; this will be more extensively described later.

In the winter *Fusarium* lives as saprophytic mycelia on dead organic matter like stubble and crop debris from last season or resting as thick walled chlamydospores (Nelson et al. 1994; Trail 2009). With the arrival of the spring, the fungi produce conidia for dispersal, and start growing as a phytopathogen. Rain plays an important role in dispersal; Jenkinson and Parry (1994) showed that the conidia most likely are rain splashed upward the plant, from leaf to leaf, in a series of steps involving infection of the upper plant parts until they reach the wheat head and infect. When growing in the wheat head, *Fusarium* can reduce crop yield and produce mycotoxins which can have negative health effects when ingested (Kosiak et al. 2003; Zain 2011). After the harvest, *Fusarium* remains on the crop debris, and start growing as a saprophyte again (Trail 2009).

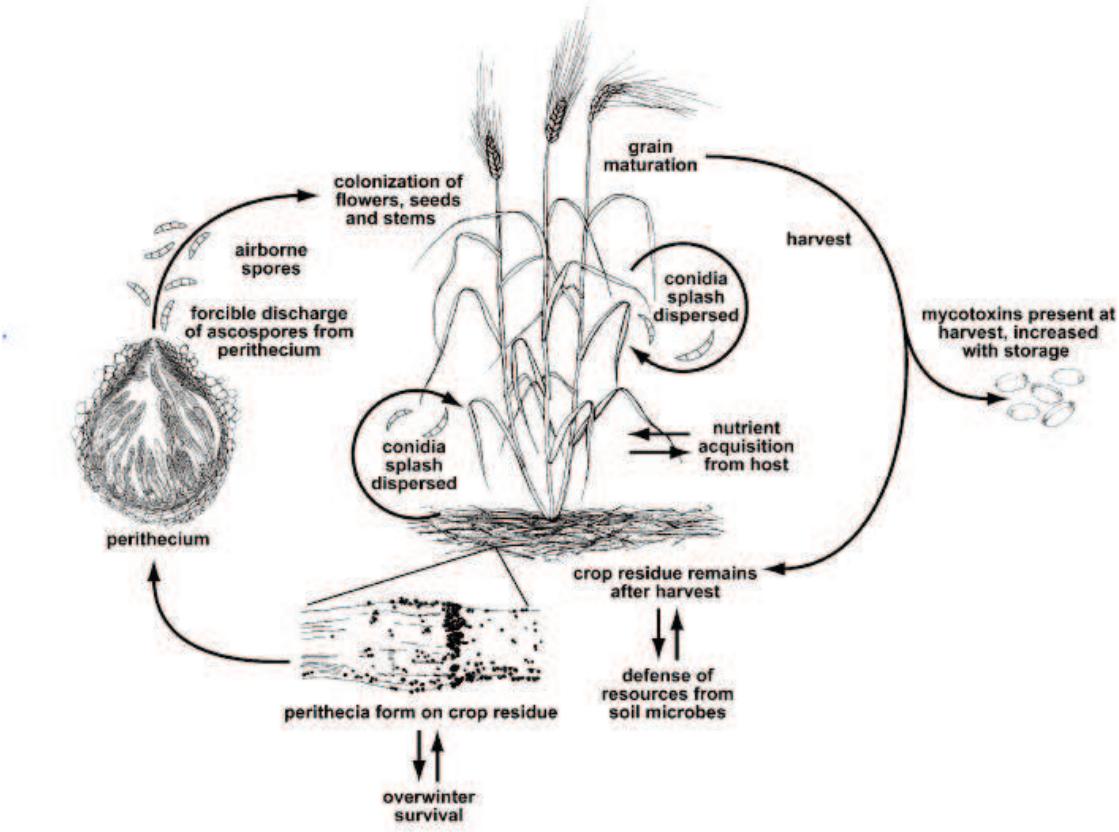

Figure 2. General life cycle of *F. graminearum* (Trail 2009). Further description in the text.

1.1.1 *Fusarium graminearum*

Fusarium graminearum is one of the most studied fungal plant pathogens and has been known as the predominant species of the *Fusarium* head blight (FHB) pathogens (Trail 2009; Xu & Nicholson 2009). Recently the top 10 fungal plant pathogens were named and *F. graminearum*

came in fourth place (Dean et al. 2012). This confirms the great impact this pathogen has on the world food production.

F. graminearum has a sexual stage in its lifecycle, even though it is haploid in the majority of its lifecycle. The sexual stage with formation of perithecium is a crucial step in the fungus' survival through the winter (Figure 2), since it is the binucleate hyphae and the perithecium initials that make up the overwintering structures (Guenther & Trail 2005). A study with genetically modified *F. graminearum* which is lacking the sexual step is showing a significant disease reduction (Desjardins et al. 2006).

To initiate sexual reproduction the production of binucleate hyphae begins. Since *F. graminearum* is homothallic, it does not need a distinct partner to develop ascospores. Small coiled cells will start to form from the binucleate cells. These cells develop further into perithecia which contains asci (Trail 2009). Warm weather conditions are ideal for maturation of perithecia that produces the ascospores simultaneously with anthesis of cereals (Goswami & Kistler 2004). The fruiting bodies mature and ascospores are forcibly discharged into the wind and spread over enormous areas (Trail et al. 2005).

Recently, several studies have found that in regions with cooler summer the amount of *F. culmorum* has decreased compared to earlier years, whereas occurrence of *F. graminearum* has increased. This increase appears be at the expense of *F. culmorum* (Kosiak et al. 2003; Waalwijk et al. 2003; Xu et al. 2005). This trend may have several possible reasons, for example: a) A genetic change in *F. graminearum*, because *F. graminearum* have the ability to reproduce by producing sexual ascospores, the ability to genetic variation is bigger than *F. culmorum* (Guenther & Trail 2005). b) A change in cropping techniques. Increase in growth of maize in circulation with wheat, may increase the *Fusarium* (Xu et al. 2005). The ploughing of fields have also been shown to play a role in *F. culmorum* development (Bateman 2005). Additionally incidents of *F. graminearum* can increase if large amounts of organic material from last season's harvest are left in the field as with reduced tillage (Brodal et al. 2012) c) A climatic change may also have an effect, as cooler areas gradually have become warmer.

To identify the exact reason for the species shift is difficult. Probably all these factors interact and contribute to the increase of *F. graminearum* in northern Europe.

1.1.2 *Fusarium culmorum*

Most FHB research has focused on *F. graminearum*. In contrast to *F. graminearum, F. culmorum* does not have a sexual stage in its life cycle and produces asexual spores (macroconidia) as the main mode of dispersal. The conidia are dispersed by wind or rain splash to wheat heads. The macroconidia have thick curved ventral and dorsal surfaces and are short and stout. *F. culmorum* also have the ability to produce chlamydospores (Wagacha & Muthomi 2007).

As mentioned earlier, *F. culmorum* has earlier been regarded as the most prevalent species in Northern Europe, but it has decreased in recent years (Wagacha & Muthomi 2007; Xu et al. 2005). This trend can reflect a change in dominance in the worlds *Fusarium* populations.

1.1.3 *Fusarium avenaceum*

F. avenaceum was first isolated from oat (*Avena sativa*) in Germany in the 19th century (Desjardins 2003), and is the most abundant *Fusarium* species found in northern Europe (Kosiak et al. 2003). Likewise to *F. culmorum,* the main mode of distribution is splash dispersal of macroconidia (Jenkinson & Parry 1994).

Because of its great genetic diversity it has been postulated that sexual reproduction in *F. avenaceum* does occur (Holtz et al. 2011). The product of sexual reproduction is ascospores produced and dispersed by perithecium. As earlier described in *F. graminearum*, the ascospores can be spread much longer distances than conidia of asexual formation (Trail et al. 2005). The long distance dispersal can explain why Holtz and co-workers (2011) recorded genetically similar *F. avenaceum* from lupin (*Lupinus angustifolius*) over surprisingly large areas. Observations of *F. avenaceum* ascospores and perithecium have only been reported twice (Booth & Spooner 1984; Cook 1967). Holtz and co-workers emphasize that the reason for this is that *F. avenaceum* is a functionally asexual species and only occasionally reproduces sexually. The great genetic diversity can also be explained by the great differences in different stages of the species life cycle. Hence the ability to live both as a saprophyte and a parasite on a wide variety of hosts will require a selection of genes for a diverse variety of traits (Holtz et al. 2011).

1.1.4 Other *Fusarium* species

The number of existing *Fusarium* species is too high for commenting all of them here. Even though, some of the most regular species found in grain in Norway will be mentioned; *F. langsethiae* is a relatively novel species, with the first reports from the 1990s (Torp & Langseth 1999). A slow growth rate makes it easily overgrown by other species and its appearance in microscope, producing only microconidia (Figure 3), closely resembles that of *F. poae.* Both factors have possibly contributed to *F. langsethiae*'s late species-recognition (Torp & Nirenberg 2004). Indeed, *F. langsethiae* was previously called "powdery poae" due to the large production of spores, giving it a powdery appearance (Torp & Langseth 1999).

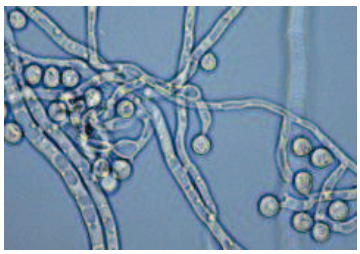

Figure 3. Microconidia of *F. langsethiae*. Photo: Jaffar Razzaghian.

1.2 Fusariosis in small grain cereals

Fusarium species are widely spread and colonizes highly variable niches around the world (Nelson et al. 1994). These niches include most genera of cultivated plants, as well as members of the Gramineae (Parry et al. 1995).

By infecting crop and causing FHB, *Fusarium* ear blight and *Fusarium* seedling blight, *Fusarium* causes great yield loss in wheat, barley and maize and this leads to large economic loss worldwide every year (Doohan et al. 2003).

1.2.1 Wheat (*Triticum aestivum*) structure

Bread wheat (*T. aestivum*) is the single most cultivated and grown food crop worldwide. It is primary grown annually in temperate climates (Willenborg & Van Acker 2008). The reproductive biology and flowering of wheat has been extensively reviewed by Vries (1971): The flowers are normally hermaphroditic, and contain three anthers and two stigmas enclosed by the glumes. Under flowering,

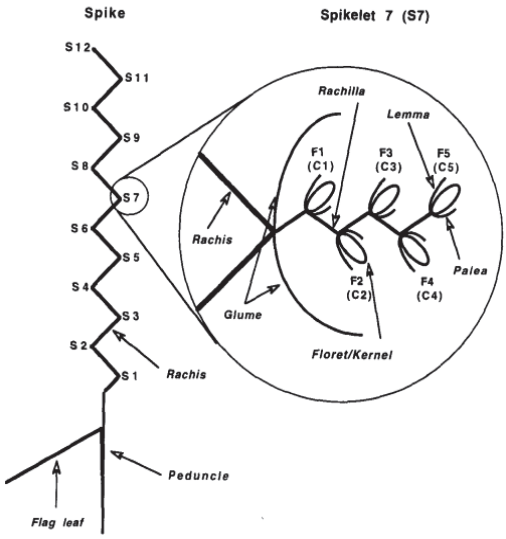

Figure 4. Wilhelm and McMasters (1996) naming scheme for wheat.

the glumes will open at a 20-35° angle and extrude the anthers, and receive pollen fertilizing the stigmas. A schematic drawing of the spike of wheat is shown in Figure 4 (Wilhelm & McMaster 1996). The upper part of the plant, the ear, is a spike with a central axis, the rachis. The spikelets are placed on the rachis and are enclosed by two glumes. Each spikelet contains several kernels (two to nine) arranged on rachilla. Every kernel is embraced by palea and lemma, with palea enclosing the ventral groove which is stretching the whole length on the ventral side of the kernel (Moragues & McMaster 2012; Wilhelm & McMaster 1996).

1.2.2 *Fusarium* head blight (FHB)

FHB is a disease where *Fusarium* is infecting the head of cereals, including wheat. The number of species causing the disease is at least 17, with the most regularly registered being *F. culmorum*, *F. graminearum*, *F. avenaceum*, *F. poae*, and *Microdochium nivale* (Parry et al. 1995). In Norway however, the most frequent isolated species have been *F. avenaceum*, *F. poae*, *F. tricinctum* and *F. culmorum* (Kosiak et al. 2003). But recently there has been a dramatically incline in *F. graminearum*, which is usually associated with warmer climates (Xu et al. 2005). *F. avenaceum* is the most prevalent *Fusarium* species in Norway and is associated with cooler climates (Kosiak et al. 2003).

Climatic factors such as temperature and humidity are of great importance for the development of FHB. Regularly, warm and humid weather provide the most optimal conditions for conidia production, growth and infection for most *Fusarium* species (Doohan et al. 2003). This is of special concern considering the climatic change the world is facing today.

The symptoms of *Fusarium* infection appear as brown water soaked spots on the cereal head in the initial infection and develop to salmon red spots as the infection is proceeding. Often, premature death or bleaching of the cereal spikelets appears (Figure 5). The underdevelopment of the spikelets and mycotoxin accumulation in the wheat heads give yield losses and contaminated grain (Parry et al. 1995). The European Union sets 650 µg/kg as maximum limit of deoxynivalenol (DON) in

Figure 5. Browning and premature bleaching of the wheat spikelets; regular symptoms of FHB (Goswami & Kistler 2004).

wheat for human consumption (Commition Regulation (EC) 2005). Limits are still to be determined for the mycotoxins HT-2, T-2, nivalenol (NIV) and enniatins in wheat.

1.2.3 Mycotoxins

Mycotoxins are secondary metabolites produced by fungi which are harmful to both animals and humans. They are often found in agricultural products and pose a threat to food safety (Marasas et al. 2008; Nelson et al. 1994). Producers of mycotoxins often belong to one of the three genera *Aspergillus*, *Penicillin* or *Fusarium* (Logrieco & Moretti 2008). There are several groups of mycotoxins. One mycotoxin group, the enniatins, has not been the object of many studies, but has gained more interest recently with the expanding population of *Fusarium* on wheat (Kosiak et al. 2003). *F. avenaceum* is a well known producer of enniatins. In addition it also produces the mycotoxins moniliformin and beauvericin. The enniatins generally have both antibiotic and phytotoxic activity. So far, little data is available on the impact this toxin has on humans when ingested (Desjardins 2006). But one study on its mutagenic/toxigenic potential found that it had no mutagenic effect, though a cytotoxic effect was found.

With regard to economic impact and scientific interest the trichothecenes are one of the major mycotoxin groups. The trichothecene producers are spanning over a wide range of unrelated genera, including *Fusarium*, *Trichotecium*, *Myrothecium* and *Stachybotrys* (Kimura et al. 2007). The trichothecenes can be divided into four groups; A-D, with A and B being associated with *Fusarium* and FHB. Group A includes the highly toxic T-2 toxin (T-2) and HT-2 toxin (HT-2). Producers of these mycotoxins include *F. langsethiae* and *F. sporotrichoides* with mycotoxin profiles similar to each other (Medina & Magan 2011; Thrane et al. 2004). Group A trichothecenes has been regarded as ten times more toxic to mammals than group B trichothecenes (Desjardins 2006). Group B trichothecenes include, among others, NIV, DON and their acetylated derivates with the major producers being *F. culmorum* and *F. graminearum* (Fink-Gremmels 2008).

F. culmorum strains can be divided into different chemotypes, depending on the mycotoxin profile (Langseth et al. 1998). The major mycotoxins produced by *F. culmorum* are type zearalenone and fusarins, and the group B trichotecens (DON, NIV, 3-acetyldeoxynivalenol (3-ac-DON) and acetyl T-2 toxin) even though, not all *F. culmorum* are able to produce type B trichothecenes (Demeke et al. 2005; Llorens et al. 2006; Wagacha & Muthomi 2007). Most

F. culmorum in Northern Europe belong to the chemotype IA, mainly producing 3-ac-DON, in contrast to isolates belonging to chemotype IB on more southern latitude producing 15-acetyldeoxynivalenol (15-ac-DON). Chemotype II producing NIV also exists in Europe, and the number has increased in recent years (Langseth et al. 1998). A survey conducted in United Kingdom suggested that there is a higher proportion of NIV producing chemotypes in the north compared to the south, were DON producers were more common. The reason for these differences in geographical distribution are unknown, possibly many factors play a part i.e. soil type, cultivar, temperature, cropping practise and alternative hosts (Jennings et al. 2004).

Similar to *F. culmorum*, *F. graminearum* also produces type B trichothecenes and can be divided into different chemotypes according to mycotoxin profile (reviewed by Goswami and Kistler (2004)). Three mycotoxin profiles are observed: NIV (NIV producers), 3ADON (DON producers also producing 3-ac-DON) and 15ADON (DON producers also producing 15-ac-DON).

The toxic effect of trichothecenes is believed to come from their ability to inhibit DNA and RNA synthesis, mitochondrial functions, cell division and membrane effects. The inhibition of these processes may come from inhibition of protein synthesis, since the dependence of that metabolic pathway is of great importance to all other functions in the cell (Rocha et al. 2005).

Trichothecene biosynthesis

Trichothecenes is the product of a complicated biosynthetic pathway consisting of multiple steps (Figure 6), which has been reviewed by Alexander and co-workers (2009) and Foroud & Eudes (2009). The first step in the pathway is catalyzed by the enzyme trichodiene synthase encoded by *Tri5*. The enzyme converts an intermediate in primary metabolism, farnesyl diphosphate, to trichodiene.

The pathway continues in a series of enzymatic steps until calonectrin is formed. Calonectrin has been isolated from T-2, DON and NIV producing Fusaria, indicating that the pathway for the different toxins is the same until this point (Alexander et al. 2009).

The NIV chemotype of *F. graminearum*, with functional expression of *Tri7* and *Tri13*, will convert calonectrin to NIV, while the DON chemotype is lacking functional copies of these genes, and will thus convert calonectrin to DON (Lee et al. 2002).

In T-2 and NIV producers the pathway proceeds with C-4 hydroxylation catalyzed by cytochrome P450 monooxygenase encoded by *Tri13*. DON producers, like *F. graminearum*, do not have a functional *Tri13*. In *F. graminearum* the process proceeds with a C-7 and C-8 hydroxylation by the *Tri1* encoded enzyme, cytochrome P450 monooxygenase. The pathway continues and ends in NIV or DON (Alexander et al. 2009; Foroud & Eudes 2009).

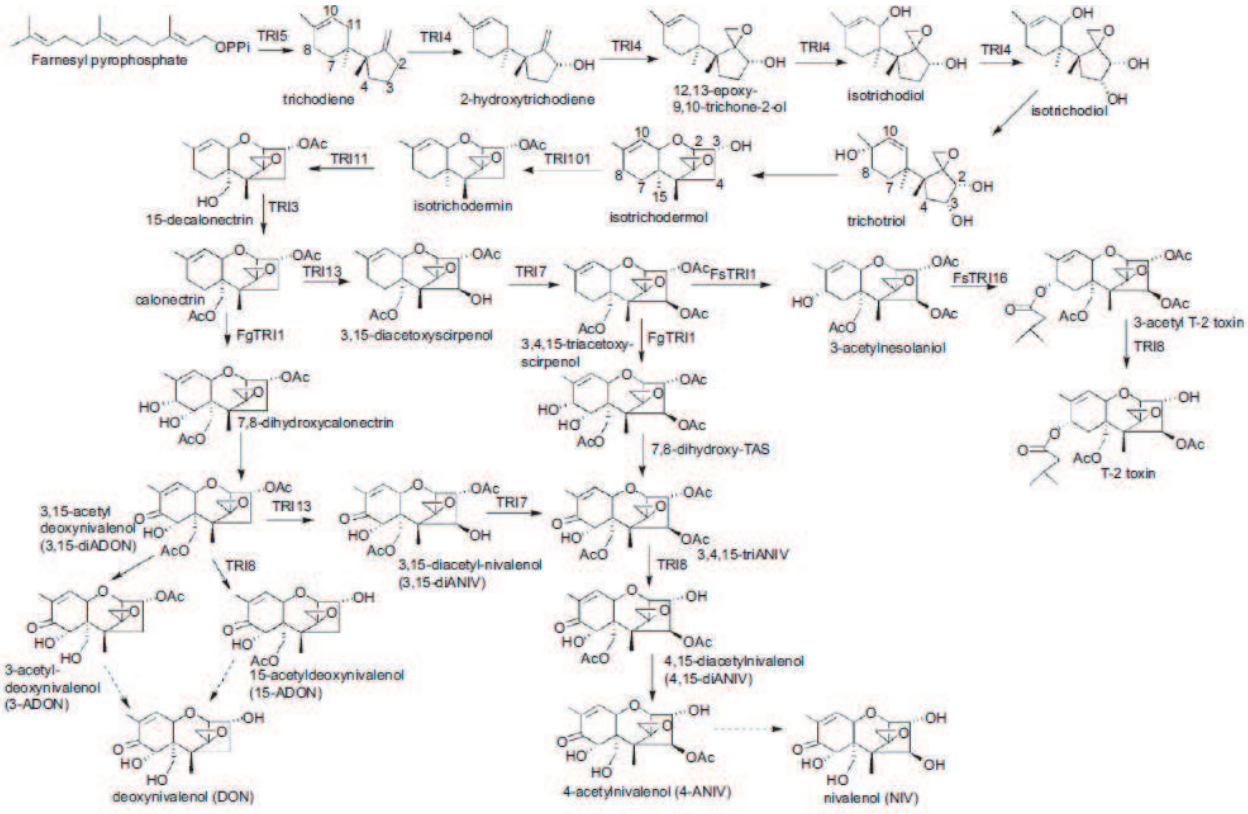

Figure 6. The proposed biosynthetic pathway of trichothecenes (Alexander et al. 2009). See text for further explanation.

1.3 Interactions

When two populations are living at the same place, and have an effect on each other, interaction between the populations takes place (Wicklow 1992). Interaction between fungi can be divided into three general groups: competition, antagonism and commensalism. If one species exploits a resource better than another, or grow faster, competition takes place. Interactions can be antagonistic when one species excludes another by directly affecting it by production of antibiotic compounds, or by parasitism. When two species are able to coexist there is commensalism (Deacon 2006). Interactions can be beneficial for both parts, as in symbiosis. The

most regular symbiosis is mycorrhiza, where fungi colonize plant roots. The fungus extracts water and nutrients from the soil and provides them to the plant. The plant gives shelter and sugars in return (Bonfante 2003).

1.3.1 Plant - pathogen interactions

Understanding the complex interactions between plants and their pathogens is extremely important in order to find a way to decrease a pathogens devastating impact on plants e.g. food crop.

To recognize its host the ascomycetes have specific receptor proteins which transmit signals from the environment to the fungi. These proteins are essential to the colonization of the host (Soanes et al. 2008). The utilization of stored energy is important in the colonization of a new host. Soanes and co-workers (2008) demonstrated by genome clustering that the phytopathogenic filamentous ascomycetes mobilize stored energy reserves previous to nutrition extracted from the plant.

Several mechanisms in the plant influence the defence of pathogens. The first line of defence is physical defence and recognition of the pathogen. In wheat, several physical components in the cuticle limit growth and intrusion of the pathogen e.g. waxy surfaces can reduce water availability limiting the fungal establishment (Walter et al. 2010).

The plant recognizes the pathogen by sensing and responding to molecules that are common to several types of microbes (Jones & Dangl 2006). When the plant have recognized the pathogen it initiates the second line of defence; the expression of resistance proteins that interfere and guard against the pathogen. The proteins do also trigger a hypersensitive response and afterwards programmed cell death. The purpose of the hypersensitive response is to prevent spreading of the pathogen further in the plant tissue by containing the pathogen. This is possible by a battery of generic plant responses i.e. changing the ion fluxes, lipid hyperperoxidation, protein phosphorylation, nitric oxide generation, and production of reactive oxygen species and antimicrobial compounds (Alfano & Collmer 2004).

A close relationship normally exists between a host and its pathogen. This relationship can be described as the gene-for-gene hypothesis which has been extensively reviewed by Takken and Joosten (2000).The hypothesis is based on that for every virulence gene (*R*) in the pathogen there is a matching avirulence (*Avr*) gene in the host. The *R* genes makes the host able to recognize the

pathogen holding *Avr* and encodes proteins defending the host from the pathogen if it overcomes the first structural defence mechanisms. This relationship exist for several pathogen-host systems including fungi and plant, however it is not been reported for *Fusarium*. For a pathogen to be able to infect it has to overcome the plant defence.

The pathogen induces several genes to defend itself such as catalases and superoxide dismutase (SOD). These enzymes inactivate the reactive oxygen species hydrogen peroxide (H_2O_2) and superoxide anion (O_2^-) respectively. In addition, several other proteins are produced to inactivate specific host proteins involved in defence. Excreted enzymes like pectin esterases, polygalacturonases, xylanases, pectate lyases and cellulases are also of importance for the degrading of the hosts cell wall, colonization process and pathogenicity (Van Sluys et al. 2002).

Most research on *Fusarium* plant interaction has been focused on the interaction between *F. graminearum* or *F. culmorum* and wheat (Walter et al. 2010). The nature of wheat resistance to *Fusarium* is complex. The great number of factors that can have an impact on resistance can be divided into several groups, with the two major groups being; type 1 resistance: resistance to the initial infection, and type 2 resistance: resistance to spreading of the pathogen through the plant (Schroeder & Christensen 1963). Both morphological and physiological factors in the plant contribute to type 1 resistance i.e. plant height, flowering timing and spikelet density (Walter et al. 2010). The wheat is most susceptible to *Fusarium* under flowering. This suggest that the initial establishment of the fungus occurs on the anthers which are extruded at this stage (Parry et al. 1995). Different wheat cultivars can vary in their anther extrusion, in both timing and flower opening, and this has been proved to correlate with FHB severity (Graham & Browne 2009). Further entry of the floret happens either passively, through natural openings such as stomata, or by direct penetration (Pritsch et al. 2000; Walter et al. 2010). *Fusarium* produces a large number of hydrolysing enzymes that probably facilitate penetration of the host cell (Walter et al. 2010). One of these enzyme groups is lipases which recently was shown to be a virulence factor in *F. graminearum* infecting wheat (Voigt et al. 2005).

Plant recognition of *Fusarium* occurs through several specialized proteins. These proteins can recognize cell wall components of the fungus like chitin or glucan. Accumulated transcripts in the wheat seeds, encoding chitinases or glucanases, follow the infection of *F. graminearum*. These enzymes could have a role in plant resistance against *Fusarium* (Pritsch et al. 2000).

When the *Fusarium* is sensed by the plant, the plant will inhibit the activity of the fungal degradative enzymes. To protect itself the plant will also reinforce its own barriers by thickening the cell walls (Walter et al. 2010).

Penetration of the plant cell immediately leads to cell death. Production of the mycotoxin DON also contributes to cell damage especially in cell membrane, chloroplast and ribosomes (Walter et al. 2010).

Whether the production of mycotoxins is necessary for the infection of plants is still an unanswered question (Ilgen et al. 2009). It has been shown that DON induction is important to suppress the plant defence and thereby enabling the fungus to penetrate through the rachis node and spread to other parts of the plant (Jansen et al. 2005). At this stage the induction of DON is most likely triggered by the host (Ilgen et al. 2009; Jansen et al. 2005). Boenisch and Schäfer (2011) suggest that similar host factors affect the DON induction during the initial phase of infection when the pathogen penetrates the cuticle.

1.3.2 Fungal - fungal interaction

Fungi can interact in antagonistic interactions when one species excludes another by directly affecting it by production of for example antibiotic compounds and thereby inhibiting the other. They can also inhibit each other by hyphal interference, as in some Basidiomycetes where one organism makes contact with the other resulting in death for the latter. Another antagonistic interaction is parasitism where the hyphae of one fungi makes contact with another and extracts nutrients from it (Boddy 2000). When two species are able to coexist there is commensalism. These interaction types can however grade into each other because fungal behaviour can vary according to different situations (Deacon 2006).

Several species of *Fusarium* often appear as a complex on the same plant, which makes it likely to believe that the species of this complex can have an effect on each other (Xu et al. 2005).

1.4 Purpose of study

FHB research has mainly been focusing on the infection of cereals inoculated with one species of *Fusarium* and the toxins produced under the infection (Nelson et al. 1994; Parry et al. 1995). The most frequently registered species causing FHB is *F. culmorum*, *F. graminearum*, *F. avenaceum*, *F. poae*, and *M. nivale* (Parry et al. 1995). These species may appear together, infecting the same wheat ear (Xu et al. 2005). Earlier research may have led to erroneous conclusions if there is a significant rate of interaction between the species. The importance of studying them together is therefore high. The few previous studies done on this subject have focused on the interactions between *Fusarium* spp. in wheat at the ripening stage (Simpson et al. 2004; Xu et al. 2005; Xu et al. 2007a; Xu et al. 2007b). To be able to see the complete picture of the interaction, it is also important to see how these species affect each other both in earlier stages and at the ripening stage of the grains development. Secondly, it is important to understand how the mycotoxin production is affected by interactions between the species and the infection process at different stages in the grains development.

Furthermore, an *in vitro* study on culture plates was done to learn more about how *F. graminearum* and *F. avenaceum* grow and interact and if they have an inhibiting effect on each other. By using a *F. graminearum* mutant expressing green fluorescence protein (GFP) when inducing a toxin related gene, one could see if toxin production of *F. graminearum* is affected by the presence of *F. avenaceum*.

Another aim of this study was to investigate the infection of *F. langsethiae* in wheat. Through this effort one should be able to address how and if *F. langsethiae* grows in wheat and why *F. langsethiae* seldom is found in wheat in Norway, but more regularly found in oat.

Chapter 2 Materials

2.1 Buffers, chemicals and enzymes

Table 1. List over buffers, chemicals and enzymes and their suppliers

Name	Supplier
2 × TaqMan® PCR Master Mix	Eurogentec, Seraing, Belgium
Bacto agar	Bectoa, Dickinson and Company, Sparks, MD, USA
DNA ladder 1 kb	New England Biolabs, Ipswich, MA, USA
DNA ladder 100 bp	New England Biolabs, Ipswich, MA, USA
Ethanol	Kemetyl Norge AS, Vestby, Norway
Ethidium bromide	vwr®, Radnor, PA, USA
Glucose	Dechefa, Haarlem, The Netherlands
Hygromycin B Solution	Sigma®, St Louis, MO, USA
Kinetin	Sigma®, St Louis, MO, USA
Magnesium Sulfate ($MgSO_4 * 7H_2O$)	Merck KGaA, Darmstadt, Germany
Nuclease-free water	Ambion®, Austin, TX, USA
Potassium cloride (KCl)	Merck KGaA, Darmstadt, Germany
Potassium di-hydrogen phosphate (KH_2PO_4)	Merck KGaA, Darmstadt, Germany
Potassium nitrate (KNO_3)	Merck KGaA, Darmstadt, Germany
Power SYBR Green PCR Master Mix 2 ×	Applied Biosystems, Carlsbad, CA, USA
Sucrose	Dechefa, Haarlem, The Netherlands

2.2 Kits

Table 2. List over kits and suppliers.

Name	Supplier
DNeasy Plant Mini Kit	Qiagen®, Venlo, The Netherlands
Fast DNA® Spin Kit for Soil	MP Biomedicals, Santa Ana, CA, USA
Gene Elute ™ PCR Clean-Up	Sigma-Aldrich®, St. Louis, MO, USA
Spectrum™ Plant Total RNA Kit	SIGMA-ALDRICH®, St. Louis, MO, USA
Superscript® VILO™ cDNA synthesis kit	Invitrogen™, Carlsbad, CA, USA
Turbo DNA free™	Ambion®, Austin, TX, USA

2.3 Primers

Table 3. Primers and probes used in DNA quantification. All primers were supplied by Invitrogen™.

Target organism	Target DNA	Primers/probe	Sequence (5′-3′)
F. avenaceum[a]	RAPD fragment	TMAV-f	AGATCGGACAATGGTGCATTATAA
		TMAV-r	GGCCCTACTATTTACTCTTGCTTTT G
		TMAV-p	TET-CTCCTGAGAGGTCCCAGAG ATGAACATAACTTC-TAMRA
F. culmorum[b]	RAPD fragment	culmMGB-f	TCACCCAAGACGGGAATGA
		culmMGB-r	GAACGCTGCCCTCAAGCTT
		culmMGB-p	FAM-CACTTGGATATATTTCC-MGB
F. graminearum[c]	β-tubulin gene	Fgtub-f	GGTCTCGACAGCAATGGTGTT
		Fgtub-r	GCTTGTGTTTTTCGTGGCAGT
		Fgtub-p	TET-ACAACGGCACCTCTGAGCT CCAGC-TAMRA
T. aestivum[d]	Cytochrome c oxidase gene	COX554-f	GGTTGTTGCCACCAAGTCTCTT
		COX554-r	TGCCGCTGCCAACTTC
		COX554-p	FAM-CTCCTATTAAGCTCAGCCTT-MGB

[a] (Halstensen et al. 2006)
[b] (Waalwijk et al. 2004)
[c] (Reischer et al. 2004)
[d] (Divon et al. 2012)

Table 4. List of primers and sequences tested for RNA quantification. All primers were supplied by Invitrogen™.

Target organism	Target cDNA	Primers	Sequence (5'-3')
F. avenaceum	Ubiquitin conjugating enzyme gene	FavenUBCf1	TGCTTGGACATTCTGCGA
		FavenUBCf2 *	TACTATTATGGGACCCAGCGA
		FavenUBCr1 *	GCTCCACTGGTCTCGCA
		FavenUBCr2 *	GGTTAGGATCCGTCAGCATC
		FavenUBCr3	CAGAGGGTCGTCAGGGTTA
	β-tubulin gene	FavenBTUBf1	CTTCCGGCAACAAGTACGTC
		FavenBTUBr1	CGGGTCGGAAAAGCTGA
	Enniatin synthetase gene	EsynF1	CAAGTTCGCAGGAAAAGCCA
		EsynR1	CGGGTGTGGGGAAGTATT
F. graminearum	Ubiquitin conjugating enzyme gene	FgraUBCf1	ATGCTGACTTTGTTCCACAGAG
		FgraUBCr1	GGAGTCAGAAGGACCCATGA
	β-tubulin gene	FgcBTUBf1	ACCCTCTCCGTCCATCAAT
		FgcBTUBr1	CGGACATGACGGCAGAG
	Tri1 gene	FGTri1SKf	AAGGATCTACGAGAACCCTGAA
		FGTri1SKr	CTTCTCTGGCGGATTGTTGTTGT

*Primers only tested and not used in RNA quantification.

2.4 Laboratory equipment

Table 5. List of instruments and suppliers.

Method	Name	Supplier
cDNA synthesis	PCR plate, skirted	Abgene® Thermo Scientific, UK
	Domed cap strip	Abgene® Thermo Scientific, UK
	Thermal cycler, T100	Biorad, Singapore
	Bioanalyzer 2100	Agilent Technologies, Santa Clara, CA, USA
Centrifugation and homogenization	Galaxy mini	vwr™, Korea
	Biofuge pico	Kendro, Germany
	Centrifuge 5810R	Eppendorf®, Hamburg, Germany
	Fast prep®-24	MP Biomedicals, Irvine, CA, USA
	Vortex, labdancer S40	vwr™, Germany
Gel Electrophoresis	Powerpac 300	Biorad, Singapore
	Geltray Subcell® GT	Biorad, Singapore
Incubation	Infors HT	Ecotron, Durham, NC, USA
Inoculation	Sterile bench, Hera Safe	Thermo electroncoorperation, Germany
Microscopy	Binoculars, Leica CLS 150 X	Leica, Thailand
	Leica TCS SP5 Confocal	Leica, Wetzlar, Germany
	Microscope, Leica DM 2000	Leica, Wetzlar, Germany
	SEM, EVO® 50	Carl Zeiss AG, Oberkochen, Germany
	Wild M38	Heerbrugg, Switzerland
Pipetting	*ep*Motion 5070 Pipetting robot	Eppendorf®, Hamburg, Germany
qPCR	Optical 96-well Reaction Plate	MicroAmp™ Applied Biosystems, Singapore
	Optical adhesive film	MicroAmp™ Applied Biosystems, USA
	7900 Fast Real-Time PCR system	Applied Biosystems, USA

2.5 Programs and software

Table 6. Name and supplier of software.

Name	Source/Supplier
SDS v. 2.2.1	Applied Biosystems, USA
REST-MCS	Pfaffl et al (2002) (Available at http://www.gene-quantification.de/download.html#rest-mcs)
Bestkeeper v. 1	Pfaffel et al. (2004) (Available at http://bioinformatics.gene-quantification.info/bestkeeper.html)
Quantity One v. 4.5.1	Biorad®, Hercules, CA, USA

2.6 Solutions and growth media

Table 7. Recipes for media and solutions.

Solution	Recipe
0.5 % WA	5 g Bacto agar was added to 1l water. The solution was autoclaved.
1 × TBE Buffer	1 × TBE Buffer was diluted from 10 × TBE. 1 litre 10 × TBE was made with: 108 g Tris-Base 55 g boric acid 40 ml EDTA (0.5 M, pH 8)
Loading buffer	0,25 % Bromophenol blue 40 % Sucrose Diluted in sterile distilled water (SDW)
MBA	40 g Mung beans 1 l Tap water 15 g Bacto agar The beans was boiled in 1 l tap water in 23 min and thereafter filtrated in a cheese cloth. The solution was supplemented with water until 1 l and agar was added before autoclaving.
SNA	1.0 g KH_2PO_4 1.0 g KNO_3 0.5 g $MgSO_4 \cdot 7H_2O$ 0.5 g KCl 0.2 g Glucose 0.2 g Sucrose 1.0 l SDW 45 g Bacto agar The solution was stirred and autoclaved.
SNB	1.0 g KH_2PO_4 1.0 g KNO_3 0.5 g $MgSO_4 \cdot 7H_2O$ 0.5 g KCl 0.2 g Glucose 0.2 g Sucrose 1.0 l SDW The solution was stirred and autoclaved.
SNB (with Hygromycin)	1.0 g KH_2PO_4 1.0 g KNO_3 0.5 g $MgSO_4 \cdot 7H_2O$ 0.5 g KCl 0.2 g Glucose 0.2 g Sucrose 1.0 l SDW The solution was stirred and autoclaved. After autoclaving, when the solution had cooled, 600 µl Hygromycin was added.

MBA= Mung bean agar, SNA= Synthetic nutrient agar, SNB= synthetic nutrient broth, WA = Water agar.

Chapter 3 Methods

3.1 Greenhouse inoculation for molecular studies

The greenhouse experiment here described had previously been conducted by Aamodt (unpublished data). Wheat samples were sprayed with the fungal isolates of the species *F. graminearum, F. avenaceum* and *F. culmorum* alone or in pair-wise combinations. The inoculums of each species consisted of three different fungal strains mixed together. This was to avoid that the specific traits of one isolate would be interpreted as the behavior of the species. A complete list of all fungal isolates is given in appendix.

Preparation of inoculum

One ml of conidial suspensions were spread on a petri dish containing Mung bean agar (MBA) and incubated at 22 °C for 15-20 days. Conidia were then rubbed from the agar surface in sterile distilled water (SDW) with a sterile glass rod. To remove agar and mycelial fragments, the suspension was filtered through sterile cotton. Spore concentrations were determined using a Bürker hemocytometer.

Plant material

Wheat (*Triticum aestivum* cv. Zebra) was grown in a greenhouse. Ten seeds were sown in each 2 l pot, containing a mix of peat with 10 % soil. Total number of plants for each treatment was 120-140. The plants were grown in white light with a 14 h photoperiod and 60 % relative humidity. The minimum day/night temperatures were 25/18 °C. After five weeks the temperatures were changed to 20/15 °C.

Inoculation

Wheat heads were spray inoculated during anthesis (Zadoks growth stage 65), each head with approximately 0.7 ml conidial suspension. Approximately 12 – 14 pots with wheat plants were inoculated with each treatment. To lower the surface tension of the conidial suspensions, all inoculums contained 0.25 % gelatin. The heads were covered with plastic bags for four days to ensure a high humidity during the initial establishment and infection of the fungi. Plants were

inoculated with two different conidial concentrations (half dose; 0.5×10^5 and full dose 1×10^5 conidia/ml) of *F. avenaceum, F. culmorum,* and *F. graminearum,* respectively. Plants were also inoculated with pair-wise combination of the species (0.5×10^5 conidia/ml of each species). The wheat was also given a control treatment of SDW with 0.25 % gelatin. After inoculation, the pots were placed in a randomized manner. All treatments were done in three biologically independent replicates where the inoculums and plants for each repeat were prepared independently and separately in time. Four wheat heads were sampled from each treatment at time points 6, 10 and 14 days post inoculation (dpi), and stored at -80 °C until further use. Wheat heads were also sampled at the yellow ripe-stage (Zadoks growth stage 92) and stored in room temperature (RT).

3.1.1 Using DNA to quantify fungus in infected plant material

DNA extraction

Wheat sampled at the yellow ripe-stage were extracted and analyzed by H. Aamodt in the same procedure as described below (Unpublished data). The mean and standard deviation for each treatment was calculated by me, using equation I, where x is each sample value, $\bar{x}$ is the sample mean and n is the number of samples.

I
$$Standard\ deviation = \sqrt{\frac{\sum(x - \bar{x})^2}{n}}$$

DNA from wheat sampled on 6, 10 and 14 dpi were extracted and analyzed by me. For each DNA extraction, one seed was taken from each of the four different wheat heads of each treatment.

DNA extraction was conducted using Fast DNA® Spin Kit for Soil (MP Biomedicals, Santa Ana, California).

Cell lysis

1. To break the cell wall, seeds were ground to powder in liquid nitrogen. The powder was transferred to a Lysing Matrix E tube where 980 µl Sodium Phosphate Buffer together

with 122 µl MT Buffer were added. A FastPrep® instrument was used to homogenize the samples at speed setting 6.0 for 40 seconds.

2. Furthermore, the samples were centrifuged for 10 min to allow settling of pellets. This and all following centrifugation steps were run at 14 000 × g.

Protein removal

3. The supernatant was transferred to a new tube and 250 µl Protein Precipitation Solution was added. The proteins were removed by centrifugation for 5 min and the supernatant was transferred to a new tube.

Extraction

4. One ml Binding Matrix was added to the supernatant and the solution was inverted for 2 min to allow binding of DNA. The tube was thereafter placed in a rack for 3 min to allow settling of silica matrix.
5. A volume of 500 µl of the supernatant was discarded while 600 µl of the remaining liquid was transferred to a SPIN™ Filter and centrifuged for 1 min. The catch tube was emptied and the remaining mixture was added to the filter and centrifuged as before. The catch tube was emptied again.
6. The pellet was resuspended in 500 µl SEWS-M and thereafter centrifuged for 1 min. The catch tube was emptied and the centrifugation was repeated. The filter was air dried for 5 min at RT.

Elution

7. The dry pellet was resuspended in 100 µl DES (DNase/Pyrogen-Free Water).
8. To bring the eluted DNA into the clean catch tube, the filter was centrifuged for 1 min. The eluted DNA was stored at -20 °C until further use.

Fungal DNA were also extracted from isolates of the different fungal species, to be used in dilution series as a standard in quantitative PCR (qPCR). DNA from the species *F. avenaceum* and *F. graminearum* had previously been extracted (Lysøe, unpublished data). The extraction of DNA from *F. culmorum* was done by me. One fungal mother plate with synthetic nutrient agar (SNA) medium was cut into squares and transferred into 0.5 l synthetic nutrient broth (SNB) media. The media was incubated at 24 °C with 150 rpm for 3 days. To concentrate the mycelia

the media was vacuum filtrated. The mycelia was thereafter removed from the tract and kept at -20°C until DNA extraction by the DNeasy Plant Mini Kit (Qiagen®, Venlo, Netherlands).

Cell lysis

1. The fungal tissue was added liquid nitrogen and grinded to powder. Approximately 20 mg of the fungal powder was filled in a microcentrifuge tube. Thereafter 400 µl Buffer AP1 was added to the powder and the solution was vortexed vigorously. The homogenized solution was then incubated for 10 min at 65 °C and mixed occasionally.

Protein removal

2. To precipitate detergent, proteins and polysaccharides, the solution was added 130 µl Buffer AP2 and incubated 5 min on ice. This was followed by a 5 min centrifugation at 20 000 × g. The lysate was pipetted into a QIA shredder Mini spin column and centrifuged at 20 000 × g for 2 min.
3. The flow through was transferred into a new microcentrifuge tube and added 1.5 volumes Buffer AP3/E

Extraction

4. A volume of 650 µl of the solution was added to a DNeasy Mini column, followed by 1 min centrifugation at 6 000 × g. This step was repeated until the whole sample had been filtered through the column. The DNA was now bound to the column, and the flow through was discarded.
5. The column was washed two times by adding 500 µl Buffer AW; first time followed by centrifugation at 6000 × g for 1 min, second time, followed by centrifugation at 20 000 × g for two minutes to dry the membrane in the column.

Elution

6. The DNeasy spin column was transferred to a microcentrifuge tube and added 100 µl Buffer AE on the membrane, followed by 5 min incubation at RT. Thereafter the elution was conducted by centrifugation for 1 min at 6 000 × g.

The eluted DNA was stored at – 20 °C until further use.

Agarose gel electrophoresis

The quality of the extracted DNA was investigated by electrophoresis through a 0.8 % agarose gel.

Seakam LE agarose was melted in 1 × TBE buffer in a microwave oven. The liquid was cooled down to approximately 60 °C and one drop of 0.07 % ethidium bromide (EtBr) was added per 50 ml. The solution was poured into a prepared gel tray.

Samples:
1µl loading Buffer
4 µl distilled H_2O
1µl DNA
6 µl Total volume

Gels of 200 ml were run at 160 V, 100 ml gels at 65 V and 50 ml gels at 40 V. The runs were stopped when the samples were half way down the gel.

Gels were analyzed under UV light in a Molecular Imager Gel Doc XR system (Biorad Laboratories, Hercules, CA, USA)

qPCR

The amount of fungal DNA in the samples was quantified by the use of qPCR testing for *F. avenaceum*, *F. culmorum* and *F. graminearum*. To normalize the amount of extracted DNA in each sample, wheat DNA (*Cytochrome c oxidase* (*cox*)) was quantified in all the samples.

The extracted DNA samples were used to make 5 × and 50 × dilutions. The 5 × dilution was used to quantify the amount of fungal DNA, while the 50 × dilution was used to quantify plant DNA.

An *ep*Motion 5070 pipetting robot (Eppendorf, Hamburg, Germany) was used to pipette sufficient amounts of the reagents to achieve as correct volumes as possible. All samples were pipetted in technical duplicates.

The reagents were:

12.5 µl	TaqMan reaction mix
10.5 µl	Primer probe mix (0.71 pmol/µl Primers, 0.24 pmol/µl probe)
2 µl	DNA
25 µl	Total volume

Final concentrations of primer and probe were 0.3 pmol/µl and 0.1 pmol/µl respectively.

A complete list of primers and probes are given in Table 3, page 22. To make a standard curve for the plant DNA five dilutions were used (plant DNA (*cox*): 100 ng, 10 ng, 1 ng, 0.1ng and 0.01ng; fungal DNA: 1000 pg, 100 pg, 10 pg, 1.0 pg and 0.1 pg) and 2 µl dsH_2O was used as negative control for each primer set.

The 7900HT Fast Real-Time PCR System (Applied Biosystems®, California) was set in normal mode, at conditions described in Figure 7.

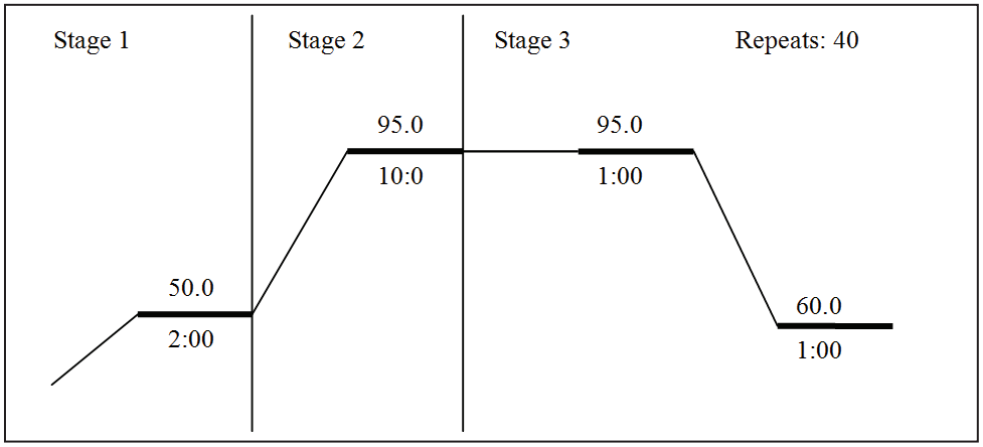

Figure 7. qPCR reaction conditions.

Data analysis

Data from the qPCR reactions were analyzed in Sequence Detection Software (SDS) version 2.2.1. The program calculated the amount of *Fusarium* and wheat DNA in the samples based on the standard curve. The means were calculated from each technical duplicate, and these values were used further. All samples having a quantification cycle (C_q) value of 36 or higher were considered as negative. Technical duplicates that ranged one C_q value or more from each other

were repeated in a new reaction. Standard deviation for the results from each treatment was calculated by use of equation I, page 26. The results were analyzed statistically in a two-ways unpaired t-test in Microsoft Excel 2007.

Mycotoxin analysis

The mycotoxin content in ground wheat sampled in the yellow ripe- stage was also examined for concentrations of DON, 3-ac-DON, NIV and enniatin B and B1. This was done by the National Veterinary Institute, Oslo, using gas chromatography–mass spectrometry (GC-MS) analysis. The data from this analysis were analyzed by me and standard deviation was calculated using formula I, page 26.

3.1.2 Expression of fungal genes *in planta*

Sample material

The previously described wheat samples from the greenhouse inoculation were used in this experiment. Only the samples inoculated with half amount *F. avenaceum*, half amount *F. graminearum* and the two species together from day 10 and 14 dpi were used. The harvesting of seeds for RNA extraction was done in the same manner as with the DNA extraction; one seed was taken from each of the four different wheat heads of each treatment.

RNA extraction

RNA was extracted using the Spectrum™ Plant Total RNA Kit (SIGMA-ALDRICH®, St. Louis, MO).

Cell lysis

1. Liquid nitrogen was used when grinding the plant tissue to powder and thereby breaking the cells. A volume of 500 µl lysis solution supplemented with 2-mercaptoethanol (1:10) was then added to the powder in a microcentrifuge tube. The mixture was vortexed for 30 seconds, incubated at RT for 5 min and thereafter

centrifuged for 5 min. This and all following centrifugation steps were run at 20 000 × g.

2. The supernatant was then filtered in a binding column to remove big particles. The filtered lysate was added 250 µl binding solution and the fluid was pipetted into a binding column and centrifuged 1 min. The nucleic acids including the RNA were now bound to the column.

DNase treatment

3. To remove DNA from the samples, an on-column DNase digestion was done. First, the RNA, bound to the column was washed by adding 300 µl Wash Solution 1 and centrifuged for 1 min. Thereafter 10 µl DNase I together with 70 µl DNase digestion buffer was added to the column. The samples were then incubated for 15 min in RT to digest the DNA.

Washing

4. A volume of 500 µl Wash Solution 1 was added to the column, it was centrifuged for 1 min and the residual liquid in the catch tube was removed. The previous step was repeated twice with Wash Solution 2 and centrifuged for 30 seconds both times. Thereafter, the column was dried by centrifugation for 1 min.

Elution

5. To elute the RNA, 50 µl Elution Solution was added to the column; it was incubated for 1 min and thereafter centrifuged for 1 min. If RNA gain was expected to be low (< 100 µg/µl), the elution stage was repeated, adding the elution solution already in the catch tube from the previous elution.

The RNA was then stored at -80°C until further use.

DNase treatment

To digest and remove all DNA still present in the RNA samples, a second DNase treatment was carried out. DNase treatments were repeated until no DNA was detected in qPCR. The kit used for DNase treatment was Turbo DNA free™ (Ambion®, Austin, Texas).

5 µl 10 x turbo DNase buffer and 1 µl turbo DNase was added to the eluted RNA in an e-tube. The tube was mixed gently and incubated at 37 °C in 20-30 min to start the digestion of DNA. After the incubation, DNase deactivation reagent was added, and incubated for 5 min, mixing occasionally. The tube was then centrifuged at 10 000 × g for 1.5 min and the RNA was transferred to a new tube.

qPCR test for DNA

To ensure that no DNA was left in the RNA samples after the DNase treatment, the samples were tested by qPCR. The conditions for the qPCR are described in Figure 7 (page 30). For samples still containing DNA, the DNase treatment was repeated until no samples ranged under 37 C_q in the qPCR.

Samples:

12.5 µl	TaqMan reaction mix
10.5 µl	Primer probe mix (0.7 pmol/µl Primers (*COX554- f + r*), 0.24 pmol/µl probe (*COX554-p*))
2 µl	DNA
25 µl	Total volume

Final concentration of primer and probe were 0.3 pmol/µl and 0.1 pmol/µl, respectively.

Investigation of RNA quality and quantity

The quality and quantity of RNA were examined by spectrophotometry using Agilent 2100 Bioanalyzer.

- A volume of 550 µl RNA 6000 gel matrix was centrifuged in a spin filter at 1500 × g for 10 min at RT. RNA 6000 Nano dye concentrate was equilibrated to RT for 30 min and 1µl of the dye was mixed with 65 µl filtered gel. The solution was centrifuged at 13 000 × g for 10 min.
- A RNA 6000 Nano chip was placed in the priming station, and 9.0 µl of the prepared gel-dye mix was pipetted in the well marked **(G)**. Thereafter the plunger was positioned at

1 ml, the priming station was closed and the plunger was pressed down until it was held by the clip. After 30 sec, the clip was released for 5 sec and thereafter pulled back to 1 ml position. The priming station was opened and 9 µl gel-dye mix was pipetted to the two wells marked G.

- A volume of 5 µl RNA 6000 Nano marker was added to all the 12 sample wells and the well marked ladder. One µl ladder was also added in its respective well.
- Sample wells were added 1 µl sample. The sample wells that had no sample were added 1 µl RNA 6000 Nano marker. The chip was removed from the priming station and placed in the IKA vortexer and vortexed for 1 min at 2400 rpm. The chip was then run in the Agilent 2100 bioanalyzer.

RNA quality and quantity of all samples were also examined in Nanodrop in two technical replicates.

cDNA synthesis

The RNA samples were analyzed by the reverse transcription quantitative polymerase chain reaction (RT-qPCR). First, a reverse transcriptase reaction was done to synthesize cDNA with the Superscript® VILO™ cDNA synthesis kit (Invitrogen™, Carlsbad, California). The amount of RNA added to the reaction was calculated so that the concentration of RNA was the same in all reactions (103 ng RNA/µl).

1. Samples were combined on ice:

4 µl	VILO™ Reaction Mix
2 µl	10X Superscript® Enzyme Mix
(14-X) µl	DEPC-treated water
X µl	RNA
20 µl	Total volume

Samples were placed in a PCR machine (Thermal cycler T100, Biorad®, serial-no.: 621BR041309). The PCR machine was programmed with the following condition steps:

2. 10 min at 25 °C
3. 60 min at 42 °C
4. The reaction was terminated by 5 min at 85 °C.

To investigate if the cDNA synthesis reactions were successful, the samples were run in an 1 % agarose gel electrophoresis. Gels were prepared as previously described. The samples not showing the expected smear on the gel were synthesized again, repeating the cDNA synthesis.

Samples:

1 µl	loading Buffer
3 µl	distilled H_2O
2 µl	cDNA
6 µl	Total volume

The cDNA samples were stored at -25°C until further use.

cDNA clean up

Accumulation of short fragment cDNA, inhibiting the RT-qPCR in one sample was attempted removed with Gene Elute ™ PCR Clean-Up (Sigma-Aldrich®, Saint Louis, Missouri).

- A GenElute plasmid mini spin column was added 0.5 ml of the Column Preparation Solution and centrifugated at 12 000 × g for 30 seconds. The eluate was discarded.
- The cDNA (approx. 20 µl) was added 100 µl Binding solution, mixed and transferred to the binding column. The column was centrifuged at maximum speed for one min and the eluate was discarded.
- The column was replaced into the collection tube and 0.5 ml diluted wash solution was added to the column. This was followed by a centrifugation of the column at 12 000 × g for 1 min. The eluate was discarded.
- The centrifugation was repeated, only this time for 2 min and without addition of wash solution.
- The collection tube was replaced by a new one and 50 µl Elution solution was added to the column. The column was incubated at RT for 1 min.
- To elute the cDNA the column was centrifuged for 1 minute at maximum speed. The cDNA was now present in the collection tube.

The cDNA was stored at -20 °C until further use.

RT-qPCR assay design

Normalization for the amount of RNA in each sample was done by including two housekeeping genes (HKG) for each fungal species tested. One of the HKGs was encoding Ubiquitin conjugating enzyme (UBC*).* Alternative primer pairs for these genes had been constructed (Klemsdal, unpublished data) (A complete list over primer names and sequences is given in Table 4, page 22). To decide which primer pair was the best, the primers specificity was tested by using all *F. avenaceum, F. graminearum, F. culmorum* and wheat as template. Efficiency were tested in 10 × dilution series of *F. graminearum* and *F. avenaceum* genomic DNA and cDNA in qPCR. The primer efficiency was calculated by equation II (Adams 2006), the slope of the standard curve had previously been calculated by the SDS 2.0 program. One primer pair for each species was proven adequate, and thus further used in the RT-qPCR.

II $$Efficiency\,\% = 10^{\frac{-1}{slope}} - 1 \cdot 100$$ (Adams 2006)

Two additional primer pairs that previously had been tested for each species were also used in the assay. These pairs were targeting the HKG *β-tubulin* (Anstensrud, unpublished data).

The primers used for *F. avenaceum* was *FavenUBC f1+ r3* targeting UBC and *FavenBTUB f1+ r1* targeting *β-tubulin*. For *F. graminearum* it was *FgraUBC f1+ r1* targeting UBC and *FgcBTUB f1+r1* targeting *β-tubulin.*

Two genes were chosen as target genes, both involved in toxin synthesis; *Enniatin synthetase* in *F. avenaceum* and *Tri1* in *F. graminearum*. Efficiency and specificity for these primers had previously been tested (Anstensrud, unpublished data).

Reverse Transcription - qPCR

The RT-qPCR assay was used to analyze for the expression of the two genes involved in mycotoxin synthesis *Esyn* and *Tri1* (primer pairs in the same order: *Esyn F1+ R1* and *FGTri1SK f + r*). A set of HKGs for each species was also included to normalize for the amount of RNA in the samples. The HKGs were UBC and *β-tubulin.*

All samples tested with the same primer set were run on the same plate and at least one test of HKG was run on each plate. There were also two wells for each primer pair on each plate without cDNA but with only dsH_2O to function as a negative control. All samples were in

technical duplicates on the plate. The qPCR was run in normal mode, with the conditions described in Figure 8.

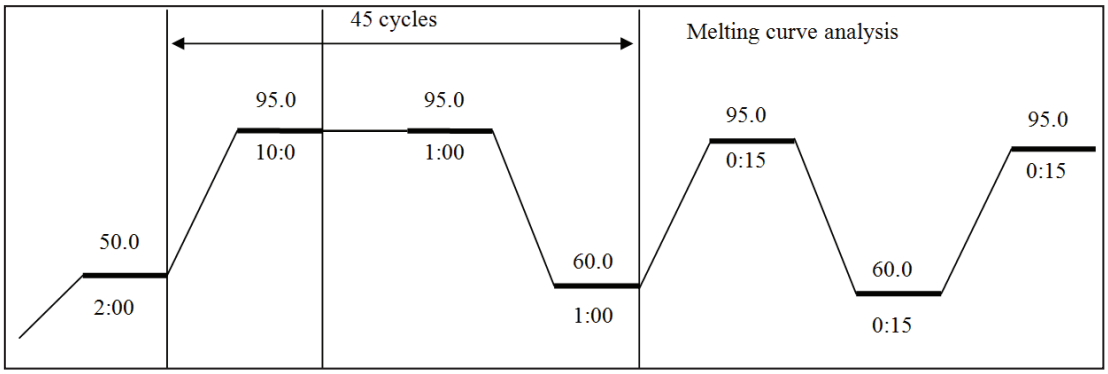

Figure 8. Conditions in RT-qPCR with melting curve added.

Samples:

10 µl	2 × SYBR reaction mix
1.3 µl	Forward primer (3 pmol/µl)
1.3 µl	Reverse primer (3 pmol/µl)
5.4 µl	dsH_2O
2 µl	cDNA
20 µl	Total volume

Final primer concentration was 2.0 pmol/µl.

Data analysis

Data from the RT-qPCR reaction were analyzed in SDS 2.0. Technical duplicates that ranged one C_q value or more from each other were repeated in a new reaction. The mean of the technical duplicates were calculated and used further in the calculations.

To determine the stability of the HKGs the excel-based program named BestKeeper (version 1, (Pfaffl et al. 2004)) was used. The program does a pairwise correlation analysis of the HKG pairs to determine the expression stability, which makes it possible to see if they are suitable to use as standardization for the amount of RNA in each sample (Pfaffl et al. 2004). The program also calculates a weighted expression index for each sample template of the non-regulated control

genes. These values were used as C_q-values in relative expression software tool (REST©, version 2) to calculate relative expression values (R-value) of the target genes. The program calculates R based on efficiency in the PCR reaction (E) and the crossing point (C_q) difference (Δ) of the target sample, versus control according to equation III (Pfaffl et al. 2002). One sample from the earliest time-point (10 dpi) when the fungi was growing alone was used as reference sample.

III

$$R = \frac{E_{target}^{\Delta C_q(control- \ sample)}}{E_{reference}^{\Delta C_q(control- \ sample)}}$$ (Pfaffl et al. 2002)

Standard deviation of each treatment was calculated by use of equation I, page 26. The results were analyzed statistically in a two-ways unpaired t-test in Microsoft Office Excel 2007.

3.2 Fungal material and preparation of inoculums

The interaction between *F. avenaceum* and *F. graminearum* was investigated by using *TRI5*prom::*GFP* reporter mutant of *F. graminearum,* previously described by Ilgen and co-workers (2009). The *TRI5*prom::*GFP* strain is constitutively expressing the DsRed gene under the control of the glycerol-3-phosphate dehydrogenase (*gpd*) promoter of *Aspergillus nidulans*. The constitutive expression of DsRed makes it possible to differentiate the *F. graminearum* mycelia from *F. avenaceum*. The *Gfp* is fused to the endogenous promoter of *Tri5* encoding the key enzyme trichodiene synthase. This enzyme catalyzes the first step in the trichothecene biosynthetic pathway, resulting in the production of the mycotoxins NIV and DON (Marin et al. 2010).

Conidia for the following experiments were produced by cutting squares of agar from fungal SNA mother plates and inoculating them in 0.5 l SNB media at 24 °C with 150 rpm for 3 days. The species used were *F. avenaceum* (6A)*, F. langsethiae* wild type (WT) (9821-16-1 (IBT9951)), *F. langsethiae* GFP (9821-16-1 (IBT9951)), *F. graminearum* WT (140/08) and *F. graminearum TRI5*prom::*GFP* (Fg8/1). The SNB media with *F. graminearum* TRI5prom::*GFP* inoculated was also added 30 µl/mg hygromycin. Each suspension was filtered through four layers of sterile cheese cloth to remove mycelia. To concentrate the conidia, they were centrifuged at 1811 × g for 35 min at 10 °C in 50 ml centrifuge tubes. The concentrated conidia were washed two times (three for *F. graminearum TRI5*prom::*GFP*) by repeatedly filling the

centrifuge tube with SDW and centrifuging 1811 × g for 35 min at 10 °C. Conidia concentrations were examined using a Bürker Hemocytometer. Suspensions were kept at 4 °C until further use.

3.2.1 *In vitro* co-inoculation

Conidial suspensions of *F. avenaceum* and *F. graminearum TRI5*prom::*GFP* were diluted to 10^6 conidia/ml from the previously described stock suspensions and combinations of the two species, 100 µl *F. avenaceum* in addition to 100 µl *F. graminearum TRI5*prom::*GFP*, were spread out on petri dishes containing SNA media in three replicates. In addition one control sample was made for each species, containing 200 µl *F. avenaceum* or *F. graminearum TRI5*prom::*GFP*.

The fungal growth and induction of *Gfp* by the *Tri5* promoter was investigated in a confocal laser scanning microscope (CLSM) in time lapse series, scanning every third hour. All the microscopy was done using a 40 × dry objective. The non-fluorescent fungi were detected with bright field (BF) microscopy. The DsRed fluorescence was excited at 561 nm wave length and emission was detected in the interval from 588 to 650 nm wavelength. The GFP was excited at 488 nm wavelength with an Argon laser at 20 % and emission detected in the range of 500-540 nm.

3.2.2 Histological characterization of *F. langsethiae* infection in wheat

Plant material

A new greenhouse experiment was set up by Hege Divon to provide infected wheat for the inoculation of *F. langsethiae*. Five seeds were sown in each 1.5 l pot, containing a mix of peat with 10 % soil. The plants were grown in white light with a 16 h photoperiod and 50 % relative humidity. The day/night temperatures were 25/18 °C at sowing, and within the first month the temperatures were gradually changed to 18/14 °C and 75 % relative humidity.

Inoculation

Inoculation and the following sampling and analyses were done by me. Conidial stock suspensions of *F. langsethiae* were diluted with SDW containing 0.1 % Tween 20 to a working concentration of 10^6 conidia/ml. A control suspension was also made, containing only SDW and 0.1 % Tween 20.

Wheat heads were sprayed with approximately 4.7 ml conidial suspension of the different treatments (*F. langsethiae* and control) about one week after anthesis (Zadoks growth stage 69-77), and heads were covered with transparent plastic bags for 6 days to keep the relative humidity high.

Sampling and sample preparation for Scanning Electron Microscopy (SEM)

Wheat heads were harvested at 6 and 14 dpi. Spikelets were separated from the head, cut in pieces and fixated in 4 % paraformaldehyde.

To prepare the sample material for SEM, it was dehydrated in a series of ethanol concentrations. First 30, 50 and 70 % ethanol in PBS buffer each step lasting 30 min, then 2 x 3 min, each in 80, 90 and 96 % ethanol, thereafter 2 x 5 min in 100 % ethanol. The drying procedure was then terminated by critical point drying (CPD) to exchange ethanol to CO_2. Afterwards, the samples were mounted on stubs and coated with gold-palladium in the sputter coater before visualizing in SEM operating with 25 kV.

3.2.3 Detached leaf assay (DLA)

DLA was conducted based on the techniques of Imathiu and co-workers (2009).

The fungal isolates used were *F. langsethiae* WT, *F. langsethiae* GFP and *F. graminearum*. Wheat (*T. aestivum* cv. Zebra) was grown 14 days in natural light at RT. The second leaf (L2) on the main stem was harvested and cut into pieces, approximately 4 cm long.

Petri dishes containing 0.5 % water agar (WA) containing sterile filtered kinetin (10 mg/l) were prepared. Kinetin (30 mg) was dissolved in 300 µl 1M NaOH, and was diluted to 2 ml with 96 % ethanol to maintain solubility. The solution was filter sterilized and added to 3 l WA-medium after autoclaving. To prevent pH from rising, 300 µl 1M HCl was added to the medium. pH in the final solution was 7.0.

The leaf pieces were placed on the petri dishes with the abaxial side down touching the agar and 10 µl or 5 µl conidial suspensions were added to the middle of the non-wounded or wounded leaf, respectively. Each fungal isolate was inoculated on 10 non-wounded and 10 wounded leaves. Wounding of the leaves were done by piercing a pipette tip (1 mm) in the middle of the

leaf. In addition a negative control suspension containing 10 µl dsH_2O was made, inoculating 3 wounded and 3 non-wounded leaves.

The petri dishes were incubated at RT in a sealed box containing moist filter paper to ensure high humidity.

The leaves were investigated at 3 and 7 dpi. At 7 dpi, the lesion sizes were measured, and standard deviation was calculated by equation I (page 26). Similarity in lesion length of *F. langsethiae* WT and *F. langsethiae* GFP was tested statistically by calculating a two-ways unpaired t-test in Microsoft Office Excel 2007. Leaves infected with *F. langsethiae* GFP were fixated in 4 % paraformaldehyde for later investigation of fungal growth in CLSM with 20 × and 40 × dry objective. The GFP was excited at 480 nm wavelength with an Argon laser at 20 % emission and detected in the 500-540 nm range.

Chapter 4 Results

4.1 Greenhouse inoculation studies of *Fusarium* spp. in wheat

To investigate the effect different species of *Fusarium* might have on each other during wheat colonization; wheat was inoculated with one, or combinations of different *Fusarium* species (*F. avenaceum, F. culmorum* and *F. graminearum*). Both DNA and RNA were extracted to investigate the amount of fungus and the genes expressed in the different species of *Fusarium* when they grew alone and together with another species.

4.1.1 Using DNA to quantify fungus in infected wheat

DNA was extracted from infected wheat heads harvested at 6, 10 and 14 dpi. The DNA quality was evaluated with agarose gel electrophoresis (Figure 9).

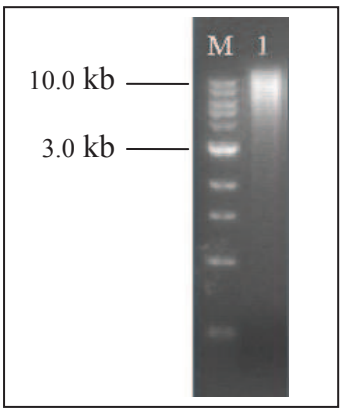

Figure 9. Examples of genomic DNA isolated from *Fusarium* infected wheat (lane 1). The quality was considered sufficient for further examination in qPCR. M is a 1 kb DNA ladder used as molecular marker.

The amount of each *Fusarium* species was quantified by qPCR at 6, 10 and 14 dpi. The primer pairs used (one pair for each *Fusarium* species and for wheat) had previously been designed and optimized by others (Table 3, page 22). The assay was designed to quantify the amounts of *Fusarium* in the wheat samples using the standard curve method.

Examples of standard curves and amplification plots from qPCR are given in Figure 10. The standard curve of the primer pairs had the following slopes; *F. avenaceum* assay: -4.04 slope, 0.99 R^2; *F. culmorum* assay: -3.64 slope, 0.99 R^2; *F. graminearum* assay: -3.53 slope, 0.99 R^2 and wheat COX assay: -3.43 slope, 0.99 R^2 (all standard curves with a R^2 value close to 1 indicating a linear slope). The amount of plant (based on the *cox* gene) and *Fusarium* DNA was normally in the range of the standard curve (fungi: 0.1 - 1000 pg, plant: 0.01 ng - 100 ng).

F. culmorum was often found in higher amounts than 10 ng DNA per reaction. To exclude the possibility of inhibition of the qPCR due to inhibitory substances at amounts of DNA greater than 10 ng from the wheat, a sixth point of 100 ng DNA was added to the standard curve. The resulting standard curve was linear (0.99 R^2), indicating that there was no inhibition.

All samples were tested with specific primers for all the *Fusarium* species included in the greenhouse experiment. This was done as a precaution to test if samples were contaminated with other species of *Fusarium* than those that were intended in that specific sample. For example; a sample infected only with *F. avenaceum* spores was tested for both *F. graminearum* and *F. culmorum* in addition to *F. avenaceum*.

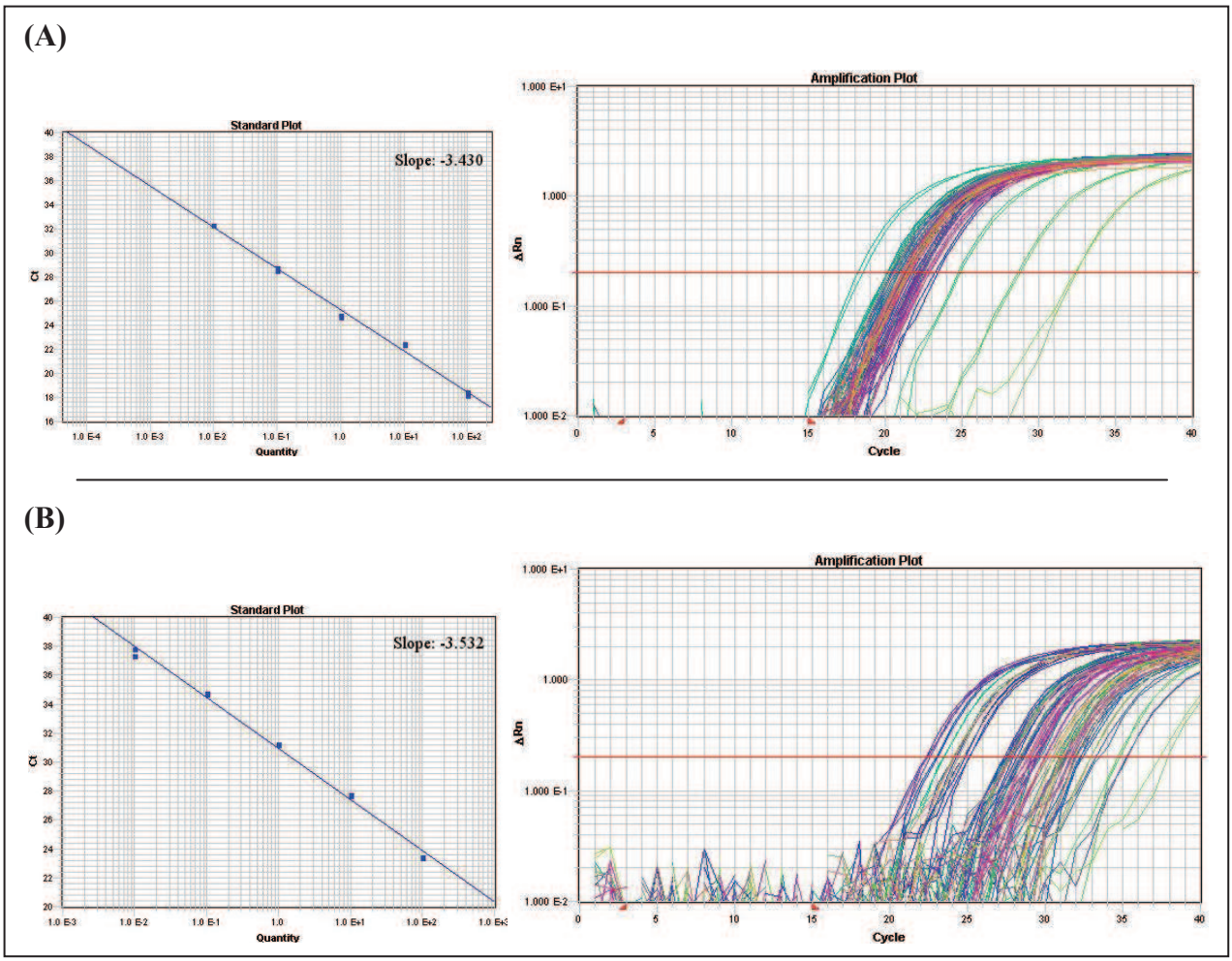

Figure 10. Examples of standard curve and amplification plot for (A) *cox* primer pair and probe (*COX554-f, r* and *p*) used for the quantification of wheat DNA, and (B) *F. graminearum* primers and probe (*Fgtub-f, r* and *p*) (B). In the standard curve quantity i.e. log(pgDNA) (x-axis) is plotted against Ct (C_q) value (y-axis). In the amplification plot cycle number (x-axis) is plotted against fluorescence signal (ΔRn) (y-axis).

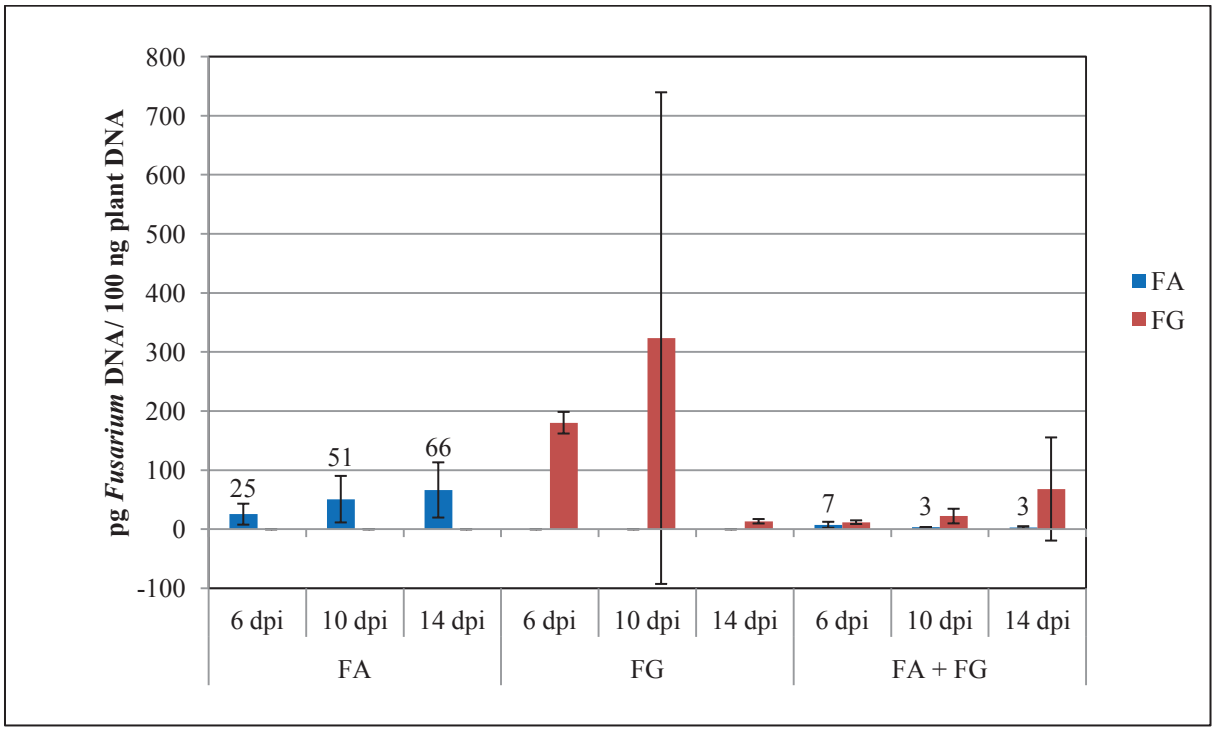

Figure 11. Amount of fungal DNA when *F. avenaceum* and *F. graminearum* grew alone and together, quantified with qPCR. There were three biological replicates at the time points 10 and 14 dpi, and two at 6 dpi. Amount of *F. avenaceum* is also indicated by the numbers over the bars. The arrows represent standard deviation calculated by equation I, page 26. (FG = *F. graminearum*, FA = *F. avenaceum*)

Both fungal and plant DNA were quantified in the samples of extracted DNA. The amounts of fungal DNA indicate the amount of fungus in the samples. The plant DNA was quantified to function as a normalization factor for the total concentration of DNA in each sample.

The amount of fungal DNA when two species, *F. avenaceum* and *F. graminearum,* were growing together and alone (inoculated with half dose, 0.5×10^5 conidia/ml) are given in Figure 11. Results from full dose inoculation are given in appendix. Each sample is the mean of two technical replicates in qPCR, and three biological replicates (mean of 6 numbers) except at 6 dpi where only two biological replicates were available. Even though the variation between the replicates is high, the amount of fungal DNA of both species is smaller when growing together than alone. When *F. avenaceum* was growing together with *F. graminearum*, the amount of fungal DNA from 6 to 10 dpi was decreasing. For *F. graminearum* the opposite trend was apparent; the fungal DNA was increasing from 6-14 dpi in co-inoculation samples. However, none of these differences in DNA quantities were proven significant when tested in an unpaired two-ways t-test in Microsoft Office Excel 2007.

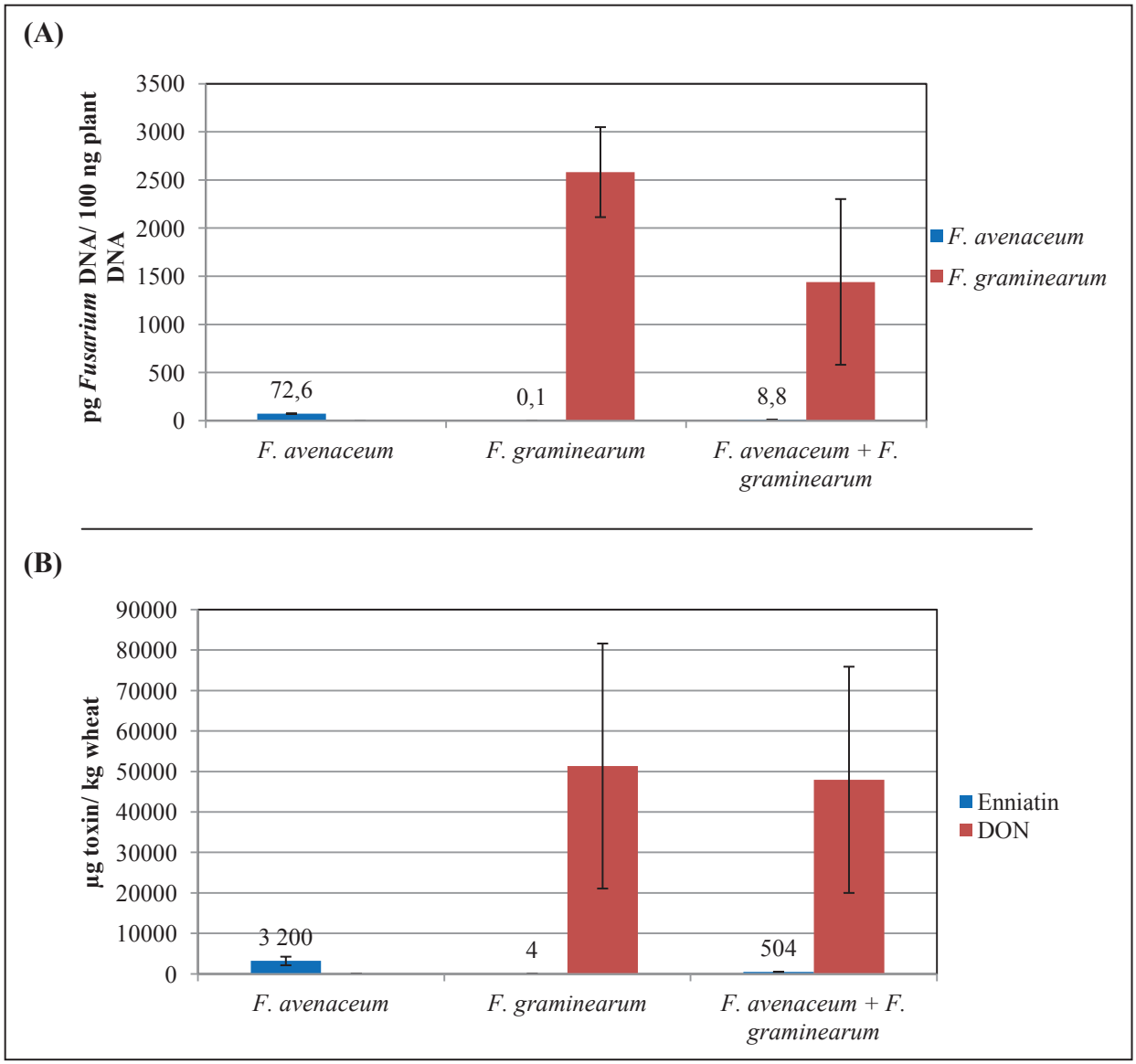

Figure 12. Quantification of *Fusarium* and mycotoxin amount with qPCR and GC-MS, respectively, in wheat at the ripening stage, approximately 50 dpi. (A) Amount of fungal DNA, and (B) toxins in wheat samples at the ripening stage. Each DNA and toxin value is the mean of three biological replicates with error bars indicating standard deviation. Under anthesis the wheat was inoculated with either *F. avenaceum* or *F. graminearum* alone or a combination of the two species.

In addition to the early time points, it was interesting to see the outcome of fungal DNA from co-inoculated wheat at the ripening stage (Zadoks growth stage 90, 30-40 days after anthesis). Wheat samples from the greenhouse experiment earlier described inoculated with *F. graminearum* and *F. avenaceum*, alone or in combination, were tested for amount of fungal DNA at growth stage Z 90. DNA values as quantified by qPCR for these samples are given in Figure 12A. All DNA values are a result of three biological replicates, each with technical duplicates (total mean of six values). The amount of both *Fusarium* species decreased when they

grew together, although the amount of *F. avenaceum* is much less than *F. graminearum* under both circumstances.

Fusarium can produce different amounts of mycotoxins under different circumstances. To see if the mycotoxin content was different when *F. avenaceum* and *F. graminearum* grew together compared to when growing alone, the amount of mycotoxins in the infected samples were analyzed, enniatins and DON, from *F. avenaceum* and *F. graminearum*, respectively (Figure 12B). The mycotoxin analysis was done on the same wheat samples that were quantified for fungal DNA. The amount of enniatins in the wheat decreased when *F. avenaceum* grew together with *F. graminearum*. On the other hand the amount of DON was to a certain extent stable in both treatments with *F. graminearum*.

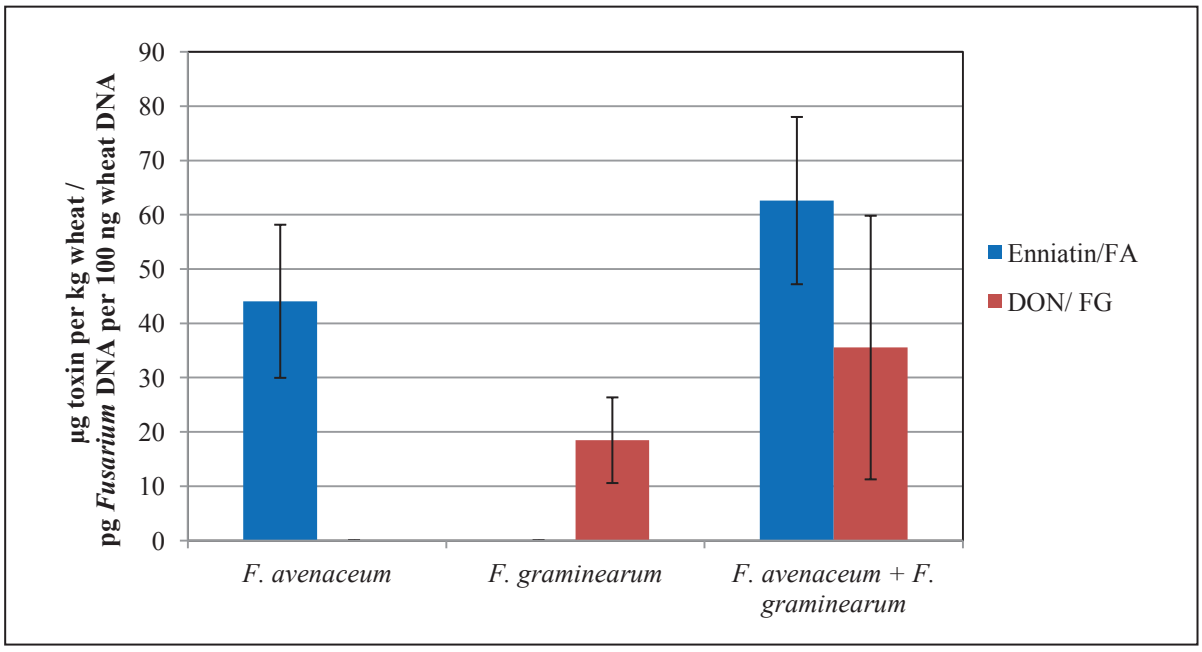

Figure 13. Amount of toxin per unit *Fusarium* DNA from wheat sampled at the ripening stage, approximately 50 dpi. (FA = *F.* avenaceum, FG = *F. graminearum*). Each bar is µg toxin/kg wheat divided on the normalized *Fusarium* DNA (shown in Figure 12A) which is the mean of three biological replicates. Error bars indicate standard deviation.

From Figure 12 it is apparent that both the amount of *Fusarium* and mycotoxin is decreasing when *F. graminearum* and *F. avenaceum* grow together. To validate whether the decrease in mycotoxin amount is proportional to the decrease of fungus, the amount of toxin produced per unit *Fusarium* was calculated (Figure 13). Interestingly, the production of toxin is increasing when the two *Fusarium* species are growing together. Some enniatin and *F. avenaceum* DNA was registered in the wheat infected with *F. graminearum* alone but the amounts were extremely small compared to that of *F. graminearum* (Figure 12).

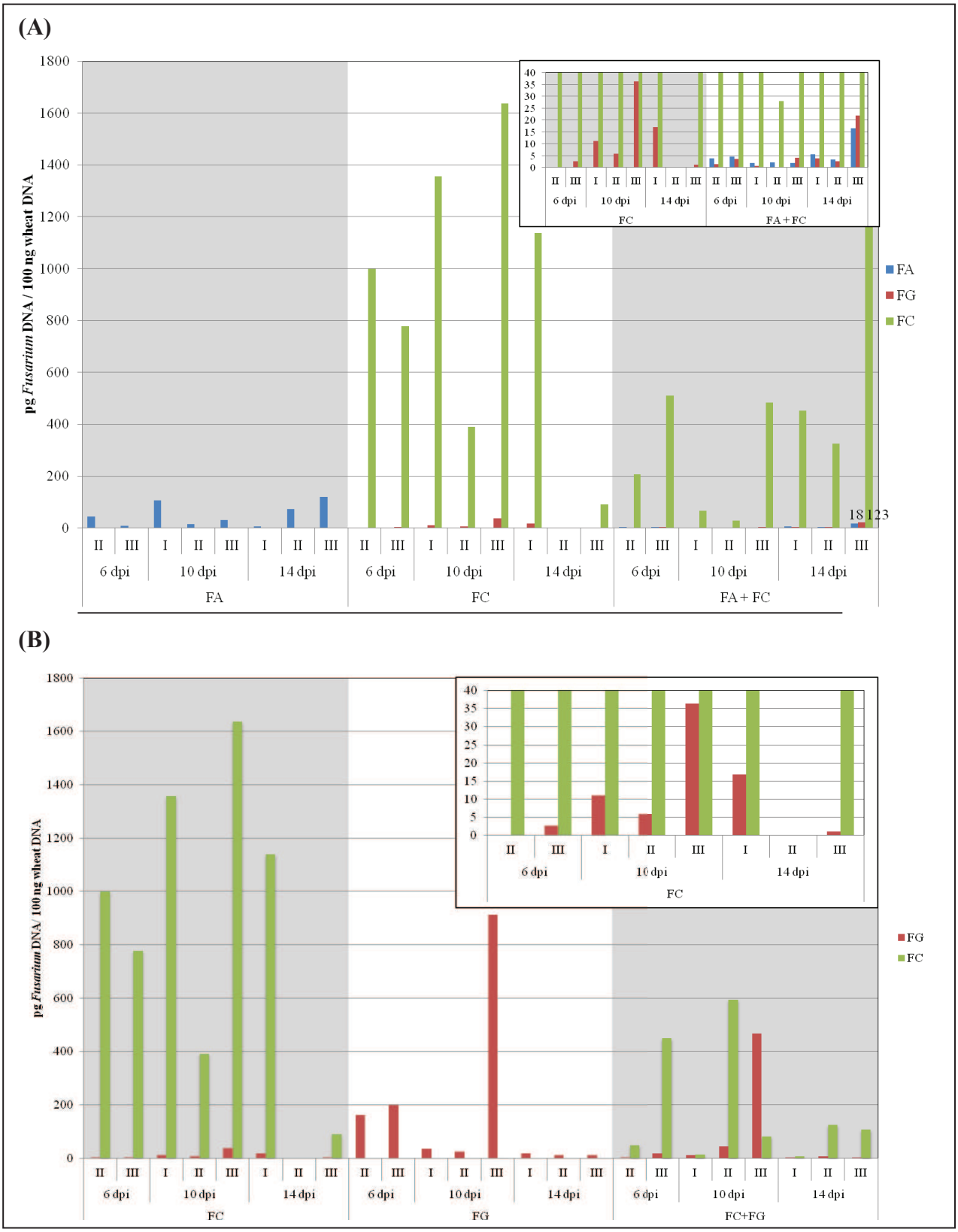

Figure 14. Examination of *Fusarium* DNA in spray inoculated wheat ears at 6, 10 and 14 dpi using qPCR. (A) *F. avenaceum* and *F. culmorum* growing alone or together. (B) *F. culmorum* and *F. graminearum* growing alone or together. The experiment was carried out in three biological repeats, given by the roman numerals. (FA = *F. avenaceum*, FC = *F. culmorum*, FG = *F. graminearum*).

Samples inoculated with *F. culmorum* alone (inoculated with half dose0.5 × 10^5 conidia/ml) or in combinations with either *F. avenaceum* or *F. graminearum* at 6, 10 and 14 dpi is shown in Figure 14. Full dose inoculations of the species are given in appendix. There are three biological replicates of each sample (given in roman numerals) except at day 6 when there was only two. Because of the relatively high variance between the biological replicates they are shown separately. In almost all samples inoculated with only *F. culmorum*, *F. graminearum* was also detected in relatively high amounts. This contamination by *F. graminearum* makes it impossible to know the amount of conidia in the *F. culmorum* inoculums, thus these samples were not analyzed further.

4.1.2 Expression of fungal genes involved in mycotoxin production

When to species grow on the same place, utilizing the same resources, they may have an effect on each other. The toxin production may be affected, either positively or negatively. To investigate this, two mycotoxin related genes were quantified from fungi *in planta*. The relative expression of the two toxin genes (*Tri1* and *Esyn* in *F. graminearum* and *F. avenaceum*, respectively) was quantified from infected wheat samples at 10 and 14 dpi.

Examination of quantity and quality of RNA

The quantities and qualities of extracted RNA were analyzed by Agilent 2100 Bioanalyzer. Examples of electropherograms are given in Figure 15. The RNA content in the samples ranged from 103-882 ng/µl, which was considered high enough for synthezising cDNA by reverse transcription. From one of the samples it was not possible to extract good quality RNA, probably due to degradation of the RNA. The extraction was done three times and still no RNA was extracted, therefore the sample was discarded. The two peaks of fluorescence (FU) at 40-50 seconds (s) are mainly plant 18S and 25S ribosomal RNA (rRNA) (Hepburn & Ingle 1976) (Figure 15). Since the amount of plant will be much greater than the amount of *Fusarium* in each sample, it is hard to say if the *Fusarium* rRNA is visible in the electropherograms. As fungi have 28S rRNA instead of 25S it is possible that the small peak at 50 s is fungal 28S rRNA (Deacon 2006). The peak of fluorescence at 22 s is the 50 bp DNA lower marker used to compare all samples. The low molecular weight particles (shown at approximately 25-30 s) are most likely micro RNA (miRNA), small RNA particles rather than degraded RNA.

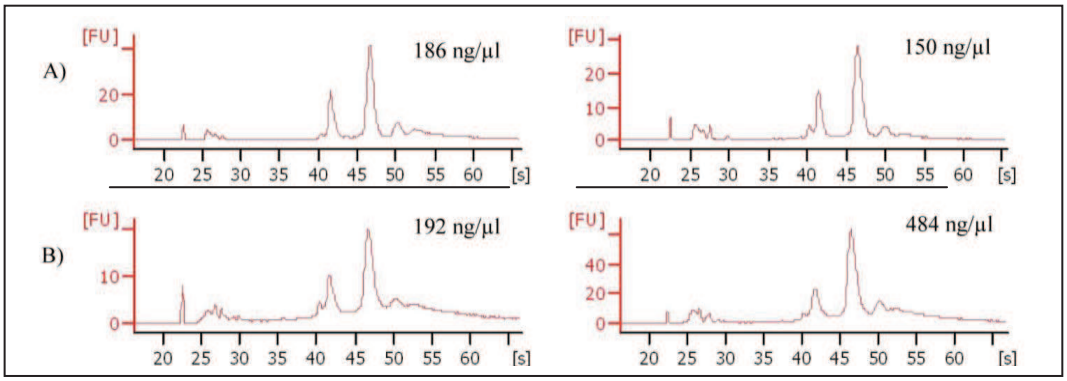

Figure 15. Examples of electropherograms of different fungal and plant RNA-samples. RNA concentration is given for each sample. (A) Samples from 10 dpi. (B) Samples from 14 dpi. The fluorescence (FU) (y-axis) is plotted against seconds (s) (x-axis).

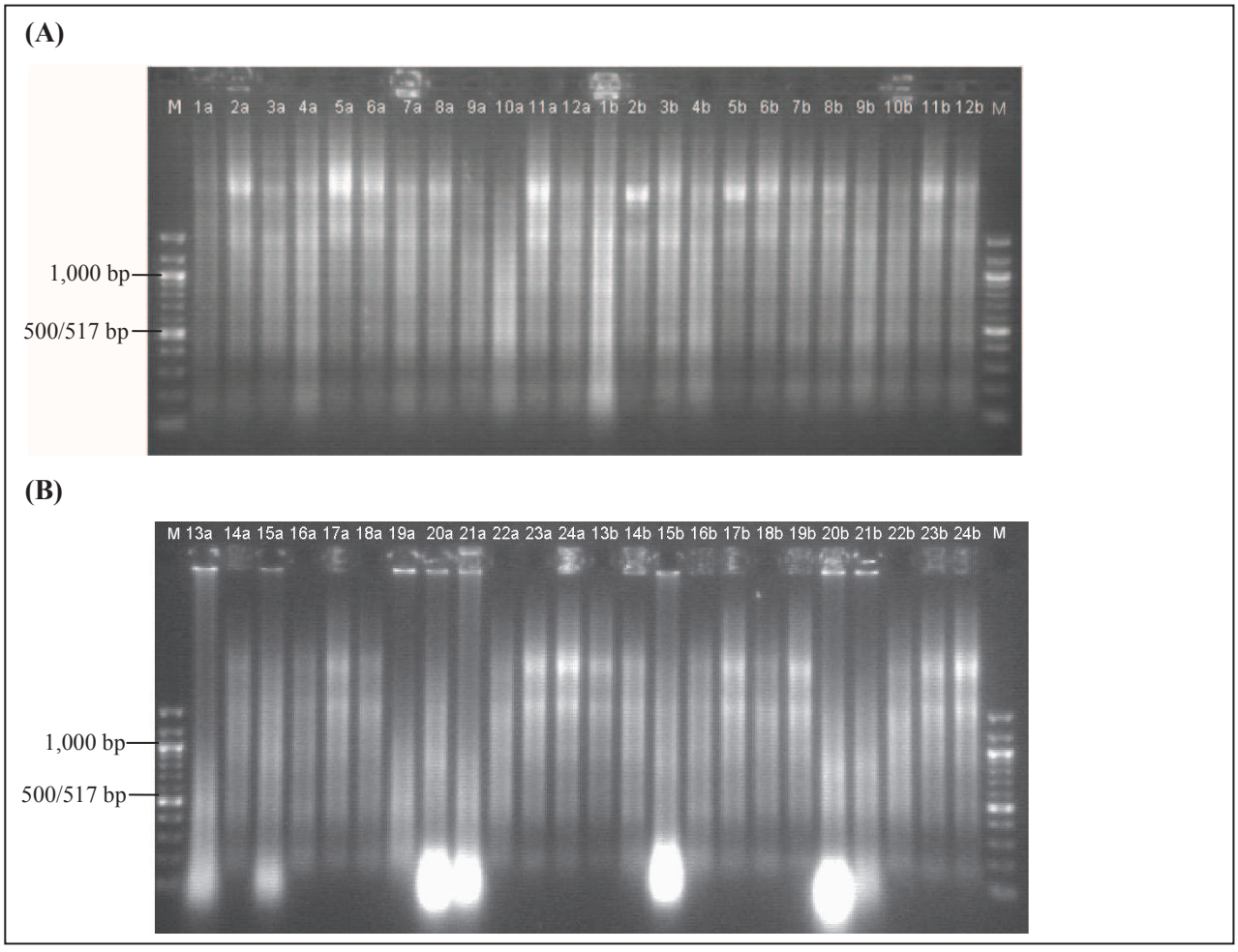

Figure 16. Agarose gel electrophoresis of cDNA reverse transcribed from (A) 10 dpi RNA and (B) 14 dpi RNA samples. Both gels contain 3 µl cDNA from different samples in all numbered wells (1-24) and a 100 bp ladder in well M. The letters a and b corresponds to two separate cDNA synthesis reactions of the same sample. These were pooled before qPCR. Some accumulation of small fragment RNA in well 13 a; 15a and b; 20 a and b; 21 a and b.

A reverse transcription reaction was conducted to synthesize cDNA from the RNA samples. The cDNA sample quality was examined on agarose gel shown in Figure 16. As expected for cDNA the synthesized bands were evenly distributed over the gel for most of the samples, with exception of sample 13, 15, 20 and 21. These samples had an accumulation of small fragment size cDNA in the bottom of the gel. When tested in qPCR (*FavenUBCf1 + r3*) these samples did not give any detectable signal. Upon new extraction and cDNA synthesis one of the samples still had an accumulation of small fragment size cDNA (Figure 17) and almost no distribution of bigger fragment size cDNA in the gel. Even after cDNA purification, there was no detectable signal in qPCR, so the sample was discarded.

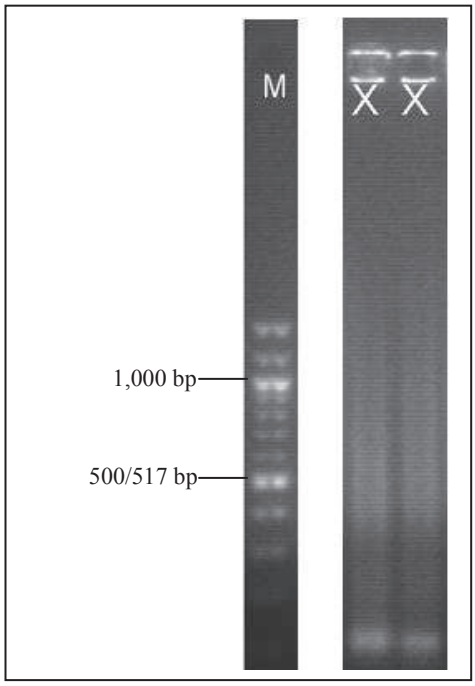

Figure 17. Accumulation of small fragment size cDNA (X) after second cDNA synthesis and a 100 bp ladder (M).

Gene expression analyzes by qPCR

Gene expression in *F. avenaceum* and *F. graminearum* growing alone or in combinations on wheat was analyzed. The expression of two genes involved in toxin production genes *Esyn* (enniatin in *F.* avenaceum) and *Tri1* (in the DON pathway in *F. graminearum*) were quantified to investigate if the presence of another *Fusarium* species had an effect on the expression. Two HKG encoding β-tubulin (BTUB) and UBC were used for normalization of the expression.

Three primer pair-candidates were tested for UBC in *F. avenaceum* (*FavenUBC f2 + r1, FavenUBC f1+r2, FavenUBC f1+r3*), while one pair was tested for UBC in *F. graminearum* (*FgraUBCf1+r1*). For a complete list of tested primers see Table 4, page 22.

The primers were tested in qPCR using DNA and cDNA as a template. The primer specificity of three of the chosen primers was satisfying (Table 8). They showed high specificity by binding to the target DNA, and not to other sequences. From the specificity tests the dissociation curves for all primer pairs show a single peak, indicating that the primers, with one exception, did not create primer-dimers or other unspecific binding Figure 18. The exception was *FgraUBCf1+r1* which also bound to *F. culmorum* in addition to the target sequence in *F. graminearum*. The efficiency for each primer pair was tested with amplification of cDNA in five 10-times dilution series and calculated by equation I (see RT-qPCR assay design p. 36). The primer pairs with the best efficiencies were *FgraUBCf1 +r1* (96%) and *FavenUBCf1 +r3* (96 %) (Table 9). Despite the low specificity of *FgraUBCf1+r1*, the primer pair was used further due to high efficiency, and the fact that the samples investigated did not contain *F. culmorum.* All other primer candidates were discarded and these two primer pairs were used further in the assay.

Table 8. Specificity of primer pairs tested in qPCR. (+) indicates binding to the DNA sequence, (-) indicates no binding.

	FavenUBCf2+ r1	***FavenUBCf1+r2***	***FavenUBCf1+r3***	***FgraUBCf1+r1***
F. avenaceum	-	+	+	-
F. graminearum	-	-	-	+
F. culmorum	-	-	-	+
T. aestivum	-	-	-	-

Table 9. Slope and R^2 of primer pairs after testing on cDNA in qPCR. Efficiency (%) is calculated by equation I (see RT-qPCR assay design, p. 36)

Primer pair	**R^2**	**Slope**	**Efficiency %**
FavenUBCf1+r2	0.99	-3.62	89
FavenUBCf1+r3	0.99	-3.43	96
FgraUBCf1+r1	0.99	-3.43	96

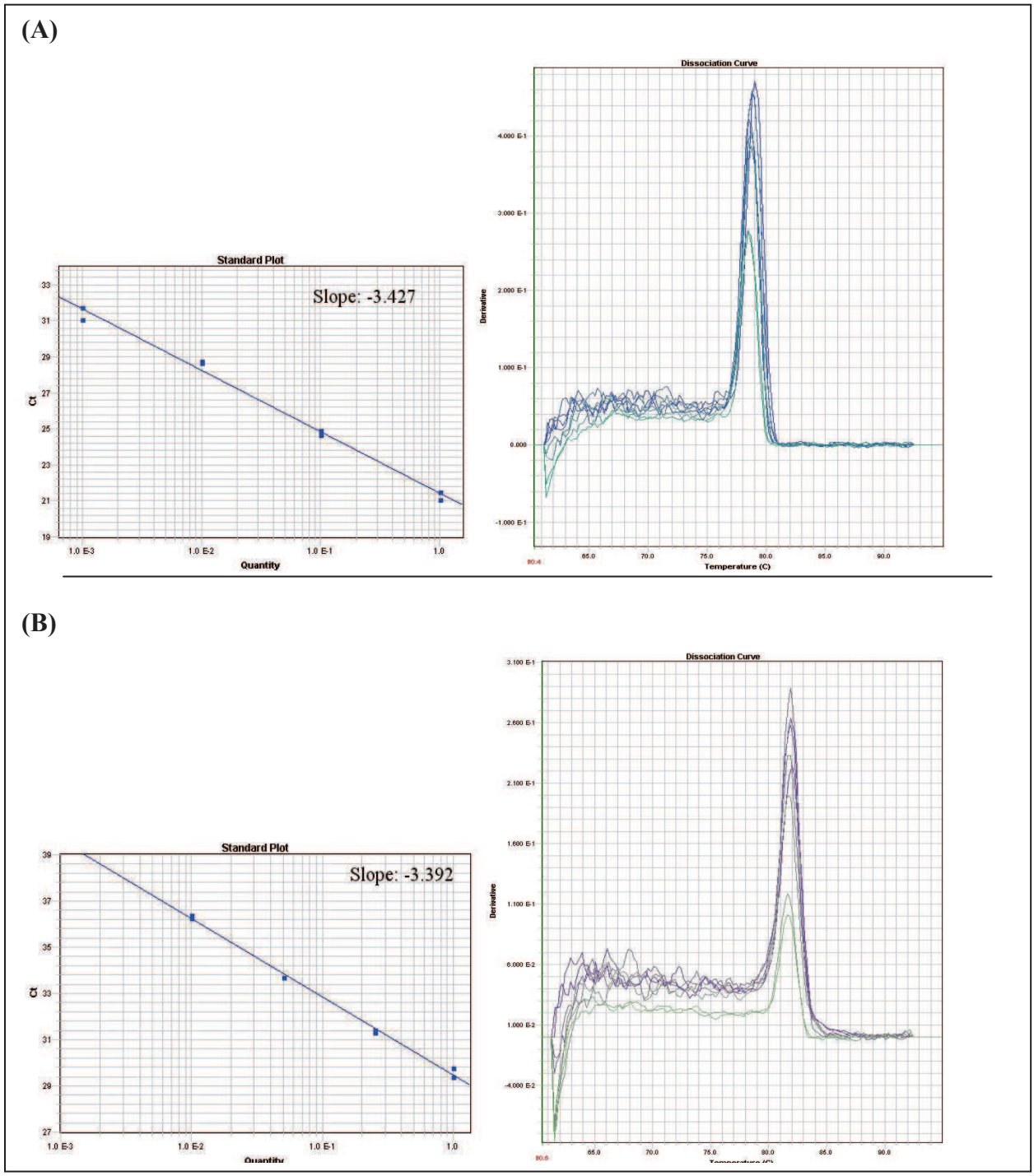

Figure 18. Standard curve and dissociation curve for HKG primer pairs for (A) *F. avenaceum UBC* (*FavenUBCf1 +r3*) and (B) *F. graminearum UBC* (*FgraUBCf1 +r1*). In the standard curve cDNA quantity (x-axis) is plotted against C_t (C_q) value (y-axis). In the dissociation curve temperature (x-axis) is plotted against derivative (the rate of change in fluorescence as a function of time) (y-axis).

The remaining primers used in the assay had previously been designed and optimized by others (Christin Anstensrud, unpublished data). Primer sequences and efficies are given in Table 4, page 22.

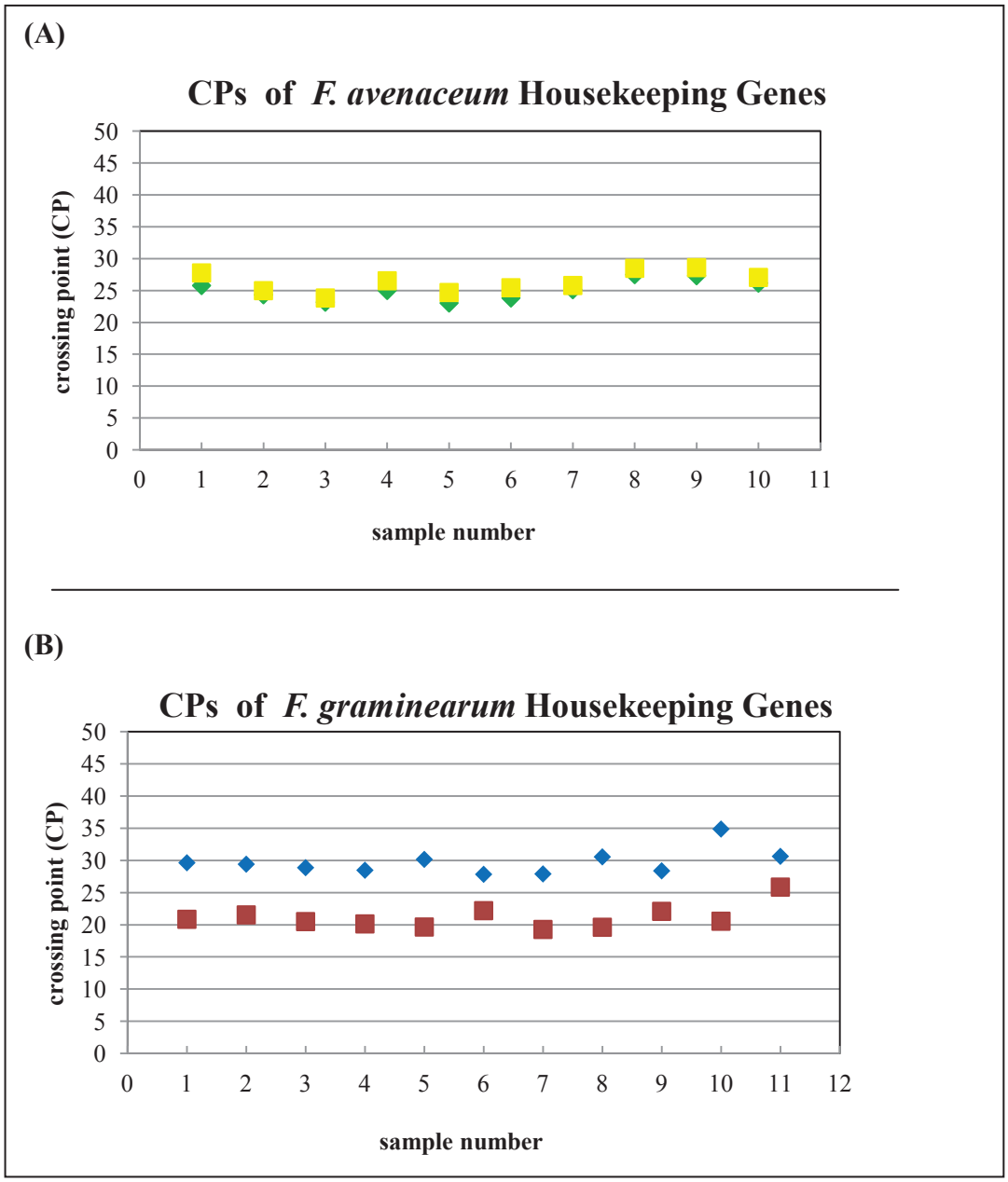

Figure 19. Bestkeeper values for HKG (A) *F. avenaceum UBC* (◆) and *β-tub* (■), and (B) *F. graminearum UBC* (◆) and *β-tub* (■) generated in the BestKeeper program (Pfaffl et al. 2004). Crossing point in RT-qPCR is plotted against sample number.

The HKGs for *F. avenaceum* and *F. graminearum* were analyzed in BestKeeper (Pfaffl et al. 2004) and are given in Figure 19. The stable expression of *UBC* and *β-tub* from infected wheat samples at different time points and inoculated fungi indicates that the chosen HKGs are suitable for normalization of the target gene expression.

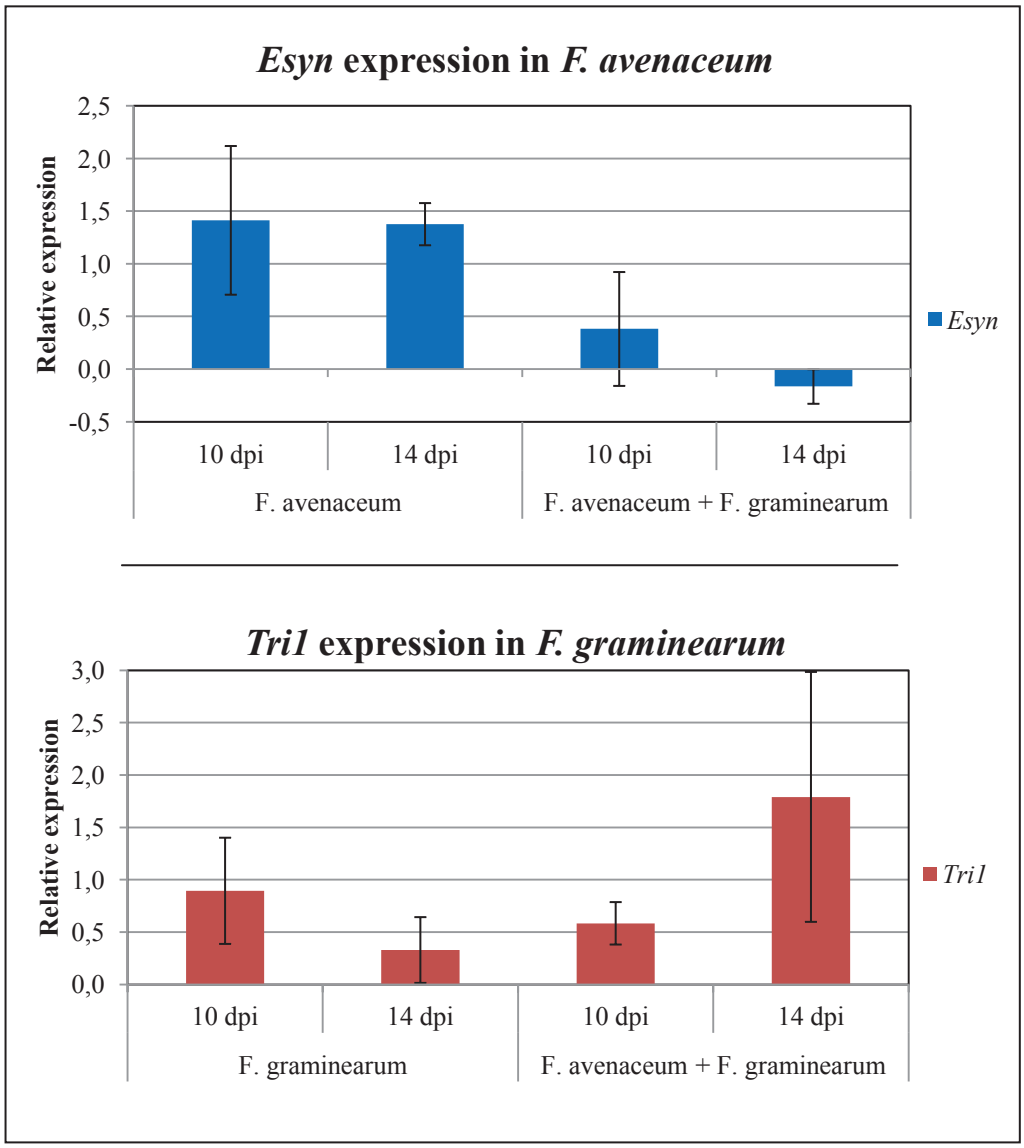

Figure 20. Relative expression value of the two target genes, esyn and tri1 at 10 and 14 dpi. Error bars indicate standard deviation. The expression is normalized to the expression of HKG using BestKeeper factor (Figure 19). Expression for each sample is calculated relative to one biological replicate at 10 dpi when *Fusarium* was inoculated alone.

The relative expression levels of *tri1* and *esyn* are expressed as Log to the base 10 (log10) and are given in Figure 20. For each of the target genes, expression is relative to one biological replicate at 10 dpi when the *Fusarium* was inoculated alone. The value of the reference sample in *Esyn* was 39 C_q and 26 C_q in *Tri1*. Each bar is the result of three biological replicates, run in technical duplicates (mean of six values, with the exception of 14 dpi when *F. avenaceum* and *F. graminearum* grew together, and when *F. avenaceum* grew alone (only two biological replicates).

Expression of *Esyn* and *Tri1* varied both with respect to condition and time point. At 14 dpi the expression of *Esyn* was decreasing when *F. avenaceum* grew together with *F. graminearum.* The decrease in expression was found to be statistically significant($p < 0.05$), when tested in a two ways unpaired t-test (Microsoft Office Excel 2007). The opposite trend to the *Esyn* expression was apparent of the expression of *Tri1* when *F. graminearum* grew together with *F. avenaceum*, although this increase was not significant ($p > 0.05$). The same trends were apparent at 10 dpi both for *F. avenaceum* and *F. graminearum*, however they were not significant.

4.2 Microbiological studies

4.2.1 *In vitro* co-inoculation

The molecular studies, earlier described, showed that the fungal species *F. avenaceum* and *F. graminearum* did have an effect on each other at 10 and 14 dpi. The earlier stages of contact however, were not covered in that experiment, since the first samples were taken at 6 dpi. To investigate their development and the effect the two species have on each other, the *F. graminearum TRI5*prom::*GFP* and *F. avenaceum* were grown together *in vitro* on solid medium. The development from conidia to hyphal structures was studied approximately every third hour using CLSM. The *F. graminearum TRI5*prom::*GFP* mutant is constitutively expressing DsRed which makes it possible to differentiate its hyphae from *F. avenaceum* when co-inoculated. In addition, the mutant is expressing *gfp* when the *tri5* promoter is activated.

Germination of the two species *F. avenaceum* and *F. graminearum TRI5*prom::*GFP* was clearly different (Figure 21). Spores of *F. graminearum TRI5*prom::*GFP* started rehydrating and swelling at 3 hours post inoculation (hpi), and nearly all spores had germinated when reaching 6 hpi. *F. avenaceum*, on the other hand did not start germinating until 6 hpi, and approximately all spores had germinated by 12 hpi. When germinating, *F. graminearum TRI5*prom::*GFP* in close to all occasions produced two or three hyphae from one macrospore. *F. avenaceum* in contrast, did only produce one.

F. avenaceum and *F. graminearum* TRI5prom::GFP showed a diverse growth pattern when compared to each other (Figure 21A). When spores of *F. graminearum TRI5*prom::*GFP* were lying alongside each other they germinated in a star shaped manner, avoiding themselves (Figure 21B). The hyphae of *F. graminearum TRI5*prom::*GFP* were more robust looking and did not grow in a straight forward pattern as did *F. avenaceum*. When it comes to invasive growth,

*F. graminearum TRI5*prom::*GFP* immediately grew invasive into the agar after spore germination (Figure 21C), while *F. avenaceum* did not start invasive growth until 20 hpi.

There was no obvious sign of interaction between *F. graminearum TRI5*prom::*GFP* and *F. avenaceum*. The two species did not have a growth pattern that showed an avoidance of each other, and *F. graminearum TRI5*prom::*GFP* did not activate *gfp* production in close proximity of *F. avenaceum*.

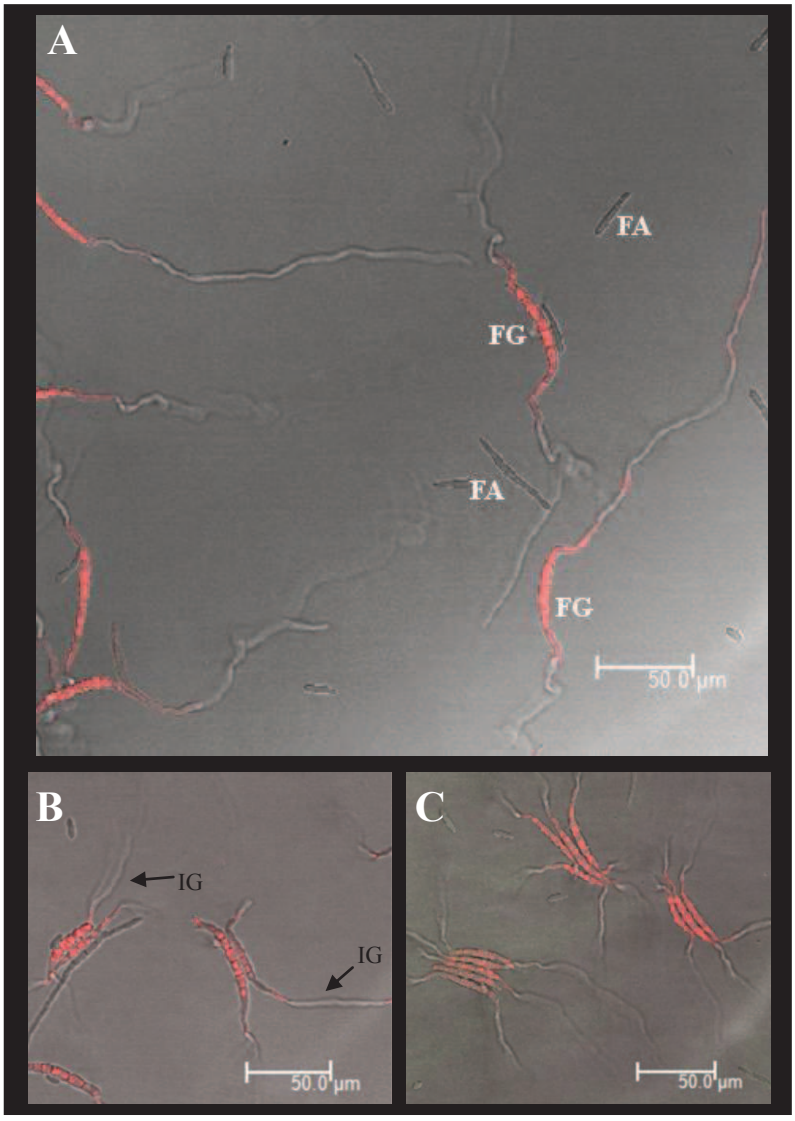

Figure 21. *F. graminearum TRI5*prom::*GFP* strain growing on SNA together with *F. avenaceum*. CLSM overlay images of DsRed fluorescence and BF. (A) 12 hpi, growth of *F. avenaceum* and *F. graminearum TRI5*prom::*GFP* is clearly different. (B) Invasive growth (IG) in *F. graminearum TRI5*prom::*GFP* at 6 hpi, (C) *F. graminearum TRI5*prom::*GFP* avoids itself when conidia are germinating adjacent to each other, 9 hpi. FA, *F. avenaceum*; FG, *F. graminearum*. Bar = 50.0 µm in all figures.

*F. graminearum TRI5*prom::*GFP* is producing *gfp* when the *tri5* promoter is induced. Induction did not seem to depend on close proximity to *F. avenaceum* (Figure 22). However, induction of *tri5* was found in microconidia, regardless of *F. avenaceum* presence, at 3 hpi and halted at 6 hpi when germ tubes of *F. graminearum* started forming. This trend was also seen in the control samples were *F. graminearum TRI5*prom::*GFP* was inoculated alone (data not shown).

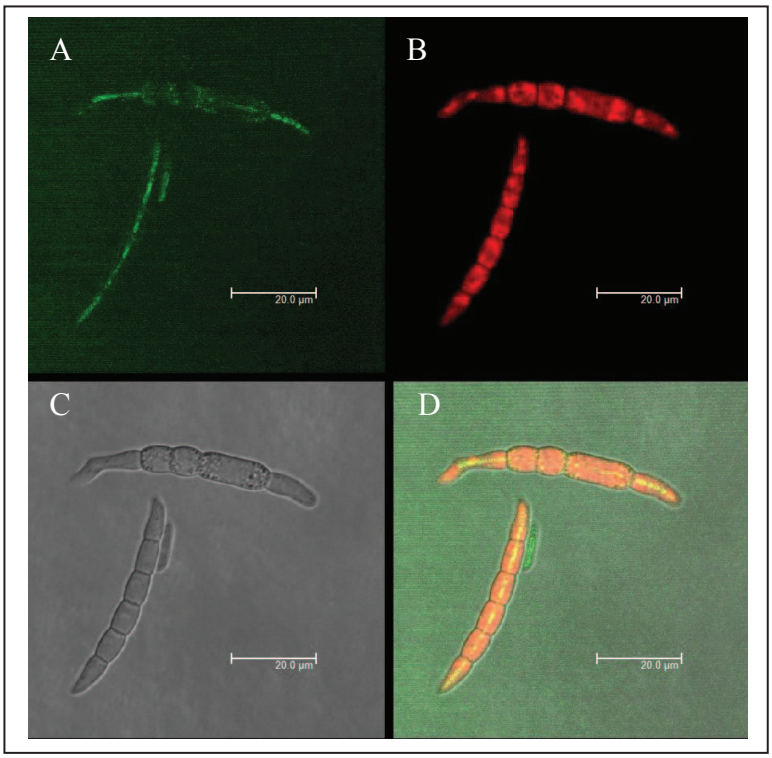

Figure 22. CLSM picture of *F. graminearum TRI5*prom::*GFP* growing on SNA at 3 hpi. GFP expression from the *tri5* promoter is activated. A) Emission of green fluorescence at 500-540 nm wavelength (GFP), B) emission of red fluorescence at 588 to 650 nm wavelength (DsRed), C) Bright field microscopy (BF) and D) Overlay of A-C. Bars = 20.0 µm.

4.2.2 Histological studies of *F. langsethiae* colonization of wheat grain

F. langsethiae infection is not a large problem in wheat in Norway, however infections do occur. To investigate possible explanations for the lower rate of infection in wheat and the possible infection routes, a spray inoculation experiment was carried out and the infected wheat grains were later investigated with SEM.

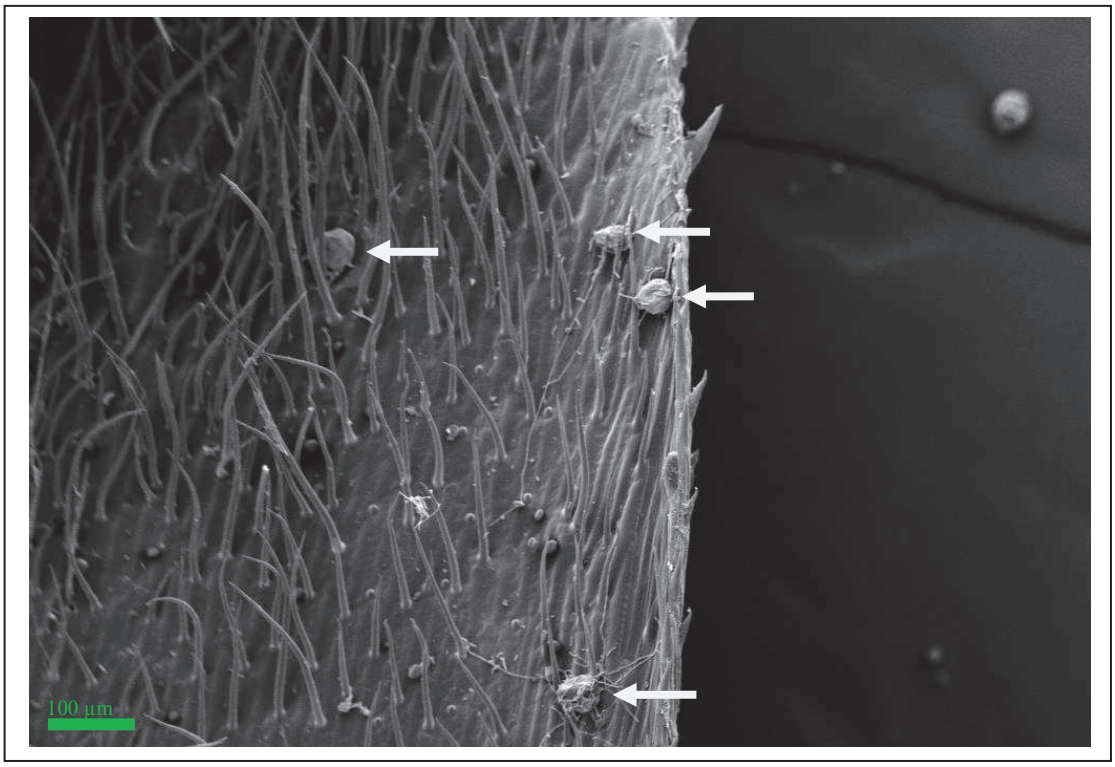

Figure 23. Wheat heads were spray inoculated at Zadoks growth stage 69-77 and investigated with SEM at 10 dpi. Fungal growth by *F. langsethiae* (indicated by the arrows) was closely associated with pollen grains on the adaxial side of lemma. Bar = 100 µm.

At 10 dpi the growth of *F. langsethiae* was found on the inside of lemma (adaxial side), mostly associated with pollen grains (Figure 23). The growth was mostly located to the edges close to the apical part of the spike. At 14 dpi, growth had spread onto the caryopsis (Figure 24), most hyphae observed being loosely associated with the cell wall. When growing in close association with the caryopsis, the hyphae looked wrinkled and lysed (Figure 24A).

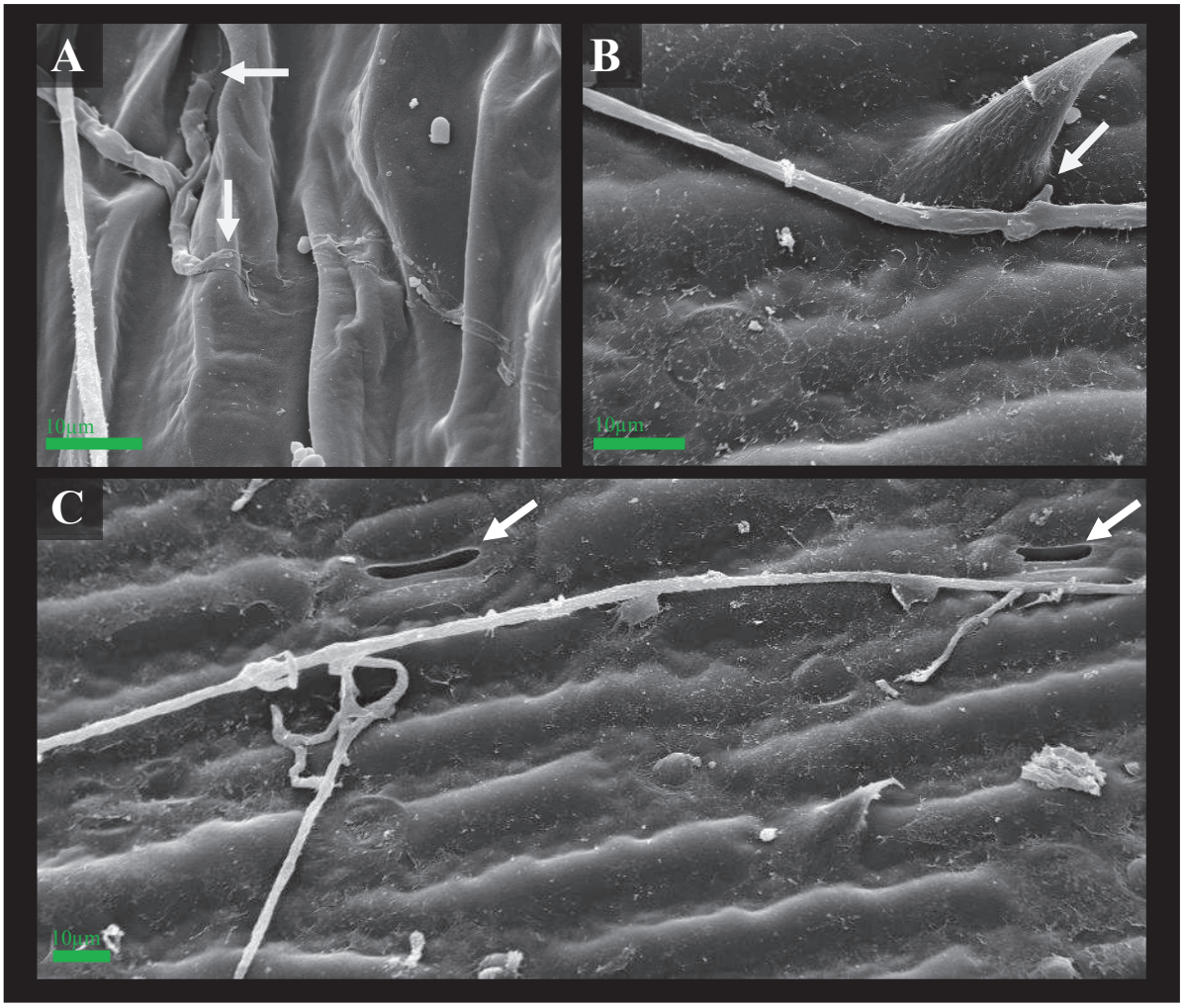

Figure 24. Wheat was inoculated with F. langsethiae at Zadoks growth stage 69-77 and investigated with SEM at 14 dpi. A) Lysis of hyphal structures on the caryopsis when in close association with the plant cells (marked with arrows). B) Structure resembling a foot structure or infection hyphae is marked with arrow. The picture is taken from palea, in the region overlapping with lemma. C) The fungi did not enter the cells through stomata. Stomata are marked with arrows. The picture is also from the overlap region. Bars = 10 µm

Structures resembling penetration pegs were seen on *F. langsethiae* growing on palea, on the overlap area with lemma (Figure 24B). The fungi grew past stomata, without entering (Figure 24C). No entering through stomata was observed in this particular study.

4.2.3 Investigation of infection routes of *F. langsethiae* in wheat

F. langsethiae is seldom found infecting wheat, and it has been shown that wounding of leaves is essential to get a successful infection on detached leaves (Imathiu et al. 2009). To investigate if, and how *F. langsethiae* is able to infect wheat a DLA was performed. To be able to detect and examine *F. langsethiae* on the infected leaves by CLSM a *F. langsethiae* mutant was used which is constitutively expressing GFP.

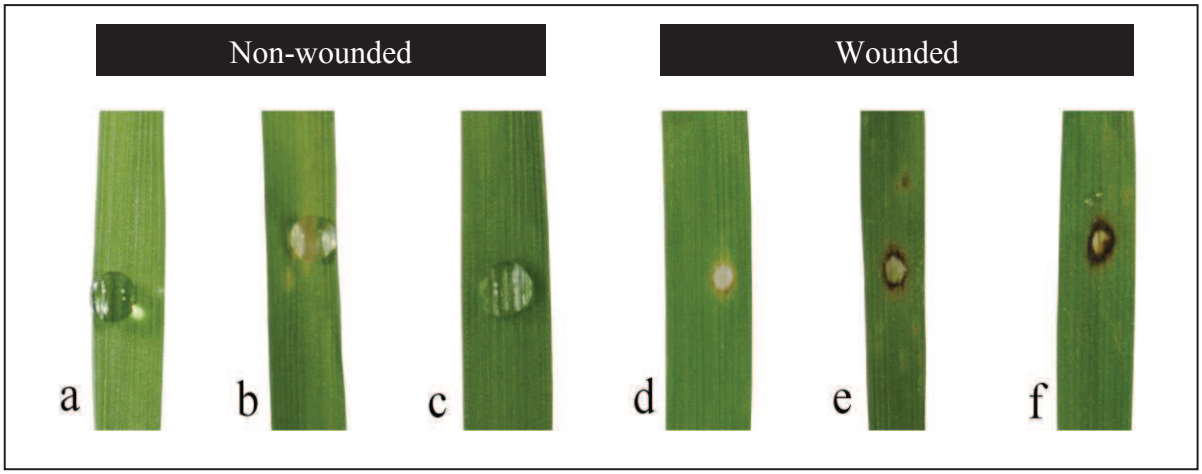

Figure 25. Detached wheat leaves 7 dpi at RT. (*a-c*) are non-wounded leaves, inoculated with SDW, *F. langsethiae* WT and *F. langsethiae* GFP, respectively. *d- f* are wounded leaves treated with SDW, *F. langsethiae* WT and *F. langsethiae* GFP.

Examples of inoculated detached leaves, wounded and non-wounded are given in Figure 25. At 7 dpi *F. langsethiae* GFP and WT had caused lesions in all the wounded leaves. The control leaves did not have any lesions. Lesions appeared as a brown ring encircled by a yellow area, indicating by dead plant cells (necrosis) and a loss of chlorophyll (chlorosis), respectively.

There was no significant difference ($p > 0.05$) between lesion lengths on leaves inoculated with the transformed *F. langsethiae GFP* compared to those inoculated with *F. langsethiae* WT (Figure 26). *F. langsethiae* WT and *F. langsethiae* GFP also had a similar appearance when grown on culture plates with SNA.

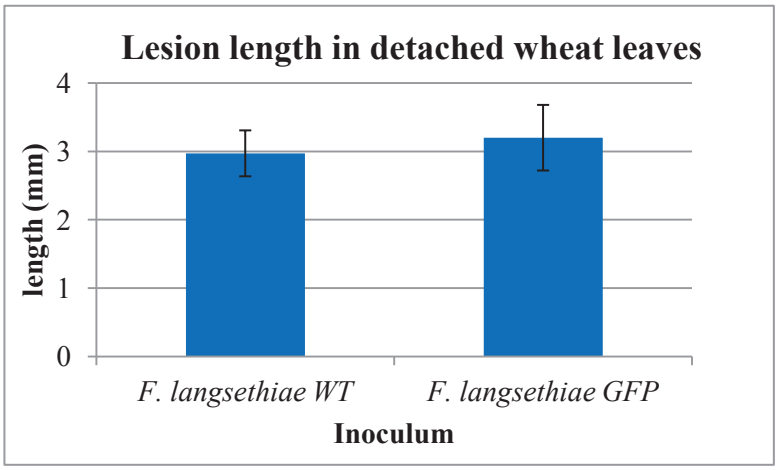

Figure 26. Mean length of lesions on detached wheat leaves wounded and inoculated with either *F. langsethiae* GFP or *F. langsethiae* WT. Each bar is the mean of 8 measurements and standard deviation is indicated by the error bars.

No visible lesions were found on non-wounded leaves or on the control leaves inoculated with SDW, at 7 dpi. Some did, however have a small chlorotic patch directly under the droplet of conidia, but it had not spread as was apparent in the wounded leaves. Chlorosis did also appear on parts of leaves that curled up and thus lost contact with the WA containing kinetin. This usually happened in the tips of the leaf, and is not close to the lesions caused by *F. langsethiae.*

All leaves, both wounded and non-wounded, inoculated with *F. graminearum*, were completely overgrown and macerated at 6 dpi (data not shown).

Detached wheat leaves infected with *F. langsethiae* GFP were investigated in CLSM at 7 dpi. Wounded leaves did clearly have more fungal growth than non-wounded leaves. The fungal growth was centred where the spores had been placed. Although *F. langsethiae* GFP was growing over a larger area in the wounded than in the non-wounded leaves, there was some growth on the non-wounded leaves as well (Figure 27).

The fungal growth in the non-wounded leaf was accumulated where the drop of spores had been placed, although some hyphae were growing away from the inoculation spot (Figure 27A). These hyphae seemed to consist of several hyphae coiled up together. The fungal growth also seemed to assemble around and over the stomata of the leaf (Figure 27B). There was also observed growth along the vascular tissue and some hyphae crossing the tissue (Figure 27C).

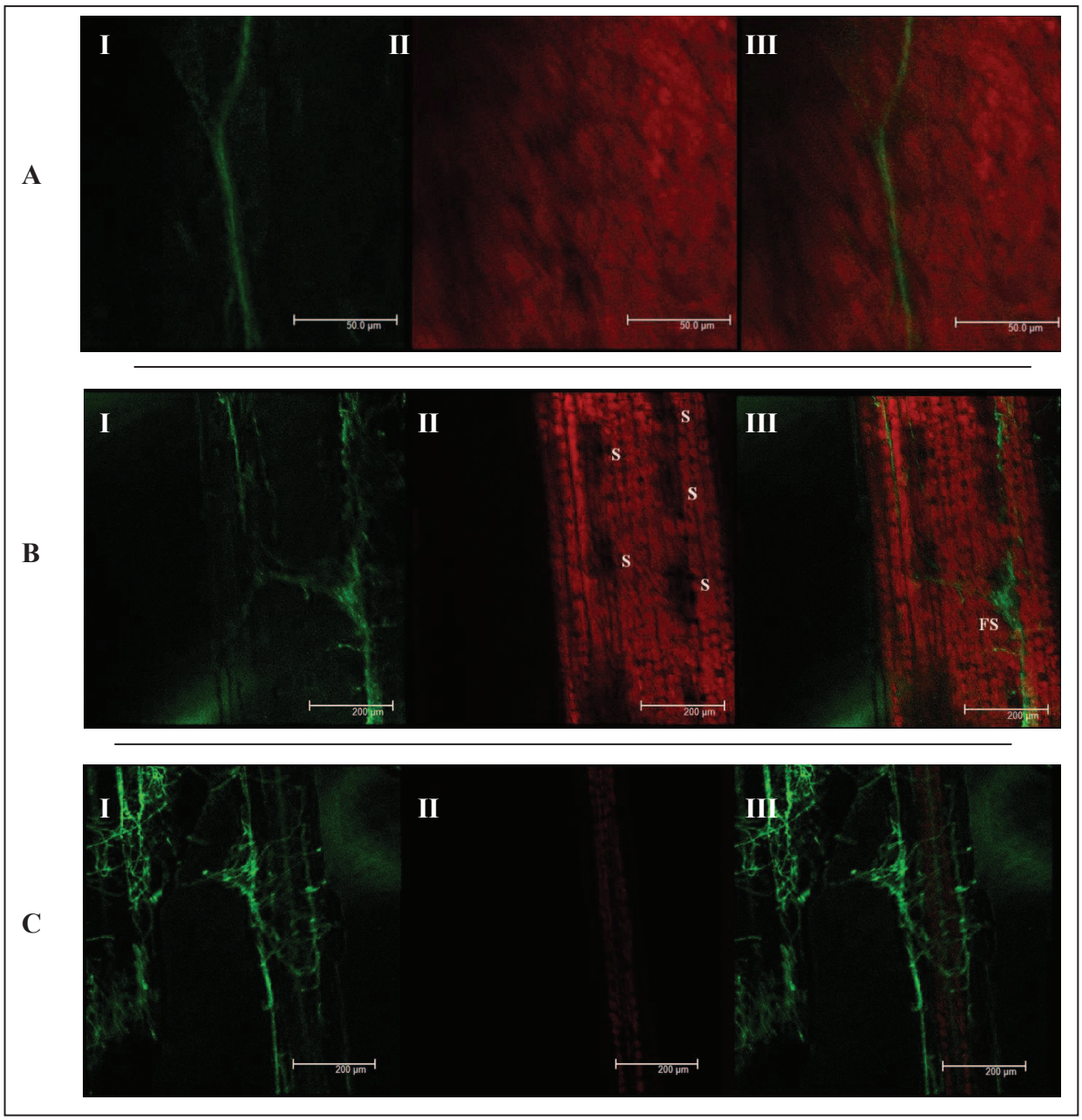

Figure 27. CLSM pictures of *F. langsethiae* GFP growing on detached non-wounded wheat leaves at 7 dpi (A) Runner hyphae. Bar = 50 µm. (B) Accumulation of fungal growth (**FS**) over stomata (**S**) of the leaf. Bar = 200 µm. (C) Growth alongside the vascular tissue in the wheat leaf. Bar = 200 µm. I) Green fluorescence emission at 500-540 nm (GFP) of *F. langsethiae* GFP is detected, II) autofluorescence from the wheat chlorophyll at 580-620 nm, and III) is an overlay of I and II.

Chapter 5 Discussion

5.1 Greenhouse inoculation studies

Three biological replicates were used in the study, in each of these; wheat material from four heads had been pooled together. In retrospect, more representative results for both fungal DNA quantification and gene expression studies could have been obtained if the number of biological replicates had been higher or several extractions could have been done from each of the three biological replicates. However, this study gives an indication of the trends of interaction between *Fusarium* species, and can make a good foundation when designing new experiments.

5.1.1 Using DNA to quantify fungus in infected plant material

MP Biomedicals states that "the FastDNA® SPIN Kit for Soil gives rapid isolation of PCR-ready genomic DNA from soil samples". This experiment strongly supports that the statement also applies for wheat samples with *Fusarium*.

The amount of fungal DNA extracted in the samples from 6, 10 and 14 dpi was highly variable between biological replicates, even though the amount of plant DNA was more stable. Samples at the ripening stage gave less variation in the biological replicates. This may indicate that the initial establishment of the fungus in the plant is highly variable and may relay on "luck", hence the *Fusarium* have to land on a favorable spot to be able to infect. In samples from the ripening stage, given longer time to establish and grow, the *Fusarium* reached its saturation point and evened more out between the biological replicates. The factors that have an influence in the establishment of the fungus should be identified to get more knowledge of the infection process and why the infection is worse in some cases than others. Xu and co-workers (2007a) also reported of highly variable replicates when they inoculated wheat with *Fusarium* in combinations of different species. They stated that the reason may be differences in the condition of the wheat plants or differences in quality/strength of the inoculum. It is highly likely that there were some differences in the wheat plants also in this study. The plants were planted at different time periods, in the controlled environment of a greenhouse, with temperature regulation by air ventilation. However, with the changing weather conditions in Norway, with warm summers, it is highly possible that the amount of sun exceeded the ventilation capacity of the greenhouse, i.e. the temperature raised in some periods (each biological replicate). Differences in strength/quality

of the inoculums are more unlikely, since the inoculums were prepared with the same procedure by the same person each time. In addition all inoculums where saturated, thus containing more than enough spores.

In some of the samples a trend of decrease in normalized fungal DNA material were seen from 6 to 14 dpi. The growth and cellularization of the developing grain at this time is rapid (Rogers & Quatrano 1983). Growth of the floret and the increase in plant biomass will therefore exceed formation of *Fusarium*. When the plant DNA was used as normalization factor for the amount of DNA in the samples, growth of the plant was not taken into account. Thus, it is expected to see the decrease in fungal material. To overcome this problem another approach could have been used. In similar studies with *Fusarium* infecting wheat, the DNA concentration of the samples were adjusted to a common concentration prior to quantification of the *Fusarium* (Halstensen et al. 2006; Keller et al. 2005; Xu et al. 2007a). The studies were all investigating wheat at the ripening stage. In the present study, however, the wheat differed in age stage. Therefore the best way of normalizing the samples was to use plant DNA, but taking care to compare results only from the same age.

qPCR quantification

Quantification of fungal DNA by the use of qPCR with TaqMan® probe is a highly specific and sensitive method. Probe assays are advantageous because they do not detect primer-dimer products (Kubista et al. 2006).

From the results presented here it seems that the growth of *F. avenaceum* at 6 dpi increased in the presence of *F. graminearum* compared to when growing alone (Figure 11). This suggests that there was some degree of competitive interaction between the two species, and that *F. avenaceum* was slightly more competitive at 6 dpi than *F. graminearum* under the conditions tested. *F. graminearum* exceeded its growth alone in the co-inoculation with *F. avenaceum* only at 14 dpi. At 14 dpi *F. graminearum* growth of one of the biological replicates exceeded its growth alone and growth of *F. avenaceum* was clearly inhibited.

F. graminearum has traditionally been looked at as the more competitive and aggressive species of the FHB pathogens (Brennan et al. 2003; Xu et al. 2007a). It is therefore not surprising to see its domination over *F. avenaceum* when the two species grew together. *F. avenaceum* was found in a higher amount at 6 dpi, than at the later timepoints. This trend may

be explained by different temperature preferences for colonization; *F. avenaceum* is often associated with cooler areas than *F. graminearum* (Kosiak et al. 2003). *F. avenaceum* had an advantage for colonizing in an early stage, due to its temperature requirement being closer to the greenhouse temperature. *F. graminearum* might have needed more time to establish due to non optimal temperature in addition to the pressure from the other species, thus grew more rapidly at 10 and 14 dpi. This trend agrees with the result Xu and co-workers (2008) obtained from a study investigating the FHB complex relationship with environmental conditions. The amount of *F. graminearum* increasing with time is also confirmed in the wheat samples investigated from the ripening stage, *F. graminearum* establishing itself as the more dominant species compared to *F. avenaceum*. The dominance of *F. graminearum* can be a contributing factor to the recent increased prevalence in northern Europe (Brodal et al. 2012; Kosiak et al. 2003; Waalwijk et al. 2003; Xu et al. 2005). An interesting study would be to follow the fungal development also in later timepoints during development towards the ripening stage. This could give insight in the further growth of the two species, to see whether the growth continues to develop, or if it evens out as the wheat go into the ripening stage.

Production of the mycotoxins enniatins and DON produced by *F. avenaceum* and *F. graminearum*, respectively, were measured per unit fungi (Figure 13). The amount increased when the fungi were growing together. This is in agreement with what Xu and co-workers (2007a) found when co-inoculating *Fusarium* on wheat. The reason for the increase may be the competition between the species in respect to resources imposing a nutritional stress on the fungi. Toxigenic fungi tend to produce more toxins when exposed to stress (Champeil et al. 2004). In addition, the toxins might not originate from the caryopsis. Only the most aggressive fungi will grow on the caryopsis, while the less aggressive are growing on the glumes. The toxins may also originate from these fungi. Studies have reported that toxins can be translocated from glumes to grain (Doohan et al. 1999).

Contaminated inoculums

Almost all samples inoculated with *F. culmorum* did show high values of *F. graminearum*. The plants with different treatments were randomized in the greenhouse, so some problems of cross-contamination between neighboring plants are likely, as seen in control samples (Figure a, page i in Appendix). Contamination of *F. graminearum* in *F. culmorum* infected plants was, however, so high and specific, that a contamination of inoculum prior to infection is suspected. The option

of airborne contamination of *F. graminearum* is also possible, since this species produce ascospores. These spores are forcibly discharged into the air, and are therefore able to travel long distances (Trail et al. 2005). There is a possibility that these spores have come through the air vents of the greenhouse and contaminated the wheat. But since the contamination of the control samples were so small, and that of the *F. culmorum* inoculum was so specific, this is not likely. Because of the contamination with *F. graminearum*, results from the samples inoculated with *F. culmorum* will be difficult to interpret. Therefore, these samples will not be discussed further.

5.1.2 Expression of fungal genes *in planta*

The kit used for RNA extraction was chosen based on former experience (Anstensrud, unpublished data); therefore no other kits were tested. The Spectrum™ Plant Total RNA Kit for soil proved to be a reliable kit, with high RNA yield. When testing with Bioanalyzer, most RNA samples also proved to be of high quality. Samples from 14 dpi however, showed more variation in RNA quantity and quality than those from 10 dpi. The reason for this might be an increase of inhibiting substances in the grain from 10 to 14 dpi. The grain endures an enormous development the first days after anthesis. For example from day 10-12 after anthesis cellularization starts and the B-type starch granules start developing and the lipid content is also changing rapidly (Bonfante 2003; Stokes et al. 1986). The change in lipid content and increase of starch in the grain might have caused the difficulties of extracting RNA from samples collected at 14 dpi. In samples proven more difficult to extract, the amount of sample was reduced as recommended by the user guide. This usually gave higher RNA yield, except for one sample which was discarded. The RNA in this sample was probably degraded prior to the extraction. Because of its unstable nature, the importance of inhibiting RNase activity to prevent degradation is high. RNases exist in most organisms and can therefore easily contaminate the RNA sample and degrade the RNA (Frostegård & Bakken 2006).

Some samples did have an accumulation of low molecular compounds when cDNA was run on agarose gel and were also not detectable in RT-qPCR. The difficulties with the samples might have several reasons: a) in the second DNase treatment, not column based, it was difficult to remove all RNA from the pelleted DNase Inactivation Reagent. In the supplied laboratory manual it was stated that this reagent possibly could inhibit the RT-PCR. Probably, an on column DNase treatment would have been better. But this kit was chosen because it was found to be the most efficient in removing DNA (unpublished data, Klemsdal). b) The RNA was not denaturated

before cDNA synthesis. In many cDNA synthesis protocols a denaturation step is included (typically 65 °C in 5 min), however this was not included in the SuperScript® VILO cDNA synthesis procedure. A high degree of secondary structures in the RNA may have inhibited the RT-reaction. By denaturing the RNA previous to the reaction, this problem could have been solved.

When looking at amounts of messenger RNA (mRNA) it is important to consider the possibility that the mRNA not necessarily will be translated into a protein or a functional protein (Waalwijk et al. 2004). However, the mRNA amount in the cell gives an indication of which processes are induced.

It was apparent that the relative expression of *Esyn* was decreasing over time and *Tri1* was increasing under co-inoculation and decreasing when *F. graminearum* was inoculated alone. When looking at reference values for Figure 20 there was a much higher expression of *Tri1* than *Esyn*. This may reflect the importance for these toxins in the infection process. Since *Esyn* has a lower expression, enniatins are likely less important in the infection and establishment on the host compared to *Tri1*. Earlier studies have shown that DON production in *F. graminearum* might be triggered by the host and is important to suppress the plant defense (Ilgen et al. 2009; Jansen et al. 2005). The decrease of *Tri1* expression when *F. graminearum* was inoculated alone was thus most likely a response to the host and might be because the initial infection period was over. Unlike the expression of *Tri1*, the *Esyn* expression was more stable when *F. avenaceum* was growing alone. The role of enniatins in infection of plants is unclear, and may depend on the host (Xu & Nicholson 2009). When growing alone, the expression of *Tri1* was reduced from 10-14 dpi. The variable expression of *Esyn* and *Tri1* at the early stages (10-14 dpi) might reflect the large development in the grain at that time, making a transforming environment (Bechtel & Wilson 2003).

Esyn expression was stable, but low when *F. avenaceum* was co-inoculated. The *F. avenaceum* DNA decreased dramatically when co-inoculated with *F. graminearum* and was almost not detectable in wheat at the ripening stage. *F. avenaceum* might be so weak that it is not able to produce enniatins. When growing alone, the expression was stable, but higher than when co-inoculated. *Tri1* expression shows the opposite trend to *F. avenaceum* and increased from 10-14 dpi when growing together with *F. graminearum*. When seen in relation to the toxin analyzes in wheat from the ripening stage, it was apparent that toxin production per *Fusarium* unit was increasing in both species when co-inoculated. The increase in *F. avenaceum* that was seen in this stage may mean that *F. avenaceum* needed more time to establish itself.

5.2 Microbiological studies

5.2.1 *In vitro* co-inoculation

Growing fungi *in vitro* on culture plates is a fast and cheap method compared to a greenhouse experiment, even though isolated from its normal environment it only gives an indication about what happens *in planta*. In addition, the fluorescence from *F. graminearum TRI5*prom::*GFP* made it easy to differentiate the two species from each other and locate *Tri5* induction. The use of SNA as a growth medium in the experiment was ideal given its low autofluorescence. It was evident that *F. graminearum* grew faster than *F. avenaceum*, even though it was impossible to do exact measurement of the growth of *F. graminearum* because it was invasive. In retrospect, if the experiment was to be repeated, a cellophane film would be added between the spores and the growth medium. This would have prevented invasive growth, while still allowing nutrients to slip through.

The growth and sporulation of *F. avenaceum* was much slower than that of *F. graminearum TRI5*prom::*GFP*. In addition, *F. avenaceum* did only develop one germ tube, while *F. graminearum TRI5*prom::*GFP* often germinated with several from each spore. This pattern shows the less aggressive nature of *F. avenaceum* compared to *F. graminearum TRI5*prom::*GFP*. The differences may partly be caused by the difference in spore morphology of the two species. *F. graminearum* has a much higher potential of storing nutrients because of its bigger spore size, thus also having the ability to develop several germ tubes and grow faster without external nutrition. *F. avenaceum* has smaller spores, thus may need external nutrition earlier in the infection. Because SNA is a poor growth medium it would probably limit the growth of both species, and *F. avenaceum* may be most affected because of its smaller nutrient store. These findings agree with several other studies, finding *F. avenaceum* a slower and less aggressive pathogen compared with *F. graminearum* (Brennan et al. 2003; Hudec 2007; Xue et al. 2006). *F. graminearum* germinated in a star shaped pattern, avoiding itself. This pattern of negative autrophism would be beneficial; hence more nutrients can be extracted if spreading over a larger area.

Tri5-induced GFP was observed for a short period around spore germination in *F. graminearum TRI5*prom::*GFP*, probably as a result of changes in the primary metabolism. Mycotoxins are products of secondary metabolism (Bennett & Klich 2003), but also end products of precursors from the primary metabolism (Keller et al. 2005). It has been hypothesized that the secondary metabolism only exists to remove accumulated products from the primary metabolism (Deacon

2006). Thus, the production of mycotoxin is highly dependent on the biochemical reactions in the primary metabolism. One of the intermediates in primary metabolism is acetyl-coenzyme A (acetyl-CoA). This molecule can go through the isoprenoid pathway ending with farnesyl pyrophosphate (Peplow et al. 2003). Farnesyl pyrophosphate is the substrate of the enzyme encoded by *Tri5* in the trichothecene biosynthesis pathway. The short term induction of *Tri5* in *F. graminearum TRI5*prom::*GFP* (Figure 22) during spore germination when the spore is going from a dormant state, to metabolizing stored nutrients to form a germ tube(Thevelein 1984), may have resulted in an abundance of acetyl-CoA, the substrate of the isoprenoid pathway, resulting in a short term induction of *Tri5*.

5.2.2 Histological study of *F. langsethiae* infection routes in wheat ears

F. langsethiae is regularly detected in oat in Norway, but is of unknown reasons somewhat more seldom found in wheat. A study on *F. langsethiae* infection in out was conducted to see how/if infection on wheat differs from infection on oat (unpublished data, Bøe).

From the results presented here it was apparent that *F. langsethiae* is able to grow on and infect wheat. Most of the fungal growth was concentrated on and in close proximity to pollen grains, with only scanty growth elsewhere. This indicates that pollen does have a large impact on determining the development of the fungus and the outcome of the infection with *F. langsethiae* in wheat. During anthesis, wheat extrudes its anthers and releases the pollen carriers. This locates the anthers to the outside of the spike (Waines & Hegde 2003). In a similar study, with oat, it was observed that the pollen remained trapped between palea and lemma (Bøe, unpublished data). Earlier research have shown that anther extrusion is one determinant for the ability to resist *Fusarium* infection (Graham & Browne 2009), and that selection for anther extrusion can improve FHB resistance in wheat (Skinnes et al. 2010). *F. langsethiae* depending on pollen to grow can be one of the factors explaining why *F. langsethiae* is more common in oat than wheat.

The small microconidia of *F. langsethiae* may explain its sparse growth apart from in association with the pollen grains, and why *F. langsethiae* appears to be a weaker pathogen compared to other *Fusarium* species. Microconidia contain less nutrient reserves than macroconidia of i.e. *F. graminearum* and may therefore depend more on host nutrients to have the energy to infect. The same pattern is seen in two other closely related fungal species; *Botrytis cinerea* and *B. fabae*. *B. fabae* with the larger spores has the ability to invade leaf tissue with only using its own nutrient reserves. While *B. cinerea* with smaller spores, however, needs exogenous nutrients

to be able to invade (Deacon 2006; Wastie 1962). Thus, *B. cinerea* has evolved as a generalist, producing a large amount of small spores, but depending on external nutrients to infect the host. *B. fabae* on the other hand produces a smaller amount of spores, where each of them has larger chance of infecting the host (Deacon 2006; Wastie 1962).

Structures resembling a foot structure or infection hyphae were seen. Penetration of cells was not observed, but it is likely to believe that *F. langsethiae* penetrates the cells. Similar structures which have been found to penetrate have been observed earlier in *F. avenaceum, F. culmorum* and *M. nivale* (Kang & Buchenauer 2000; Kang et al. 2004; Kang et al. 2005).

β-1,3-Glucanases and chitinases are well known defense enzymes secreted by wheat; these hydrolytic enzymes target chitin and β-1,3-glucan in the hyphal cell wall (Mauch et al. 1988). A higher production of these substances in the caryopsis cells may be the reason for the lysis of *F. langsethiae* hyphae. This has earlier been shown in a study where chitinase where able to lysate hyphal tips of *F. solani* (Selabuurlage et al. 1993). However, the fact that lysis does not happen to other closely related *Fusarium* species growing on the caryopsis is noteworthy (Kang & Buchenauer 2000; Kang et al. 2005). More research needs to be done on this phenomenon to draw any conclusions.

5.2.3 Detached leaf assay (DLA)

Wheat infection by *F. langsethiae* was also investigated using DLA. Some of the leaves in this experiment were wounded before inoculating with *F. langsethiae*. This was to allow the fungi to overcome the penetration barrier. The length of the lesion developing on the leaves is an indicator of the fungal pathogenicity and aggressiveness.

All wounded leaves inoculated with *F. langsethiae* in this experiment developed lesions, confirming the pathogenicity of *F. langsethiae*. In the unwounded leaves, no lesions developed, indicating that *F. langsethiae* was unable to penetrate the leaf and needs a wound to be able to grow. Wounded leaves had an area with chlorosis on the leaf tissue around the necrotic patch; the same also found directly under the conidia droplet of some non-wounded leaves. This agrees with the results from a DLA conducted by Imathiu and co-workers (2009), who found chlorosis in leaves inoculated with *F. langsethiae*. The chlorotic patches may originate from the secretion of the mycotoxins T-2 and HT-2, produced by *F. langsethiae* (Thrane et al. 2004). T-2 and HT-2 have earlier been shown to cause, among other symptoms, both chlorosis and necrosis in wheat

tissue, and mycotoxins have been reported to interfere and inhibit plant's defence mechanisms (Jansen et al. 2005; McLean 1996). Thus it is likely to believe that T-2 and HT-2 can be causing the chlorosis spots.

The *F. langsethiae* GFP mutant maintained its wild type like behaviour. Lesions from leaves with the *F. langsethiae* GFP mutant did not differ significantly from *F. langsethiae* WT, confirming this. Furthermore, as the autofluorescence of the leaves is not overlapping with the emission wavelength used to locate *F. langsethiae* GFP, infected detached leaves were investigated using CLSM. Several different mycelial structures were seen on the leaves investigated with CLSM. Some hyphae were seen growing out of the colony. The leaf surface is an environment scarce of nutrients, so the hyphae growing out of the colony were possibly in search of nutrients. Growth of *F. langsethiae* accumulated around stomata. This accumulation may indicate that stomata could be an entering point of *F. langsethiae* into the cell. Pritsch and co-workers (2000) also found indications of hyphae entering through stomata when investigating wheat spikes inoculated with *F. graminearum*. The same has also been observed for *F. culmorum* (Kang & Buchenauer 2000). Another possibility is that the stomatal cells somehow provide nutrients for the fungus, either by being easily penetrable or by accumulation of favourable nutrients, leading to an accumulation in fungal growth in their vicinity.

Extensive growth along the vascular tissue was seen. Growth associated with the vascular tissue has also been reported for *F. graminearum* by others (Jansen et al. 2005). In the plant, nutrient and fluid transport occur through the vascular tissue. Hence this tissue will contain large amounts of nutrients (Jung & Park 2007). It is therefore likely that fungal growth will accumulate in these areas.

5.3 Concluding remarks

Results from this study demonstrate that *F. graminearum* and *F. avenaceum* are likely to compete for nutrients or infection sites on wheat heads both in early stages after infection and at later stages. The result of this competition is an increase in the production of the mycotoxins enniatin and DON and a decrease in *Fusarium* mycelia.

Furthermore, the findings suggest that there is a close resemblance between *F. langsethiae* and other *Fusarium* spp. in the FHB complex. Stomata could possibly be a route for infection or a nutrient source when *F. langsethiae* colonize wheat. In the ears, however, stomata were not favored as growth spot, but instead fungus spread via germinating spores on the anthers, growing

between palea and lemma and entering the caryopsis. The slow growth of *F. langsethiae* favors the impression that this fungus is a weak pathogen. The shriveled and sometimes degraded hyphae growing on the caryopsis were possibly caused by hydrolytic enzymes secreted from the wheat cells. This phenomenon needs to be investigated further, but may explain why *F. langsethiae* seldom is found in wheat.

The *in vitro* experiment was inconclusive, and did not give any indication of competition or interaction. This may be caused by conditions differing too much from what was found during *in vivo* experiments, or may be because the growth of the fungi had not yet come to a stage to allow competition.

5.4 Future aspects

More work should be done to investigate at the interaction between other *Fusarium* species on wheat. The similar behavior between the *Fusarium* spp. makes it reasonable to believe that a similar interaction will happen between other species of *Fusarium*. A greenhouse experiment inoculating plants with other regularly found *Fusarium* spp. should be done to see if this is the case. When doing these experiments it is important to take specific care to avoid cross contamination of samples.

The expression of *Esyn* was demonstrated to vary from 10-14 dpi. This may indicate that enniatins are involved in the infection process of *F. avenaceum* in the plant. This could be investigated further by creating a knock-out mutant of *F. avenaceum*, unable to express the *Esyn* gene. Inoculating such a mutant on wheat heads and see if it is able to infect the wheat, would make it possible to see if enniatins have a similar role in the infection and spreading for *F. avenaceum* as DON does for *F. graminearum*.

More studies needs to be done on *F. langsethiae* on wheat to investigate molecular mechanisms influencing aggressiveness. And investigate the reason for the lysis when growing on the caryopsis.

Chapter 6 Literature

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Appendix

A. Fungal isolates

Table a. List over fungal strains used in the different experiments. DLA= Detached Leaf Assay.

Species	Strain	Experiment
F. avenaceum	6A	RNA/DNA quantification, *in vitro* co-inoculation,
	11A	RNA/DNA quantification
	21A	RNA/DNA quantification
F. culmorum	9C	DNA quantification
	14C	DNA quantification
	27C	DNA quantification
F. langsethiae	9821-16-1 (IBT9951)	DLA
F. langsethiae GFP	9821-16-1 (IBT9951)	DLA
F. graminearum	2007-059	RNA/DNA quantification
	2008-028	RNA/DNA quantification
	2008-140	RNA/DNA quantification, DLA
*F. graminearum TRI5*prom::*GFP*	Fg8/1	*in vitro* co-inoculation

B. Control samples

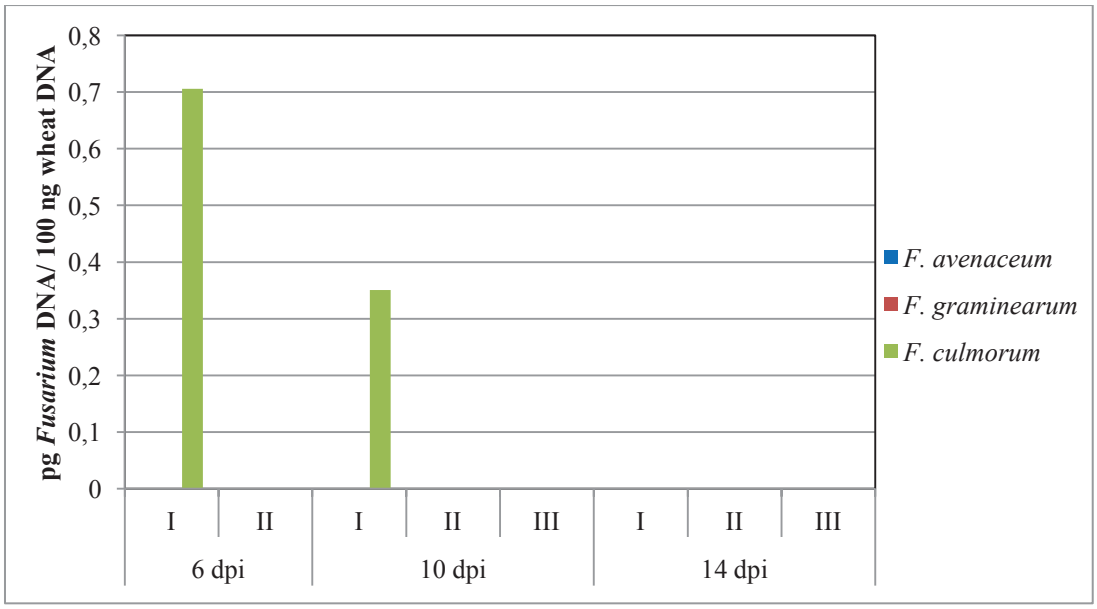

Figure a. Control samples sprayed with a conidial solution with 0.25% gelatine in SDW under anthesis and sampled at 6, 10 and 14 dpi. Small amounts of fungal DNA were detected with qPCR. Biological replicates are given in roman numerals.

C. DNA amounts from full dose inoculations of wheat

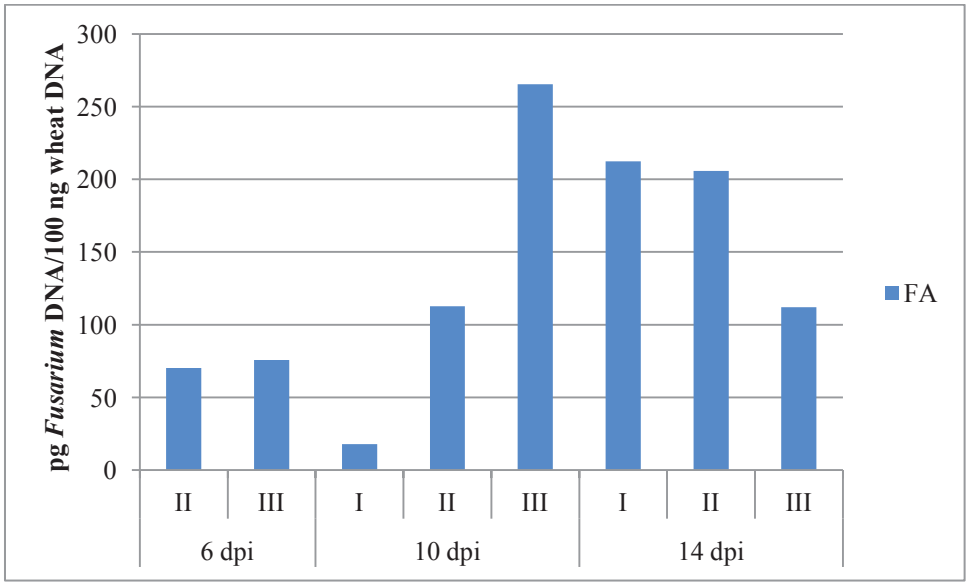

Figure b. amount of *Fusarium DNA* at 6, 10 and 14 dpi in samples inoculated with full spore amount (1x 10^5 conidia/ml) of *F. avenaceum*. The biological replicates are given in roman numerals. FA=*F. avenaceum.*

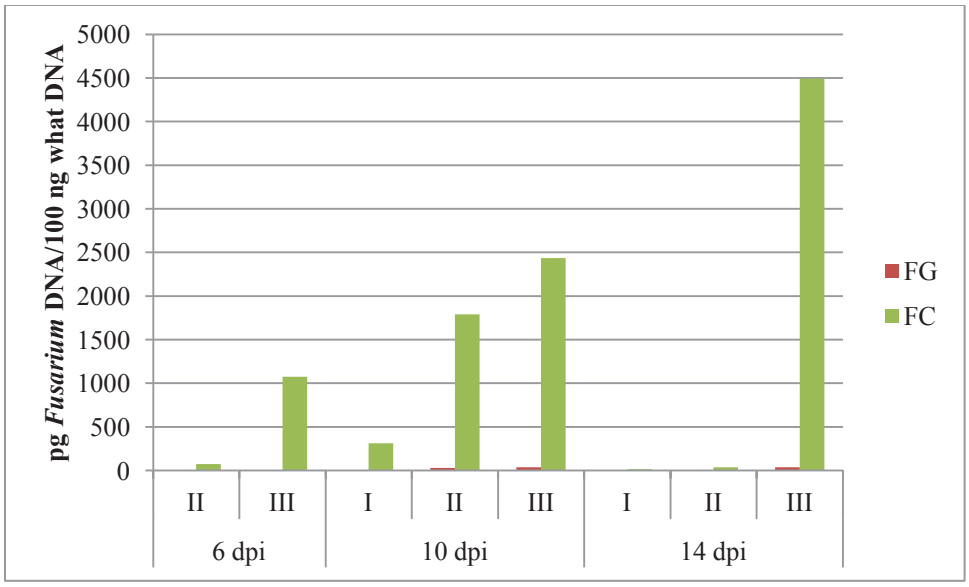

Figure c. Amount of *Fusarium* DNA at 6, 10 and 14 dpi in samples inoculated with full spore amount amount (1x 10^5 conidia/ml) of *F. culmorum*. The biological replicates are given in roman numerals. FG = *F. graminearum*, FC=*F. culmorum*.

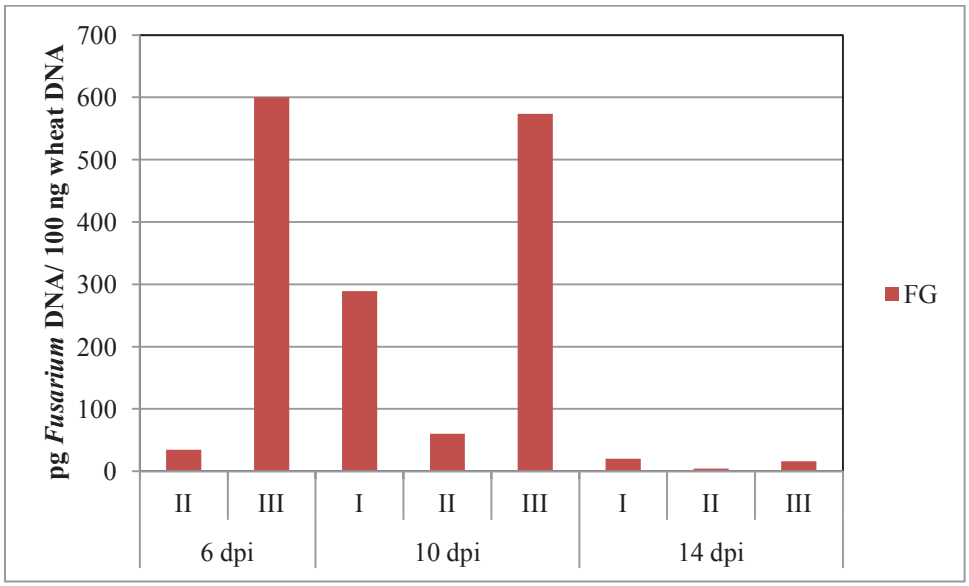

Figure d. Amount of *Fusarium* DNA at 6, 10 and 14 dpi in samples inoculated with full spore amount (1x 10^5 conidia/ml) of *F. graminearum*. The biological replicates are given in roman numerals. FG = *F. graminearum*.