In planta and *in vitro* studies of interactions between different *Fusarium* species and the infection of *Fusarium langsethiae* in oats

In planta og *in vitro* studier av samspillet mellom ulike *Fusarium* arter og infeksjon av *Fusarium langsethiae* på havre

Lise Bøe

NORWEGIAN UNIVERSITY OF LIFE SCIENCES
DEPARTMENT OF CHEMISTRY, BIOTECHNOLOGY AND FOOD SCIENCE
MASTER THESIS 60 CREDITS 2012

Preface and acknowledgment

First of all, thank you to the Research Council of Norway, who sponsored the research of this master thesis. Many working hours in the laboratory at Bioforsk Plant Health have resulted in the present study, and I would like to thank the many kind and caring people for their help regarding everyday problems. Their great working environment is something I will miss being a part of.

Special thanks go to my supervisors at Bioforsk Plant Health; Sonja S. Klemsdal and Hege H. Divon. To me, their guidance, inspiration and scientific input have meant a lot. They have both given me great opportunities and it has been a privilege to work under and learn from Sonja and Hege. I would also like to thank my supervisor at UMB, Professor Arne Tronsmo for good help and advice.

My thanks also go to my fellow master students at Bioforsk Plant Health, especially Christin Anstensrud and Martha M. N. Tveit. Their collaboration and friendship have been both valuable and encouraging. At last, my sincerely thanks and appreciation go to my family for their endless support.

Ås, August 2th 2012

Lise Bøe

Abstract

The genus *Fusarium* includes a diverse range of ascomycete fungi. Some species are known to cause *Fusarium* head blight (FHB) and other diseases on small grain cereals like oat, wheat and barley all over the world. Species of *Fusarium* are not only plant pathogens, but also mycotoxin producing agents, and this makes *Fusarium* species relevant to human and animal health. During infection, pathogens interact with each other as well as with the plant, and the object of this study was to gain more knowledge of these processes.

The interactions between *F. avenaceum, F. langsethiae, F. culmorum* and *F. graminearum* during early infection of oats (6, 10, and 14 days post inoculation) were investigated. Oat panicles were spray inoculated with fungal inoculums and the amounts of *Fusarium* DNA was quantified by real-time quantitative PCR. The trends showed that *F. avenaceum* was not affected by *F. graminearum,* positively affected by *F. culmorum* and somewhat negatively affected by *F. langsethiae* when co-inoculated on the plant. *F. langsethiae* was in general negatively affected in all combinations. Interactions between *F. langsethiae* expressing green fluorescence protein (*Fl*-GFP) and the other *Fusarium* species were investigated *in vitro* during the first 26 hours of interactions in a co-inoculation experiment using confocal laser scanning microscopy (CLSM). This enabled us to differentiate *F. langsethiae* from the other species. The trends in this study indicated greater total hyphal length (spore + germtube) of the species when co-inoculated pair wise compared to when grown alone. Invasive growth was also found to be delayed when the species were co-inoculated compared to when grown alone.

A gene expression study using four different target genes in *F. langsethiae* during early infection of oat, demonstrated higher expression of the putative genes; *Sod, Nps4* and *Fl-est17042* during infection compared to expression in mycelia alone. Using histological characterization, it was shown that the fungus is able to enter the plant through natural openings such as stomata, but that it might be depended on a nutrient source outside itself for successful establishment on the plant.

The interaction between *F. langsethiae* and oats was demonstrated in a detached leaf assay, where oat leaves were inoculated with droplets of fungal inoculums. It was shown that the plant produced O_2^- radicals as a response to the pathogen, and that *F. langsethiae,* in turn, produced high amounts of SOD during infection, possibly making it more resistant to the reactive oxygen species (ROS). *F. langsethiae* is a relatively newly identified specie and its infection process in oats and interactions with the plant are poorly understood. This study greatly advanced our understanding of this plant pathogen.

Sammendrag

Slekten *Fusarium* inkluderer en rekke ascomycete sopparter. Noen av disse er kjent for å forårsake aksfusariose og andre kornsykdommer på for eksempel havre, hvete og bygg over hele verden. *Fusarium* arter er ikke bare plantepatogener, men også mykotoksinprodusenter. Dette gjør *Fusarium* artene relevante i forhold til menneske- og dyrehelse. Under infeksjon oppstår både samspill mellom *Fusarium* artene og mellom patogenen og planten, og målet med dette studiet var å oppnå mer kunnskap om disse prosessene.

Samspillet mellom *F. avenaceum*, *F. langsethiae, F. culmorum* og *F. graminearum* ble studert under tidlig infeksjon av havre (6, 10 og 14 dager etter inokulering). Havre ble sprayinokulert med soppinokulum, og mengde *Fusarium* DNA ble kvantifisert ved bruk av real-time kvantitativ PCR. Trendene viste at *F. avenaceum* ble lite påvirket av *F. graminearum*, positivt påvirket av *F. culmorum* og noe negativt påvirket av *F. langsethiae* når artene parvis ble inokulert på planten. *F. langsethiae* var generelt negativt påvirket i alle kombinasjonene.

Samspillet mellom *F. langsethiae,* som uttrykker grønn fluorescerende protein (*Fl*-GFP) og de andre *Fusarium* artene ble studert i ett co-inokulerings eksperiment der konfokal laser skanning mikroskop ble brukt. Dette gjorde det mulig å skille *F. langsethiae* fra de andre artene. Trendene i dette studiet indikerte en større total hyfelengde (spore + germtube) når artene ble co-inoculert parvis sammenlignet med når de vokste alene. Det ble også vist at invasiv vekst var forsinket når artene var co-inokulert sammenlignet med når de vokste alene.

En genekspresjonsstudie undersøkte fire gener hos *F. langsethiae* under tidlig infeksjon av havre, og demonstrerte at genene *Sod*, *Nps4* og *Fl-est17042* var høyere uttrykt under infeksjon av havre sammenlignet med uttrykket i mycel alene.

Ved bruk av histologisk karakterisering, ble det vist at soppen hadde evnen til å vokse gjennom naturlige åpningene i planten (stomata), men at den mest sannsynlig er avhengig av en næringskilde på utsiden av seg selv for vellykket etablering på planten.

Samspillet mellom *F. langsethiae* og havre ble demonstrert i et blad assay hvor havreblader ble inokulert med dråper av soppinokulum. Det ble vist at planten produserte O_2^- radikaler som en respons på patogenen, og videre at *F. langsethiae* hadde produsert høy mengde SOD under infeksjon, noe som muligens gjør soppen mer resistent mot reaktive oksygen forbindelser.

F. langsethiae er relativt nylig identifisert og dens infeksjonsprosesser i havre og samspillet med planten er lite forstått. Dette studiet utvidet i stor grad vår forståelse om denne plantepatogenen.

Contents

Chapter 1 Introduction

1.1 The genus *Fusarium*

The genus *Fusarium* includes a diverse range of fungi (Doohan et al. 2003) that exhibit a notable level of physiological, morphological and cultural variation. *Fusarium* species are extensively distributed in soil, common in tropical and temperate regions but also found in areas with harsh climatic conditions like desert, alpine and arctic areas (Nelson et al. 1994).
These ascomycetes are also regularly associated with plant roots either as parasites or saprophytes (Nelson et al. 1994). Some *Fusarium* species are also known to cause *Fusarium* head blight (FHB) and other diseases on small grain cereals (Parry et al. 1995). Their widespread distribution may be due to their ability to colonize diverse ecological niches in most geographical areas of the world. This explains the remarkable degree of variation to be found in the *Fusarium* genus (Nelson et al. 1994).

Regarding taxonomy, the genus *Fusarium* is complex (Stakheev et al. 2011), and difficulties occurred in the development of a stable and widely accepted taxonomic system for the genus due to huge degree of variation (Nelson et al. 1994). To group species with similar morphological characteristics, the genus has been divided into sections (Nelson et al. 1994). *F. graminearum* and *F. culmorum* is to be found in the section called *Discolor*, *F. avenaceum* is a member of the *Roseum* section and in the *Sporotrichiella* section, *F. langsethiae* is found (Stakheev et al. 2011). The phylogeny of *Fusarium* species has been studied, and Figure 1 gives an overview of the relation between some of the *Fusarium* species.

When it comes to spore morphology, *Fusarium* species may produce three types of spores, but not all species produce all three types (Nelson et al. 1994). One type of spores is macroconidia that are usually produced in a sporodochium, but can as well be produced on monophialides and polyphialides in the aerial mycelium. Regarding size and shape, some conidia are intermediates and have been referred to as both macroconidia and mesoconidia, but the term mesoconidia is questionable (Nelson et al. 1994).

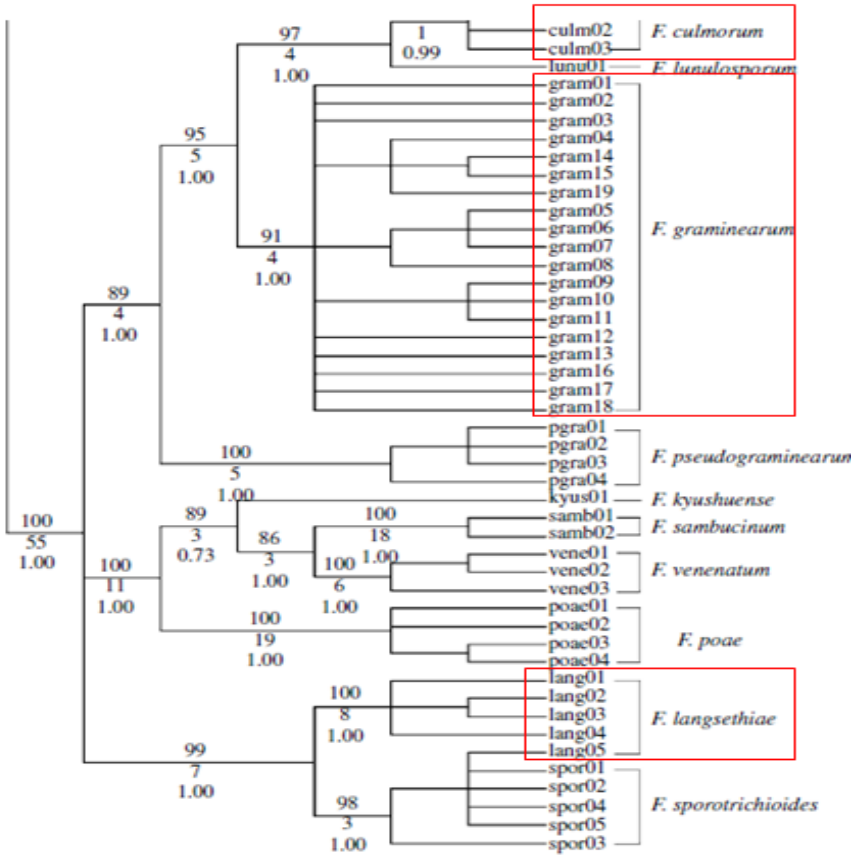


Figure 1: The phylogenic relation between some *Fusarium* species. Modified from Kristensen et al. (2005).

The macroconidia of *Fusarium* species are of different shapes and sizes, but relatively consistent for a given specie when growing on natural substrates and under standard conditions. This makes the morphology of the macroconidia a key feature for characterization of *Fusarium* species (Nelson et al. 1994). The banana or canoe shaped macroconidia are often related to many of the *Fusarium* species (Figure 2).

Figure 2: The general macroconidia shape of *Fusarium* species. Photo: Jafar Razzaghian (Bioforsk).

The second type of spores is microconidia produced in the aerial mycelium in false heads only or in false heads and chains on either monophialides or polyphialides (Nelson et al. 1994). In *Fusarium* taxonomy, the presence or absence of microconidia is a primary character. Microconidia are of various shapes and sizes and if microconidia are present, the shape and the mode of formation is the considered features (Nelson et al. 1994).

Clamydospores are the third type of spore *Fusarium* species can produce. Clamydospores are thick-walled and filled with lipid-like material. These spores can survive over winter in soil when a suitable host is not available. A primary character in *Fusarium* taxonomy is the presence or absence of clamydospores, and when present they may be formed singly, in pairs, in chains or in clumps (Nelson et al. 1994).

Species of *Fusarium* also differs when it comes to reproduction and whether they have a sexual stage or not. Environmental conditions like temperature, spore density and water potential influence the germination of *Fusarium* conidia (Stakheev et al. 2011). A switching between phases of growth and reproduction of filamentous fungi are highly regulated and influenced by physiological and environmental conditions. Spore production is triggered by factors like nutrient source, lights, metals, lipid signals and the chemistry of the plant host (Brodhagen & Keller 2006).

1.2 *Fusarium* species

F. graminearum

The dominant specie *F. graminearum* is in many parts of the world a widespread and destructive agent causing FHB (Goswami & Kistler 2004; Ilgen et al. 2009; Miedaner et al. 2008), but areas with warm and humid climate is where it has been predominating. However, the distribution of *F. graminearum* have seemed to change (Wagacha & Muthomi 2007), and reports from Germany, the Netherlands, Italy and Norway have been more common in the recent years (Hofgaard et al. 2009; Wagacha & Muthomi 2007). One reasons for this might be the climatic changes (Waalwijk et al. 2003) and moist and warm weather during flowering periode can increase the chances of infection by *F. graminearum* (Hofgaard et al. 2009). Head blight disease caused by *F. graminearum* on wheat is initiated by the landing of airborne spores on flowering spiklets (Trail 2009). The life cycle of *Fusarium* species have mainly been studied using *F. graminearum* in wheat as an example. The general life cycle is showed in Figure 3.

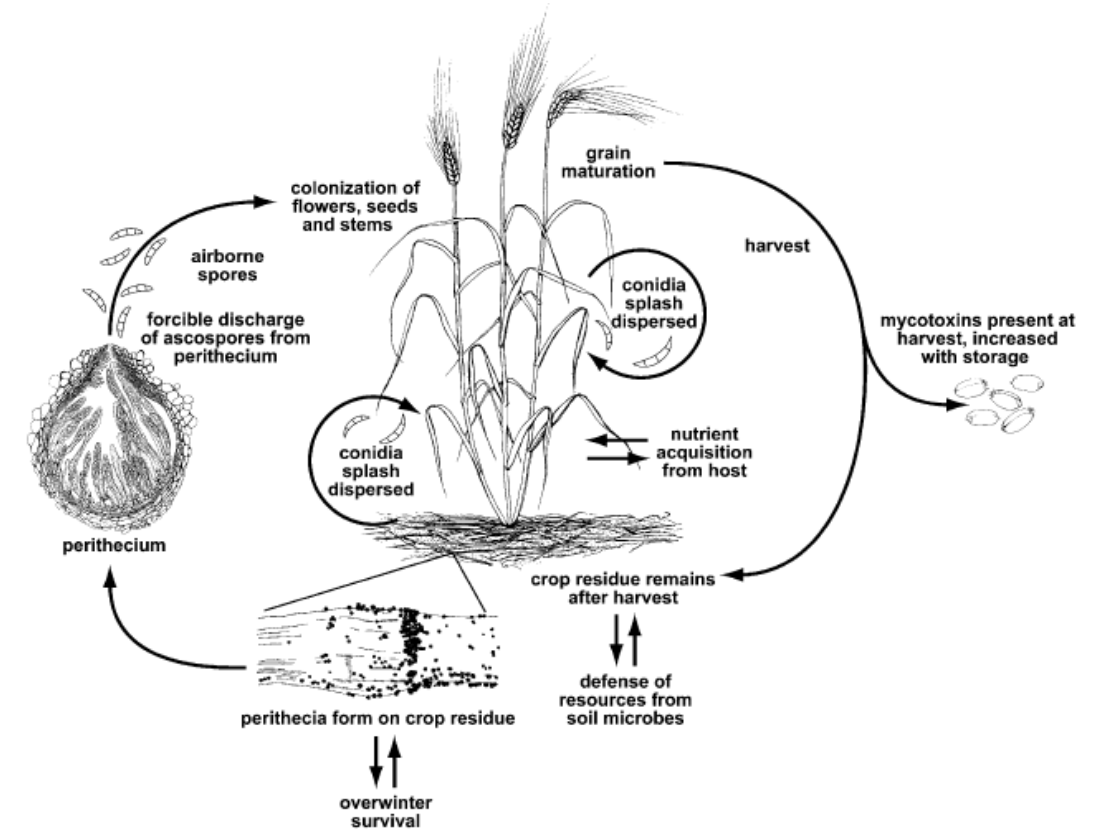


Figure 3: The life cycle of *F. graminearum* on wheat (Trail 2009).

F. graminearum is mostly haploid. The fungus overwinters as saprophytic mycelia and the asexual spores (conidia) are produced on the surface of infected plants or on crop residue during damp periods (Trail 2009). The dispersial of the conidia can be by wind, rain or insects (short distance) to the host plant and cause diseases (Goswami & Kistler 2004).

Formation of hyphae with binucleate cells is the beginning of sexual development and the development of ascospores (sexual spores) is not depended on a sexual distinct partner due to the fact that *F. graminearum* is homothallic (Trail 2009). Coiled cells produced by the binucleate cells of *F. graminearum* are the fruiting body initials and develops into flask-shaped perithecia that are filed with asci (tubular sacs containing the ascospores) (Trail & Common 2000; Trail 2009). The ascospores are forcibly discharged into the air when the asci extend up to the mouth of the perithecium, and are considered long distance dispersal (Trail 2009). From any known inoculums source, the inoculums can be detected at kilometer distances (Munkvold 2003).

However the relative contribution of conidia versus ascospores to disease epidemiology remains unresolved. After spore dispersal and landing on a host plant, spores germinate, and the fungus enters the plant through natural openings (the base of the lemma and palea or through degenerating anther tissues) (Trail 2009). At the infection front, the fungus grows intercellularly and asymptomatically, and spreads through the xylem and pitch (Guenther & Trail 2005).

Further, the fungus spreads radially, grows intracellularly resulting in necrosis, and rapidly colonizes the tissue. This gives symptoms like water soaking and premature bleaching of head tissue (Trail 2009).

F. culmorum

Similar to *F. graminearum, F. culmorum* also belongs to the *Discolor* section and has been reported to predominate in cooler areas such as Canada and Europe, including Norway (Parry et al. 1995; Wagacha & Muthomi 2007). In some of the countries where *F. culmorum* have been predominating, *F. graminearum* have taken over as the dominate specie causing FHB. *F. culmorum* was reported to be among the four most frequently isolated *Fusarium* species from wheat, barley and oat in Norway (Kosiak et al. 2003). *F. culmorum* is capable of causing severe disease and loss of usable grain (Lacey et al. 1999). The spores produced by this fungus are short, stout and thick-walled macroconidia and abundantly and quickly formed chlamydospores that occur singly, in chains or in clumps. (Wagacha & Muthomi 2007). *F. culmorum* is asexual and is not known to produce ascospores as *F. graminearum*. Dispersal of the asexual conidia are either by rain splash or wind (short distances) to the plant hosts (Wagacha & Muthomi 2007). The infection occurs during a short period of high susceptibility during anthesis but the infection routes are not clearly understood (Wagacha & Muthomi 2007).

F. avenaceum

F. avenaceum is a member of the *Roseum* section (Stakheev et al. 2011), and is characterized by long and slim macroconidia produced in large sporodochia but rarely produces microconidia and does not produce chlamydospores. A sexual stage have been detected (Desjardins 2006). *F. avenaceum* may be spread by air, and by the reuse of transplant trays infected with the fungi (Nalim et al. 2009). *F. avenaceum* are widely distributed on a great variety of hosts (Kulik et al. 2011) and is a major component of FHB of cereals in areas with cool weather during the growing seasons such as Europe, Canada, China, and some parts of USA and New Zealand (Desjardins 2006). The specie has also been frequently reported in northern agriculture areas and is a common fungus on living and dead organic substrates like cereal grains (Yli-Mattila et al. 2002).

F. langsethiae

It is difficult to postulate when *F. langsethiae* evolved, but the first reported isolate dates back to 1990 (Torp & Nirenberg 2004). In Torp and Nirenbergs study (2004) on *F. langsethiae* they saw that the specie had a slow growth rate, sparse aerial mycelium and was easily overgrown by

other species growing more rapidly. *F. langsethiae* have similar morphological characters to *F. poae* and *F. sporotrichioides*. When it comes to mycotoxin profile, *F. langsethiae* is mostly related to *F. sporotrichioides*, but based on the morphology; it is very similar to *F. poae* (Schmidt et al. 2004), and was first called powdery poae (Torp & Langseth 1999). Torp and Adler (2004) also saw in their study that strains originally identified as *F. sporotrichioides* were later found to be *F. langsethiae*. This might by an indication of why *F. langsethiae* have not been identified earlier and that the specie is more widespread than first assumed. *F. langsethiae* is only known to produce microconidia (Figure 4) in the aerial mycelia and not sporodochial falcate conidia or chlamydospores (Torp & Nirenberg 2004). Little is known about the source of inoculums and mode of dispersal of *F. langsethiae*, and no sexual stage is known.

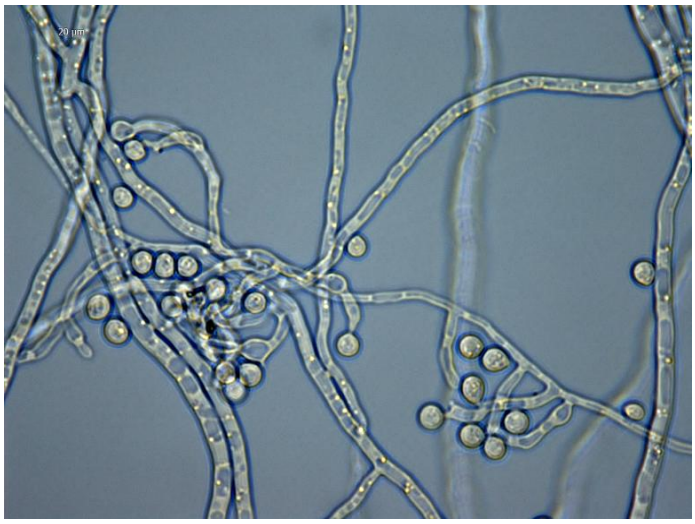


Figure 4: Microconidia of *F. langsethiae*. Photo: Jafar Razzaghian (Bioforsk).

F. langsethiae has been isolated from infected oats, wheat and barley in central and Northern Europe (Medina & Magan 2011) but oats is one of the preferred crops for *F. langsethiae* (Torp and Langseth 1999). *Fusarium* infection in wheat is less common, but the reason for this is still unknown. Whether *F. langsethiae* is an agent able to cause *Fusarium* seedling blight (FSB) in oat, wheat and other small grain cereals have been questionable until now. Studies have revealed that *F. langsethiae* have no or limited ability to infect vegetative tissue of seedlings (Divon et al. 2012), and it has not been able to cause seedling blight on oat or wheat cultivars as compared to other FSB pathogens (Imathiu et al. 2010). There is a discussion of whether the fungus should be considered a pathogen or whether it pocesses endophytic abilities due to symptomless grain and as yet unknown colonization process of *F. langsethiae* (Divon et al. 2012; Torp & Adler 2004).

1.3 Fusariosis in small grain cereals

Many species in the *Fusarium* genus are to be considered as phytopathogenic to many plants under diverse environmental conditions (Doohan et al. 2003; Stakheev et al. 2011).

When it comes to deterioration of growing and stored grain, fungi like *Fusarium* is a major concern. This is because of their ability to cause fusariose, and include diseases like root rot, foot rot, FSB and FHB (Parry et al. 1995; Walter et al. 2010).

FHB or also called *Fusarium* ear blight (FEB) is an important disease of small grain cereals and affects the ears (heads) of the host (Lemmens et al. 2004; Parry et al. 1995). It was in England in 1880s that FHB was first recorded and during the twentieth century it was considered a major threat to wheat and barley (Goswami & Kistler 2004). FHB has increased globally, and during the last decade, reached epidemic levels (Goswami & Kistler 2004). It has been shown that FHB has a negative effect on the crop and symptoms includes brownish spots, bleaching of cereal spikelets, grains smaller than normal, or premature death (Parry et al. 1995). Symptoms on wheat and barley are shown in Figure 5.

Figure 5: Symptoms of FHB on small grain cereals. A: wheat (http://www.gov.mb.ca/agriculture/crops/insects/fad64s00.html) and B: Barley (http://www.ag.ndsu.nodak.edu/aginfo/barleypath/fhbdisease.html).

Infection of cereals is of particular importance in the growing crop, and can occur from anthesis to harvest (Lacey et al. 1999). FHB is to be found in most areas where small grain cereals are grown, but conditions like warm and wet weather around anthesis are usually associated with epidemics of FHB that occurs sporadically (Parry et al. 1995). Many of the *Fusarium* species

are associated with FHB disease of small grain cereals, but *F. graminearum, F. culmorum* and *F. avenaceum* are predominating internationally (Parry et al. 1995). Another and relatively newly identofyed *Fusarium* specie is *F. langsethiae* that are mainly found in oats and barley across Europe, but with almost no symptoms on the crop. It is widespread in the Nordic regions and in England, but is less frequently found all over Europe (Imathiu et al. 2010; Torp & Nirenberg 2004).

Other cereal diseases under the term Fusariosis, is FSB and foot rot. Periods of dry weather predispose cereals to seedling blight and foot rot disease (Doohan et al. 2003). The symptoms depends on the casual pathogen, cereal cultivar and the environmental conditions (Doohan et al. 2003; Imathiu et al. 2010; Parry et al. 1995), but range from pre-emergence death to post-emergence death and lesions on stems and leaves. This can lead to poor crop establishment (Imathiu et al. 2010). FSB is predominantly caused by *F. graminearum* and *F. avenaceum* (Parry et al. 1995) which are more destructive to young seedling, and *F. culmorum* (Parry et al. 1995) which have been reported as more damaging to older seedlings (Imathiu et al. 2010).

The impact of *Fusarium* pathogens

Fusariosis in small grain cereals is a major concern all over the world. This is due to the negative effects on both grain yield and quality (Lemmens et al. 2004) which has a large economic impact (Foroud & Eudes 2009). Species of *Fusarium* are not only considered plant pathogens, but also known as agents producing mycotoxins (Placinta et al. 1999). This makes *Fusarium* species relevant to human and animal health (Torp & Nirenberg 2004). Mycotoxins are secondary metabolites of fungi capable of having acute toxic effects on the animals and humans (Rocha et al. 2005). The largest and most common group of mycotoxins produced by the *Fusarium* species are trichothecenes (Rocha et al. 2005). *Fusarium*- infected cereals like oats, maize, barley and wheat are the main source of trichothecene contamination in human and animal diet (Desjardins 2006). There are four types of trichothecenes, but *Fusarium* species only produces type A and type B (Rocha et al. 2005). Type A is highly toxic and includes T-2 and its deacetylated form HT-2 (Medina et al. 2010). The structure of T-2 is shown in Figure 6. *F. langsethiae* is known as one of the main producers of T-2 and HT-2. The study of Thrane et al. (2004) and Medina and Magan (2011) showed that *F. langsethiae* produced mainly T-2 toxins, but higher concentrations of HT-2 in oats have been detected (Langseth & Rundberget 1999). Occurrence of T-2 and HT-2 are mainly limited to cereal grains in Europe, and has over the last decade represented an increasing problem in the Scandinavian countries the UK, and France (Langseth & Rundberget

1999; Torp & Langseth 1999; van der Fels-Klerx 2010). It have also been found minor levels in Northern and central Europe (Torp & Adler 2004).

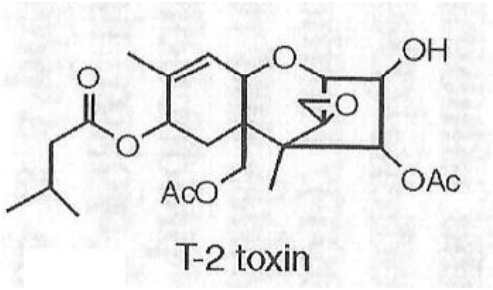


Figure 6: Structure of the mycotoxin T-2.

The most common Type B trichothecenes are deoxynivalenol (DON) and nivalenol (NIV) (Krska et al. 2007) mainly produced by *F. graminearum* and *F. culmorum* (Placinta et al. 1999). Their structures are shown in Figure 7. DON is most frequently detected in cereals in USA and Europe, and NIV is common in parts of Eastern Asia (Langseth & Rundberget 1999; Placinta et al. 1999; Ryu et al. 1996). In Norway, oat grains have been more heavily contaminated with DON than wheat and barley and the concentrations of DON have along with the reports of *F. graminearum* increased in the recent years in Norwegian oat and spring wheat (Brodal et al. 2012).

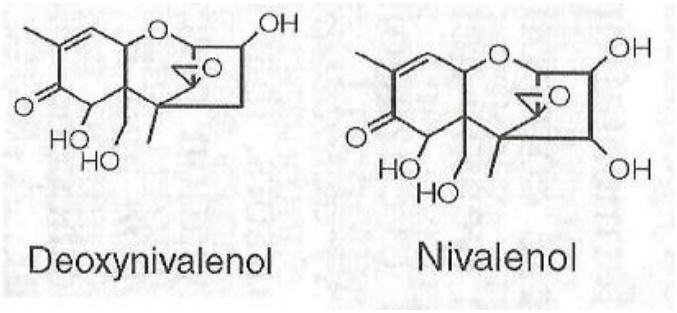


Figure 7: Structures of the mycotoxins deoxynivalenol (DON) and nivalenol (NIV).

F. graminearum and *F. culmorum* also produce zearalenone (ZEA), a mycotoxin considered a cereal storage contaminant (Velluti et al. 2000) but the concentrations are generally low (D'Mello et al. 1999). Another type of *Fusarium* mycotoxins are enniatin, which are nonribosomal and cyclic depsipeptides. Enniatin is produced by *F. avenaceum* and is an important pathogen of small grain cereals.

The *Fusarium* mycotoxins can have toxic effect to the plant host, animals and humans.
Regarding plants, trichothecenes can inhibit germination of seeds and seedling growth at concentrations down to 1-10 µM. The symptoms on the plants, plant tissue and cell line are often necrosis, chlorosis and wilting (Desjardins 2006).
The biological effect of trichothecenes in animal systems was first recognized and quantified in the 1960s and 1970s. This was shortly after the discovery of DON, NIV and T-2 (Desjardins 2006). The T-2 mycotoxin was found to be of special interest because it has been shown to inhibit protein synthesis, induce DNA fragmentation characteristic of apoptosis and has an immunosuppressive effect (Rocha et al. 2005). T-2 toxin was probably the primary agent causing the disease called Alimentary Toxic Aleukia (ATA) that many people were affected by in the former Soviet Union before and during World War II. The outbreak to this disease was due to the consumption of overwintered mouldy grain and this disease resulted in a high rate of mortality (Sarkisov 1954). The toxicity of DON and NIV can lead to growth retardation in eukaryotic cells, disruption of nucleic acid synthesis and mitochondrial function (Desjardins et al. 1993; Foroud & Eudes 2009).
The trichotecenes can inhibit diverse aspects of antibody production and cellular immunity. DON is called vomitoxin and feed containing DON at less than 2.0 µg/g can cause feed refusal and vomiting of swine, but other animals such as cows and chickens can tolerate 10-folds higher levels (Desjardins 2006).

Regulation of mycotoxin levels was mostly a national concern until the late 1990s but gradually economic communities coordinated their mycotoxin regulations (van Egmond et al. 2007). In 2003, at least 99 countries had regulations for mycotoxins in food and/or feedstuff. The regulations are primarily based on known toxic effects, hazard and exposure and specific regulations exist for the various mycotoxins. The first EU-harmonized regulations for mycotoxins in human food came into force in 1998 and they have gradually expanded to a variety of mycotoxins in different food stuff (van Egmond et al. 2007).
The limit of DON is 200 µg/kg in baby food, 500 µg/kg in bread and cakes, 750 µg/kg in pasta and maize, 1250 µg/kg in unprocessed cereals other than durum wheat, oats and maize and 1750 µg/kg in unprocessed oat and durum wheat (Commision 2006).

1.4 Plant – pathogen interactions

Plant defense

Aspiring pathogens are always challenging plants, but disease is rare due to several factors. For one, the plant may not be a specialized host for the pathogen and the pathogens requirements is not maintained (HammondKosack & Jones 1996). The fact that plants possess preformed structural barriers or toxic compounds is another factor that may restrain the pathogen infection, and a third factor is the plants ability to recognize the attacking pathogen by defense mechanisms (HammondKosack & Jones 1996). The majority of plants is resistant to most plant pathogens due to their passive protections against pathogens not specialized to attack a specific host (Dangl & Jones 2001). Plants have a protective waxy cuticular skin layer (Dangl & Jones 2001) and produce different secondary metabolites which may have antifungal activity (Osbourn 1996).

Antimicrobial secondary metabolites include saponins, phenolics, cyclic hydroxamic acids and many others. These components are produced either as part of their normal growth and development, or in response to attack by pathogens (Osbourn et al. 2000). Preformed antimicrobial compounds are present constitutively in healthy plants and represent the first chemical barriers to infection. These molecules protect plants against attack by potential pathogens. Saponin is one class of preformed antimicrobial compound and is found in many plant species. The triterpenoid avenacin saponins for example are found in oat (Osbourn et al. 2000). A number of lines of evidence indicate that avenacins do act as determinants of disease resistance in oats. Saponin deficient (*sad*) mutants were isolated from the diploid oat specie *Avena strigosa* and while the wild type *A. strigosa* line normally fails to give disease when infected with the wheat pathogen *Geaumannomyces. graminis* var *tritici*, the mutants were susceptible to this fungus and developed lesions on theis roots. The *sad* mutant also had increased susceptibility to *F. culmorum*, *F. avenaceum* and *G. graminis* var. *avenae* (Osbourn et al. 2000).

The products of the dominant or semidominant plant resistance (*R*) gene are likely to provide key components for recognition of pathogens. This recognition is hypothesized to be a result of interaction of the product of the R gene and the product derived from the corresponding dominant pathogen avirulence *(Avr*) gene (HammondKosack & Jones 1996). Another role in disease resistance is the hypersensitive response (HR) which is the death of host cells when in contact with pathogens. The HR can occur in a single cell, in several cells or can result in spreading necrotic areas, and this can accompany limited pathogen colonization (HammondKosack & Jones 1996). The signal transduction pathways required for activation and

coordination of plant defense responses is complex and there is no goal to try to explain every step, but an overview is shown in Figure 8.

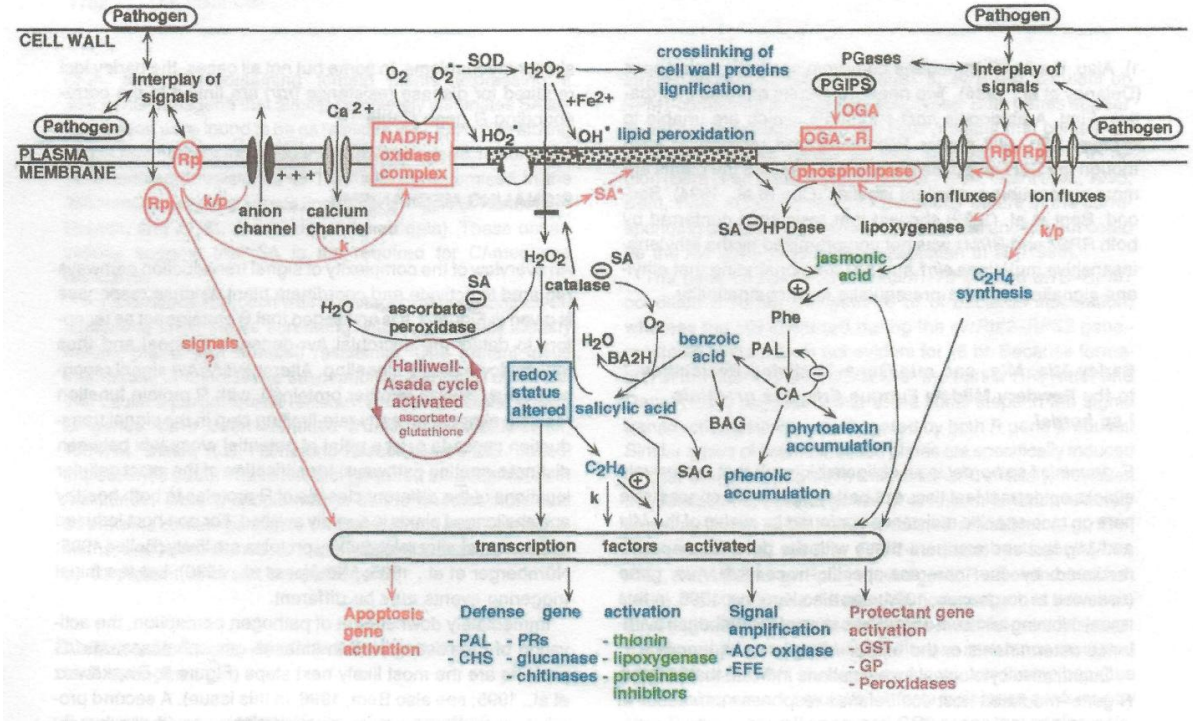


Figure 8: The complexity of signaling events in signal transduction pathways controlling activation of defense responses in plants (HammondKosack & Jones 1996).

A key role in plant defense is the production of reactive oxygen species (ROS) and as seen in Figure 8, it is often one of the first responses of the plant (HammondKosack & Jones 1996). Oxygen is an essential component for living organisms and the formation of reactive oxygen intermediates seems to be usual in aerobically metabolizing cells (Cadenas 1995). ROS are formed as a response to diverse stimuli by specialized physiological reactions: the formation of oxyradicals during the respiratory burst and the release of nitric oxide (Cadenas 1995). Several highly reactive forms of oxygen such as O_2^- (superoxide anion), H_2O_2 (hydrogen peroxide) and OH- (hydroxyl radical) are toxic at even low levels (HammondKosack & Jones 1996).

Fungal virulence

Pathogen infection and disease arise successfully if the preformed plant defenses and the activated defense responses of the plant are unsuitable and unsuccessful, or if the plants are unable to detect the pathogen (HammondKosack & Jones 1996). For the fungi or other microbes to reach the plant cells, it must try to enter the plant cuticle or the plant cell wall. This is done by producing extracellular enzymes that degrades plant cell wall components (Kikot et al. 2009). These extra-cellular cell wall-degrading enzymes (CWDE) are an important source of nutrients

for the pathogen when plant cell wall polymers are digested. The production of CWDE also enables the pathogen to penetrate, grow and infect through the plant tissue (Kikot et al. 2009). Fungi can penetrate the host cells with specialized structures like appressoria or haustoria, but species with no penetration structures must enter the host through natural openings or by infection hyphae (Kikot et al. 2009). One well studied plant pathogen is *F. graminearum* that can ensure their position of the plant by living on the plant without causing any symptoms, and then has the competitive advantage when the plants become senescent and wilts. *F. graminearum* is able to penetrate and invade a host with the help of secreted cell wall degrading enzymes. Examples of these enzymes are cutinases that might play an important role in the penetration of plant surfaces), lipases that might participate in the degradation of plant cuticle, and pectic enzymes that produce modification of cell wall structure that increase the degradation of cell wall components by other enzymes (Kikot et al. 2009).

As already told, triterpenoid avenacin saponins are found in oat, and protect against attack by pathogen (Osbourn et al. 2000). The fungus *Geaumannomyces graminis* is unable to infect oat, but causes "take all" disease in wheat and barley. The avenacin detoxifying enzyme avenacinacinase has been shown to be required for *G. graminis* var. *avenae* to infect oats (Osbourn et al. 2000) Resistance to avenacins may be a general requirement for fungi that infect oat roots.

A survey of a collection of different fungal isolates from field-grown cereal plants showed that nearly all fungi isolated from oat roots were avenacin resistant (Osbourn et al. 2000) Wheat is a non-saponin producing cereal and both avenacin-sensitive and avenacin- resistant fungi were isolated from the wheat roots (Osbourn et al. 2000). The majority of avenacin resistant fungi in the survey were able to degrade avenacin A-1 and this suggested that avenacin A-1 is likely to influence the development of fungal colonies within oat roots (Osbourn et al. 2000).

As we know, a role in plant defense is the production of reactive oxygen species (ROS). All cell components like lipids, nucleic acids, proteins and carbohydrates are sensitive to damage by ROS (Cadenas 1995), and organisms that grow in the presence of oxygen need mechanisms for coping with the toxic effects (Deacon 2006). Several vitamins and micronutrients as well as enzymes such as superoxide dismutase (SODs) are active at quenching these free radicals and are required for their enzymatic detoxification. SODs are present in the cytosolic and mitochondrial compartments which catalyse the dismutation of O_2^- to O_2 and H_2O_2. This is the first line of defense against ROS (Cadenas 1995).

Nonribosomal peptide synthetase (NRPS) are multifunctional proteins responsible for the production of small nonribosomal peptides (Oide et al. 2006). The majority of characterized NRPSs and their nonribosomal peptide products to date have been from bacteria and filamentous fungi (Lee et al. 2005; Oide et al. 2006). This synthesis has not been shown to occur in plants but the products of certain fungal NRPSs play critical roles in plant-microbe interactions (Oide et al. 2006).

1.5 Fungal -fungal interactions

There are many ways fungi can interact with each other, but there are three broad categories of interactions among fungi, although there might be overlaps between these. Species that have the ability to exclude another species by competition by growing faster and exploiting the resource more efficient represents one category. This is probably the most common type of interactions in natural environments. A second category is when species can exclude or replace another by antagonism by directly affecting the other organism via production of antibiotics or other inhibiting compounds. Species of Ascomycota and mitosporic fungi including *Penicillium, Aspergillus, Fusarium* and *Thricoderma* are some of the most common fungi that produce antibiotics and other inhibiting compounds in natural and agricultural environments. The last broad category is commensalism (coexistence where one benefit) and mutualism (mutual benefit) (Deacon 2006). The interactions among the fungi can both have an impact on each other and their host. A study using isolates of *Microdochium nivale* var. *majus, M. nivale* var. *nivale* and *F. culmorum* was conducted to try to give increased understanding of interaction (Simpson et al. 2004). The study showed that a greater disease occurred on wheat and rye seedlings when mix-inoculated with *M. nivale* var *majus* and var. *nivale* compared to inoculated alone. The seedling experiment also showed that the specie with advantage did not colonize significantly more than when inoculated alone (Simpson et al. 2004). They also saw that mixed-inoculation of *M. nivale* and *F. culmorum* led to reduced levels of *M. nivale*, and that in mixed liquid culture, both species had reduced growth relative to single inoculums control cultures (Simpson et al. 2004). A study using *F. moniliforme*, *F. proliferatum* and *F. graminearum* (Velluti et al. 2000) showed that the population of *F. moniliforme* and *F. proliferatum* were reduced by the presence of *F. graminearum* under the conditions tested. They also saw that the presence of *F. moniliforme* and *F. proliferatum* had a limited inhibitory effect on populations of *F. graminearum* (Velluti et al. 2000).

1.6 Purpose of the present study

One of the aims in the present study was to determine amounts of *Fusarium* DNA to investigate whether the interactions between the *Fusarium* species affect the DNA amounts during early infection of oats. It was also an aim to investigate the Interactions between different *Fusarium* species and how they might affect each other when co-inoculated *in vitro* on SNA plates.

A second objective was to look into the expression of different genes in *F. langsethiae* during infection of oat. The infection process of F. langsethiae in small grain cereals is not known. Therefore, one aim was to get a better understanding on how *F. langsethiae* grows and infects oats in a histological characterization study. A Detached leaf assay was used to investigate the infection process and the interactions between the fungus and the plant.

This will be useful in understanding how different *Fusarium* species interact with each other, infection processes of *F. langsethiae* and the interactions between the fungus and the plant during infection of oats.

Chapter 2 Materials

2.1 Buffers, chemicals and enzymes

Table 1: List of buffers, chemicals and enzymes and their suppliers

Name	**Supplier**
2x TaqMan® PCR Master Mix	Eurogentec, Seraing, Belgium
Agarose (SeaKem®LE)	Lonza, Basel, Switzerland
Bacto agar	Bectoa, Dickinson and Company, 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 ($MgSO_4$ x $7H_2O$)	Merck KGaA, Darmstadt, Germany
Nitro blue tetrazolium (NBT)	Merck KGaA, Darmstadt, Germany
Nuclease-free water	Ambion®, Austin, TX, USA
Paraformaldehyde	Kemetyl Norge AS, Vestby, Norway
Potassium chloride (KCl)	Merck KGaA, Darmstadt, Germany
Potassium cyanide (KCN)	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 2x	Applied Biosystems, UK
RNAse-free water	Qiagen, Venlo, The Netherlands
Sodium chloride (NaCl)	Merck KGaA, Darmstadt, Germany
Sodium phosphate (Na_2HPO_4)	Merck KGaA, Darmstadt, Germany
Sucrose	Dechefa, Haarlem, The Netherlands

2.2 Kits

Table 2: List of kits and their suppliers

Name	**Supplier**
Agilent RNA 6000 Nano Kit	Agilent Technologies, Germany
DNA-free™ Kit	Applied Biosystems, USA/Canada
DNeasy Plant Mini Kit	Qiagen® Venlo, The Netherlands
Fast DNA® SPIN Kit for Soil	MP Biomedicals, Santa Ana, CA, USA
Spectrum™ Plant Total RNA Kit	Sigma-Aldrich, St. Louis, MO, USA
Super Script® VILO ™ cDNA synthesis Kit	Invitrogen™, Carlsbad, CA, USA

2.3 Solutions

Table 3: Solutions with recipes

Solutions	**Recipes**
0.5 % water agar (WA)	5 g Bacto agar was added to 1 l water, and the solution was autoclaved.
1x TBE buffer	1x TBE buffer was diluted from 10 x TBE. 1 l 10x TBE was made with 108 g Tris-base, 55 g boric acid and 40 ml EDTA (0.5 M, pH 8).
Loading buffer	0.25 % bromphenolblue and 40 % sucrose was diluted in sterile distilled water (SDW).
Mung bean agar (MBA)	40 g mung beans, 1 l tap water and 15 g bacto agar. The beans was boiled in 1 l tap water in 23 minutes and then filtrated in a cheese cloth. Water was added to the solution to a volume of 1 l, and agar was added before autoclaving.
Synthethic nutrient agar (SNA)	1.0 g KH_2PO_4 1.0 g KNO_3 0.5 g $MgSO_4 * 7 H_2O$ 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.
Synthetic nutrient broth (SNB)	The same recipe as SNA, but no agar.
Synthetic nutrient broth (SNB) with Hygromycin B	The same recipe as SNB. The solution was stirred and autoclaved and 600µl Hygromycin B was added.
1x Phosphate Buffered Saline (PBS buffer) pH 7.4	8 g NaCl 0.2 g KCL 1.44 g Na_2HPO_4 0.24 g KH_2PO_4 1. 0 l SDW The solution was stirred and autoclaved

2.4 Laboratory equipments

Table 4: List of laboratory equipments and their supplier

Method	Name	Supplier
cDNA synthesis	PCR plate, Skrited	Abgene® Thermo Scientific, UK
	Domed cap strip	Abgene® Thermo Scientific, UK
	Thermal cycler, 621BR4130	Biorad, Singapore
	Bioanalyzer 2100	Agilent Technologies, Santa Clara CA, USA
Centrifugation and homogenization	Biofuge pico	Kendro, Germany
	Centrifuge 5810R	Eppendorf®, Hamburg, Germany
	Fast prep®- 24	MP Biomedicals, Irvine, CA USA
	Galaxy mini	VWR™, Korea
	Vortex, labdancer S40	VWR™, Germany
Gel electrophoresis	Geltray Subcell® GT	Biorad, Singapore
	Powerpac 300	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, USA
	Optical Adhesive Film	MicroAmp™, Applied Biosystems, USA
	7900 Fast Real-Time PCR system	Applied Biosystems, USA

2.5 Software

Table 5: List of software and sources/supplier

Software	Source/ Supplier
Best keeper version 1	(Pfaffl et al. 2004)
Molecular Imager Gel Doc XR System	Biorad, Italy
Quantity One version 4.5.1	Biorad®, Hercules, CA, USA
REST-MCS© version 2	(Pfaffl et al. 2002)
Sequence Detection Software (SDS) version 2.2.1	Applied biosystems, USA

2.6 Primers

Table 6: Specific primers used in the quantification of DNA

Target organism	Target DNA	primers/probes	sequence (5´-3´)
F. avenaceum[a]	RAPD fragment	TMAV-f TMAV-r TMAV-p	AGATCGGACAATGGTGCATTATAA GGCCCTACTATTTACTCTTGCTTTTG TET-CTCCTGAGAGGTCCCAGAG ATGAACATAACTTC-TAMRA
F. culmorum[b]	RAPD fragment	culmMGB-f culmMGB-r culmMGB-p	TCACCCAAGACGGGAATGA GAACGCTGCCCTCAAGCTT FAM-CACTTGGATATATTTCC-MGB
F. graminearum[c]	β-tubulin gene	FGtub-f FGtub-r FGtub-p	GGTCTCGACAGCAATGGTGTT GCTTGTGTTTTTCGTGGCAGT TET-ACAACGGCACCTCTGAGCTCCAGC-TAMRA
F. langsethiae[a]	rDNA	TMLAN-f TMLAN -r TMLAN -p	GAGCGTCATTTCAACCCTCAA GACCGCCAAATTTGGG FAM-AGCTTGGTGTTGGGATCT GTCCTTACCG-TAMRA
A. sativa Cherepanov[d]	Cytochrome c oxidase gene	COX554-f COX554-r COX554-p	GGTTGTTGCCACCAAGTCTCTT TGCCGCTGCCAACTTC FAM-CTCCTATTAAGCTCAGCCTT-MGB

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

Table 7: primers used in the gene expression study

Target organism	Target cDNA	primers	Sequence (5´-3´)
F. langsethiae	Superoxide dismutase gene	Sudis f Sudis r	TCGACATAAGCAGCCTTTCC TTGCGTATCGTTACCACCAA
	Nonribosomal peptide synthetase gene	NRPS f1 NRPS r1 NRPSf2 * NRPSr2 *	TGTGTTGTCTCGCAAATGGT AGATCGCTTGGAAAGACGAA TTCGTCTTTCCAAGCGATCT GTTACGCATCCTCGAAAAGC
	Unknown gene	Fl-est17042 f Fl-est17042 r	ATCCTACGCCTTCTCCCAGT TCGAATAGTGCCCTGGAATC
	Depudecin polyketide syntase gene	DepPKS f1 DepPKS r1	TCCTTCTTCCCAGTCGCTTA ATGTAGCGTCAGAGCGGAGT
	Ubiquitin conjugating enzyme gene	FlanUBC f2 FlanUBC r2	GGTGTCTTCTTCCTTGCGATT AGAAGAACTTTGGAGATGGTCA
	β-tubulin gene	FlanBTUB f2 FlanBTUB r2	GTCGAGAACTCTGACGAAACC GGAAACGGAGACAGGTCGTA

*Primers tested, but not used in RNA quantification

2.7 Strains

Table 8: Strains used in different experiments in the present study

***Fusarium* spp.**	**Strain**	**Experiment**
F. avenaceum	6A	Quantification of DNA *In vitro* co-inoculation
	11A	Quantification of DNA
	21A	Quantification of DNA
F. culmorum	9C 14C 27C	Quantification of DNA
	17 C	*In vitro* co-inoculation
F. graminearum	2007-059 2008-028	Quantification of DNA
	2008-140	Quantification of DNA *In vitro* co-inoculation
F. langsethiae wt	2010-058 IBT9951 IBT9956	Quantification of DNA and RNA
	9821-16-1 (IBT 9951)	*In vitro* co-inoculation Histological characterization Detached leaf assay
Fl- GFP	9821-16-1 (IBT 9951)	*In vitro* co-inoculation Histological characterization Detached leaf assay

Chapter 3 Methods

3.1 Greenhouse experiment

The greenhouse experiment explained here was previously performed before the beginning of my study (main responsible Heidi U. Aamot). Oat plants had been sprayed with fungal isolates of *F. avenaceum* and *F. langsethiae,* both alone and in pair wise combinations with each other and with *F. culmorum* and *F. graminearum,* separately. All species were used as mixtures of equal amount of three strains. A list of strains is given in Table 8, and a total list of all treatments in the greenhouse experiment is given in Table 9.

3.1.1 Production of *Fusarium* inoculums

One ml of initial conidial suspension was placed on the surface of Mung bean agar (MBA) in petri dishes and spread by tilting the dishes. Cultures were grown at 22 °C for 15-20 days and conidia were then rubbed from the surface of MBA using sterile distilled water (SDW) and a sterile glass rod. The suspensions were filtered through sterile cotton to remove fragments of mycelia and agar. Spore concentrations were determined using a Bürker hemacytometer and adjusted to working concentrations using SDW and gelatin.

3.1.2 Growth of plant material

Oat (*Avena sativa*) cv. Lena was grown in a greenhouse using 2 l pots (LOG A/S, Norway) with perlite-added "P-jord", a mix of peat with 10 % soil (LOG A/S, Norway). Ten seeds were sown per pot and total number of pots for each treatment was 12-14. The plants were grown in white light (HPI) with a 14 h photoperiod, relative humidity (RH) of 60 %, and minimum day/night temperatures of 25/18 °C. After five weeks the minimum day/night temperatures were lowered to 20/15 °C.

3.1.3 Inoculations and sampling

Oat panicles were spray inoculated during anthesis (Zadoks growth stage 60-65 (Zadoks et al. 1974)) with approximately 0.7 ml conidial suspension per oat panicle. All inoculums contained 0.25 % gelatin to lower the surface tension of the conidial suspension. The plants were then covered by plastic bags for 6 days to ensure high humidity during initial establishment and infection. The plants were given following treatments; inoculations with *F. avenaceum* and *F.*

langsethiae at two different concentrations (0.5×10^5 and 10^5 spores/ml for *F. avenaceum*, and 2×10^6 and 4×10^6 spores/ml for *F. langsethiae*), pair-wise combinations of *F. avenaceum* with *F. culmorum*, *F. graminearum* and *F. langsethiae* (0.5×10^5 spores/ml of each species, with exception of *F. langsethiae* where a concentration of 2×10^6 spores/ml) was used. The same combinations were also made with *F. langsethiae* together with *F. graminearum* and *F. culmorum.* Oat sprayed with SDW and 0.25 % gelatin were used as controls.

All treatments were done in three biologically independent repeats (i.e. inoculums were prepared independently for each repeat, and plants were sown at two weeks interval). After inoculation, the pots were placed randomly in the greenhouse. Five panicles were harvested at 6 days post inoculation (dpi), 10dpi and 14dp. Panicles from different plants and pots, but from the same treatment were wrapped in aluminum foil and stored at - 80°C in aluminum foil. Table 9 gives an overview of the different treatments used in this study.

Samples with the letter K are control samples not actively infected and only sprayed with SDW with 0.25 % gelatin. The first number in Table 9 (1, 2, 7, 8, 12, 13 and 14) represent the different treatments of *Fusarium* species given to the plants and the number following (6, 10 and 14) represent number of days after inoculation the sample was collected. Two different spore concentrations were used for *F. avenaceum* and *F. langsethiae*. Treatment number 1 and 10 had the lowest concentrations and treatments 2 and 11 had a 2x concentrations. The lowest concentrations were used in pair-wise combinations.

I started my study with randomly collecting oat seeds from several different panicles from the aluminums foil found in the -80 °C freezer. Oat seeds were then stored in 1.5 ml tubes at -80 °C. The amount of material was approximately the same for all samples (six oat seeds).

Table 9: Sample name and inoculums used to inoculate the oat-plant during anthesis for quantification of fungal DNA in oat.

Sample name	Inoculums	Sample name	Inoculums
K.6.I	Not actively infected	10.6.I	*F. langsethiae*
K.6.II	Not actively infected	10.6.II	*F. langsethiae*
K.6.III	Not actively infected	10.6.III	*F. langsethiae*
K.10.I	Not actively infected	10.10.I	*F. langsethiae*
K.10.II	Not actively infected	10.10.II	*F. langsethiae*
K.10.III	Not actively infected	10.10.III	*F. langsethiae*
K.14.I	Not actively infected	10.14.I	*F. langsethiae*
K.14.II	Not actively infected	10.14.II	*F. langsethiae*
K.14.III	Not actively infected	10.14.III	*F. langsethiae*
1.6.I	*F. avenaceum*	11.6.I	*F. langsethiae 2x*
1.6.II	*F. avenaceum*	11.6.II	*F. langsethiae 2x*
1.6.III	*F. avenaceum*	11.6.III	*F. langsethiae 2x*
1.10.I	*F. avenaceum*	11.10.I	*F. langsethiae 2x*
1.10.II	*F. avenaceum*	11.10.II	*F. langsethiae 2x*
1.10.III	*F. avenaceum*	11.10.III	*F. langsethiae 2x*
1.14.I	*F. avenaceum*	11.14.I	*F. langsethiae 2x*
1.14.II	*F. avenaceum*	11.14.II	*F. langsethiae 2x*
1.14.III	*F. avenaceum*	11.14.III	*F. langsethiae 2x*
2.6.I	*F. avenaceum 2x*	12.6.I	*F. langsethiae + F. avenaceum*
2.6.II	*F. avenaceum 2x*	12.6.II	*F. langsethiae + F. avenaceum*
2.6.III	*F. avenaceum 2x*	12.6.III	*F. langsethiae + F. avenaceum*
2.10.I	*F. avenaceum 2x*	12.10.I	*F. langsethiae + F. avenaceum*
2.10.II	*F. avenaceum 2x*	12.10.II	*F. langsethiae + F. avenaceum*
2.10.III	*F. avenaceum 2x*	12.10.III	*F. langsethiae + F. avenaceum*
2.14.I	*F. avenaceum 2x*	12.14.I	*F. langsethiae + F. avenaceum*
2.14.II	*F. avenaceum 2x*	12.14.II	*F. langsethiae + F. avenaceum*
2.14.III	*F. avenaceum 2x*	12.14.III	*F. langsethiae + F. avenaceum*
7.6.I	*F. avenaceum + F. culmorum*	13.6.I	*F.langsethiae + F. culmorum*
7.6.II	*F. avenaceum + F. culmorum*	13.6.II	*F.langsethiae + F. culmorum*
7.6.III	*F. avenaceum + F. culmorum*	13.6.III	*F.langsethiae + F. culmorum*
7.10.I	*F. avenaceum + F. culmorum*	13.10.I	*F.langsethiae + F. culmorum*
7.10.II	*F. avenaceum + F. culmorum*	13.10.II	*F.langsethiae + F. culmorum*
7.10.III	*F. avenaceum + F. culmorum*	13.10.III	*F.langsethiae + F. culmorum*
7.14.I	*F. avenaceum + F. culmorum*	13.14.I	*F.langsethiae + F. culmorum*
7.14.II	*F. avenaceum + F. culmorum*	13.14.II	*F.langsethiae + F. culmorum*
7.14.III	*F. avenaceum + F. culmorum*	13.14.III	*F.langsethiae + F. culmorum*
8.6.II	*F. avenaceum + F. graminearum*	14.6.I	*F.langsethiae + F. graminearum*
8.6.III	*F. avenaceum + F. graminearum*	14.6.II	*F.langsethiae + F. graminearum*
8.10.I	*F. avenaceum + F. graminearum*	14.6.III	*F.langsethiae + F. graminearum*
8.10.II	*F. avenaceum + F. graminearum*	14.10.I	*F.langsethiae + F. graminearum*
8.10.III	*F. avenaceum + F. graminearum*	14.10.II	*F.langsethiae + F. graminearum*
8.14.I	*F. avenaceum + F. graminearum*	41.10.III	*F.langsethiae + F. graminearum*
8.14.II	*F. avenaceum + F. graminearum*	14.14.I	*F.langsethiae + F. graminearum*
8.14.III	*F. avenaceum + F. graminearum*	14.14.II	*F.langsethiae + F. graminearum*
		14.14.III	*F.langsethiae + F. graminearum*

3.1.4 Extraction of genomic DNA

The first step towards quantifying the amount of *Fusarium* DNA in the samples was to extract genomic DNA from the collected oat grains using the FastDNA®SPIN Kit for Soil and the FastPrep® Instrument (MP Biomedicals, CA, USA). The extraction procedure is outlined below.

Cell lysis

1. Oat material (approximately 3 oat seeds from different panicles) were ground to powder by using liquid nitrogen and a porcelain mortar, and added to a Lysing Matrix E tube.
2. A volume of 980 µl Sodium Phosphate Buffer and 122 µl MT Buffer was added to the tube with oat-powder and further homogenized in the FastPrep® Instrument for 40 seconds at a speed setting of 6.0.
3. The next step was centrifugation at 13 000 x g for 10 minutes to allow settling of a pellet. All the following centrifugation steps were run at 13 000 x g.

Protein removal

4. The supernatant was transferred to a clean 2.0 ml microcentrifuge tube and then mixed with 250 µl Protein Percipitation Solution (PPS) by shaking the tubes by hand 10 times. A centrifugation was done for 6 minutes to remove the proteins and the supernatant was transferred to a new clean 2.0 ml tube.

Extraction

5. One ml resuspended Binding Matrix was added to the supernatant and inverted by hand for 2 minutes to allow binding of DNA, followed by 3 minutes incubation in a rack at room temperature (RT) to allow settling of silica matrix.
6. A volume of 500 µl of the supernatant was removed and discarded, and after resuspension of the remaining amount, 650 µl was transferred to a SPIN MT Filter and centrifuged for 1 minute. The catch tube was emptied and the remaining amount was added before a new centrifugation was performed as before. The catch tube was emptied again.
7. The next step was to resuspend the pellet in the catch tube with 500 µl prepared SEWS-M and then centrifuged for 1 minute. The catch tube was emptied and replaced, and without any addition of liquid, a new centrifugation step was performed for 2 minutes to dry the pellet.

8. The catch tube was discarded and replaced with a new, clean catch tube. The sample was then air dried for 5 minutes at RT.
9. Binding Matrix above the SPIN filter was gently resuspended in 100 µl of DNase/Pyrogen-Free Water (DES) followed by a last centrifugation step for 1 minute to bring eluted DNA into the clean catch tube.
10. The eluted DNA was transferred to a clean 2.0 ml tube and stored at -20 ºC.

DNA from all the *Fusarium* species and oat was to be used as standards in the standard curves during DNA quantification. To make these standards, spores of the different *Fusarium* species were incubated on plates with SNA medium. After a few days of incubation in RT, the SNA medium with fungal growth was cut in small pieces, put in 0.5 ml SNB medium in a 500 ml flask and placed in a shaking incubator at 150 rpm for 3 days at 24 °C. Mycelia was concentrated using a vacuum filter, and DNA was extracted using the DNaesy Plant Mini Kit (Qiagen®, Venlo, The Netherlands). The procedure is outlined below.

Cell lysis

1. Approximately 100 mg of fungal material were ground to powder in liquid nitrogen. A volume of 400 µl Buffer AP1 and 4 µl of RNase A stock solution were added to the ground fungal material and was then vortexed vigorously.
2. To lyse the cells, the mixture was incubated for 10 minutes at 65 °C and mixed by inverting the tube 2 or 3 times during this incubation.

Protein removal

3. A volume of 130 µl of Buffer AP2 was added to the lysate, to precipitate detergent, proteins and polysaccharides. After brief mixing, the solution was incubated on ice for 5 minutes and then centrifuged for 5 minutes at 20 000 x g.
4. The lysate was pipetted into a QIAshreder Mini spin column placed in a 2 ml collection tube and centrifuged for 2 minutes at 20 000 x g. The flow-through was transferred to a new 2 ml eppendorf tube and 1.5 volumes of Buffer AP3/E was added and mixed by pipetting.

Extraction

5. A volume of 650 µl of the mixture from step 4 were transferred to a DNeasy Mini column placed in a 2 ml collection tube and centrifuged for one minute at 6 000 x g. The

flow-through was discarded and this step was repeated with the remaining sample volume from step 4.

6. The DNeasy Mini spin column was placed into a new 2 ml collection tube and 500 µl Buffer AW was added and centrifigated for 1 minute at 6 000 x g. The flow-through was discarded and another 500µl of Buffer AW was added to the DNeasy Mini spin column and then centrifuged for 2 minutes at 20 000 x g to dry the column.

Elution

7. The spin column was transferred to a 2 ml microcentrifuge tube and 100 µl of Buffer AE was pipetted directly onto the DNeasy membrane, and then incubated for 5 minutes at RT. After this incubation the tube was centrifuged for 1 minute at 6 000 x g to elute.
8. Step 7 was repeated.

Extracted DNA from uninfected oat (control sample) was used as oat standards. Measurement on Nanodrop and dilution with SDW was used to get the starting concentrations already described.

One µl of the extracted DNA to be quantified was analyzed by gel electrophoresis. Agarose gels were prepared as described below.

1. A 0.8% agarose gel was prepared with Seakam LE agarose and 1x TBE buffer. The solution was mixed and heated in a microwave oven to dissolve the agarose.
2. When all agarose was dissolved, the solution was cooled down to approximately 60 °C and one drop of 0.07 % ethidium bromide (EtBr) per 50 ml was added.
3. A gel mould with loading comb was prepared and the solution with agarose, buffer and EtBr was poured in. All bubbles were removed quickly by a pipette-tip. The loading comb was removed when the gel had set, and the gel was placed in a gel chamber.
4. One µl of the extracted DNA was mixed with 4µl DSW and 1µl loading buffer. All samples were loaded into the gel wells and a DNA ladder of known size (1 kb) was used as a molecular marker.
5. Gels with the size of 200 ml were run at 160 V, 100 ml gels at 65 V and 50 ml gels at 40 V. The run was completed when the sample was half way down the gel, and the gels were then visualized under UV light in a molecular Imager Gel Doc XR System (BioRad, Italy).

3.1.5 Quantification of *Fusarium* DNA by real -time quantitative PCR

Real time quantitative PCR (qPCR) was carried out to calculate the amount of *Fusarium* DNA in the infected oat samples. Oat DNA (*cytocrome c oxidase* gene (*Cox*)) was also quantified in all the samples to normalize the amount of extracted fungal DNA in each sample. Oat and *Fusarium* DNA was quantified by TaqMan-qPCR using the Applied Biosystems 7900HT instrument with a standard 96-well block (Applied Biosystems, USA). Extracted DNA of all samples was diluted 5x and 50 x in a 96-well plate. The 5 x dilutions were used to quantify amount of *Fusarium* DNA, while the 50x dilutions were used to quantify oat DNA. To achieve as correct volumes as possible, an *ep*Motion 5070 pipetting robot (Eppendorf®, Hamburg, Germany) was used to pipette sufficient amounts of the reagents.

The target DNA and the sequences of primers and probes for each assay are listed in Table 6. The probes were labeled at the 5`ends with either reporter dye FAM (6-carboxyfluorescenin) or TET (6-carboxy-4.7.2´.7´-tetrachlorofluorescenin), and the 3`ends with either the quencher dye TAMRA (6-carboxytetramethylrhodamine) or with minor groove binder (MGB). A volume of 250 µl of forward primer (3 pmol/µl), 250 µl of reverse primer (3 pmol/µl) and 250 µl of probe (1 pmol/µl) was mixed with 300 µl of SDW to get the primer/probe mix with a volume of 1050 µl. The assay reaction volumes per sample are given below.

Real-time reaction mixture

12.5 µl	2x TaqMan master mixture
10.5 µl	Primer/probe mixture
2µl	Template (DNA)
25µl	Total volume

Final primer and probe concentrations in the real-time PCR reactions mixture were 0.3 pmol/µl and 0.1 pmol/µl respectively.

Reactions were run with the following parameters: 2 min at 50 °C and 10 min at 95 °C, followed by 1 min at 95 °C and 1min at 60 °C for 40 cycles. Amount of DNA in the samples was quantified by a standard curve algorithm using 2 µl of five 10-fold dilutions of known amounts of DNA, starting with 0.5 ng/µl for *Fusarium* DNA and 50 ng/µl for oat DNA.

Data analysis

The Sequence Detection Software (SDS) version 2.2.1 (Applied Biosystems, USA) was use to analyze the data from the qPCR reaction. All samples having a quantification cycle (Cq) value of

36 or higher were considered as negative. Technical replicates that ranged one Cq value or more from each other were repeated in a new reaction. The SDS program calculated the amount of fungal DNA in the samples based on the standard curves. The mean of the two corresponding replicates were used in further calculations. The dilution factors were included in the calculations, and to get the quantity values for the fungal DNA from ng to pg, it was multiplied with 1000. Amount of fungal DNA in inoculated grain was then calculated relative to plant DNA by dividing pg fungal DNA on the respective amount of ng plant DNA and multiplied with 100 to get pg fungal DNA / 100 ng oat DNA. The mean of the biological replicates were used to illustrate the results, and the standard deviations between the biological replicates were calculated in excel according to formula 1.

(1) $$\sqrt{\frac{\sum (x-\bar{x})^2}{n}}$$

An unpaired, two ways T-test was conducted in excel to test if any possible observed differences in DNA amount between pair-wise inoculation of two *Fusarium* species and only one *Fusarium* species were significant.

3.1.6 Extraction of RNA

The samples from the previously described greenhouse were used in this study. Oat samples infected with *F. langsethiae* (treatment 10 and 11) and control samples (K) (Table 9) were extracted for total RNA to be used in a gene expression study. The first step was to extract total RNA from collected oat grains using Spectrum™ Plant Total RNA Kit (Sigma-Aldrich, St. Louis, MO, USA). The extraction procedure is outlined below

Cell lysis

1. The samples were ground to a fine powder in liquid nitrogen using a mortar and pestle. The powder was then collected in a 2 ml tube, and to make sure that the frozen materiel did not thaw before Lysis Solution was added, the tubes were stored in liquid nitrogen until all samples was handled and ready to continue with.
2. The Lysis Solution was first supplemented with 2-mercaptoethanol (1:10) before use. For every 1 ml of Lysis Solution, 10 µl of 2-ME was added, and each DNA preparation required 500 µl of the mixture. The Lysis Solution/2ME Mixture was mixed briefly and 500 µl was added to the sample tubes. After immediate vortexing for at least 30 seconds,

the samples were incubated at RT for 3-5 minutes, and then centrifuged at 13 000 x g for 5 minutes. All the following centrifugation steps were run at 13 000 x g.

3. The next step was to pipette the lysate supernatant into a Filtration Column seated in a 2 ml Collection Tube. To remove residual debris, the samples were centrifuged for 2 minutes. After centrifugation there was still some liquid on top of the column. The centrifugation step was therefore repeated as described above.
4. All the clarified flow-through lysate was now in the 2 ml Collection Tube and to bind the RNA, 250 µl of Binding Solution was added and mixed by pipetting at least 5 times. The mixture was then transferred to a Binding Column seated in a 2-ml Collection Tube and centrifuged for 1 minute. The flow-through liquid was decanted and the tube was tapped upside down on a clean absorbent paper to drain the residual liquid.

DNase treatment

5. When the nucleic acid had bound to the binding column as described in step 4, on-column DNase digestion was conducted to remove DNA. The first step in this procedure was to pipette 300 µl of Wash Solution 1 in to the binding column and centrifuged for 1 minute. The flow-through was decanted and the Collection Tube was tapped on an absorbent paper as described earlier. The second step was to add a combination of 10 µl of DNase 1 and 70 µl of DNase digestion buffer for each sample. This combination was mixed gently by pipetting and then 80 µl was added directly to the center of the filter inside the Binding Column. The samples were then incubated at room temperature for 15 minutes.

Washing of the sample

6. A volume of 500 µl of Wash Solution 1 was pipette into the Binding Column and was then centrifuged for 1 minute. The flow-through was decanted as earlier described.
7. A second column wash with 500 µl of Wash Solution 2 was then pipetted into the column and the tubes were centrifuged for 30 seconds. The flow-through liquid was decanted and residual liquid was removed using a clean absorbent paper as earlier described.
8. Another 500 µl of Wash Solution 2 was added to the column and the tubes were centrifuged for 30 seconds. The flow-through was decanted and the 2 ml Collection Tubes were tapped on a clean absorbent paper to remove residual liquid.
9. The tubes were then centrifuged for 1 minute to dry the column.

Elution

10. The dry column was removed into a new, clean 2-ml Collection Tube and 50 µl of Elution Solution was added directly onto the center of the binding matrix inside the column. The tubes sat for 1 minute and then centrifuged for 1 minute to elute. Purified RNA was now in the flow-through eluate and was stored at – 80 °C.

3.1.7 DNase treatment

The samples were then DNase treated with DNA-free™ Kit (Applied Biosystems, USA/Canada) to get rid of residual DNA, in order to get clean RNA template. The procedure is outlined below. To the RNA, 0.1 volume of 10 x DNase I buffer (5 µl) and 1 µl of rDNase 1 was added and mixed gently.

1. The samples were then incubated at 37 °C for 20-30 minutes.
2. After incubation, 0.1 volume of resuspended DNase Inactivation Reagent was added (5 µl). The tubes was mixed well and then incubated at RT for 2 minutes. The contents were occasionally mixed during this incubation to redisperse the DNase Inactivation Reagent.
3. The last step was to centrifuge the samples at 10.000 x g for 1.5 minutes and transfer the RNA to a clean tube.

3.1.8 Test for residual DNA

Real-time quantitative PCR was done as described under section 3.1.5, using *Cox* as assay to check if there was any genomic DNA left in the samples using undiluted RNA as the template. If that was the case, the sample had to undergo new DNase treatments until all samples ranged over 37 as Cq value in the qPCR.

3.1.9 Analysis of RNA quantity and quality

The Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA, USA) and measurements on Nanodrop was used to analyze the RNA quantity and quality. The kit used with the Agilent bioanalyzer 2100 was Agilent RNA 6000 Nano Kit (Agilent Technologies, Germany), which includes chips and reagents designed for analysis of RNA fragmentation. The chip contains an interconnected set of microchannels that separates nucleic acid fragments based on their size as they are driven through it electrophoretically. The procedure is given below.

1. The first step was to prepare the gel by pipetting 500 µl of RNA Nano gel matrix into a spin filter and centrifuge at 15000 g for 10 minutes at RT.

2. To prepare the Gel-Dye Mix, the RNA 6000Nano Dye concentrate had to equilibrate to RT for 30 min. The dye was then vortexed for 10 seconds and spinned down before 1 µl was added to 65 µl of aliquot filtered gel. The solution was mixed with a vortexer and spinned down at 13 000 x g for 10 minutes at RT.
3. A new RNA 6000 Nano chip was placed on the chip priming station and 9 µl of gel-dye mix was pipetted in the well marked **(G).** The plunger had to be positioned at 1 ml and the chip priming station was then closed. The plunger was pressed until it was held by the clip and after 30 seconds the plunger was released from the clip. After 5 second, the plunger was pulled back to 1 ml position and the chip priming station was opened. A volume of 9 µl of the gel-dye mix was then added in the wells marked G.
4. The next to be placed was the RNA 6000Nano marker. A volume of 5 µl of the marker goes in all 12 samples wells and in the well marked with a ladder. One µl of the ladder was pipette in the ladder-marked well.
5. One µl of the samples was then placed in each of the 12 sample wells and the chip was placed horizontally in the adapter of the IKA vortexer and vortexed for 1 minute at 2400 rpm
6. The last step was to run the chip in the Agilent 2100 bioanalyzer.

Sample concentrations were displayed on each electropherogram and the quality of the RNA was given as RNA Integrity Number (RIN). The RIN value say something about the RNA integrity level based on a numbering system from 1 to 10. RIN value 1 is the most degraded profile and 10 is the most intact RNA profile.

3.1.10 Reverse transcription

The kit used in this reverse transcription method was SuperScript® VILO™ cDNA Synthesis Kit (Invitrogen™, Carlsbad, CA, USA). This kit provides enhanced cDNA synthesis efficiency and can be used to synthesize cDNA from total RNA. Multiple reactions were to be made, and therefore it was appropriate to make a master mix without RNA. The following was mixed together for one sample:

5x VILO™ Reaction Mix	4µl
10x SuperScript® Enzyme Mix	2µl

The amount of RNA and nuclease free water was calculated to get the same concentration (114.9 ng / µl) for all samples and final volume at 20 µl.

The next step was to run the reaction mixture in the Thermal Cycler (Biorad, Singapore) and incubate the samples at 25 °C for 10 minutes followed by 60 minutes at 42 °C and finally at 5 minutes at 85 °C. This reaction was made twice for all samples (2 x 20 µl cDNA). The cDNA was then analyzed on a 1 % agarosegel to investigate if the cDNA synthesis reactions were successful. The corresponding samples were pooled together. The biological replicates for the control samples were pooled together giving one sample for 6 dpi and one for 10 dpi. The cDNA was stored at -20 °C until use.

3.1.11 Testing of primers

Target genes used in this gene expression study were the *Sod* gene encoding Superoxide Dismutase, *Nps4* gene encoding Nonribosomal Peptide Synthetase, *Dep5 Pks* gene encoding Depudecin Polyketide Synthetase, and a gene with unknown function (*Fl-est17042*). Two housekeeping genes (HKGs) constitutively expressed were also used, *Ubc* gene coding for Ubiquitin Conjugating Enzyme and *β-Tub* gene coding for β-Tubulin. Primer pairs for the genes *Dep5 Pks*, *Ubc* and *β-Tub* were already tested by Christin Anstensrud (unpublished data). I tested primer specificity and efficiency on one primer pair for *Sod*, two primer pairs for *Nps4* and one primer pair for *Fl-est17042*. An overview of the primer pairs used in the gene expression study can be seen in Table 7.

Primer specificity

The specificity of the primer pairs were tested on cDNA from *F. langsethiae* and oat to see whether the primers only bound to the target specie (*F. langsethiae*). To obtain a final concentration of 2 pmol/µl in the reaction mixture, 1.3 µl of 3 pmol/µl forward primer and 1.3 µl of 3pmol/µl reverse primer were mixed with 10 µl of 2 x SYBR green reaction mixture and 5.4 µl of SDW for each well in a 96 wells plate. Two µl of fungal cDNA, oat cDNA or SDW was added to the mixture on the plate. All reactions were run in two technical replicates with the following parameters: 2 min at 50 °C and 10 min at 95 °C, followed by 1 min at 95 °C, 1min at 60 °C for 45 cycles and finally a dissociations curve. Primer specificity was also analyzed by a melting curve, which would detect putative unspecific binding and /or primerdimers. Data were analyzed using the SDS program.

Reaction efficiency

Dilution series of cDNA from *F. langsethiae* was used to test the reaction efficiency. Two primer pairs for the *Nps4* gene were tested using a 2 folds dilution series from a starting concentration of

1991.5 ng/µl. The primer pair for the *Sod* gene and the *Fl-est17042* gene were tested using a 5 folds dilution series with the same start concentrations. All samples were run in two technical replicates, and with the same reaction mixture and parameters as described for the specificity test. The reaction efficiency (%) was calculated using formula 2 (Adams 2006).

(2) $E = (10^{-1/slope} - 1) \times 100$ (Adams 2006)

The slope of the standard curve was given in the computer program for the real-time instrument (SDS version 2.1.1, Applied Biosystems). Those primer pairs showing satisfactory reaction efficiency were further used in the real-time expression assay.

3.1.12 RT- qPCR, gene expression analysis

The real-time assay included expression study of six different genes; *Sod, Nps4, dep5, Fl-est17042, Ubc* and *β-Tub.* 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.

The samples were run in two technical replicates and negative control reactions were run with 2µl of SDW for each primer pair. The assay reaction volumes per sample are given below.

Real-time gene expression reaction mixture

10 µl	2x SYBR green reaction mixture
1.3 µl	Forward Primer (3 pmol/µl)
1.3 µl	Reverse primer (3 pmol/µl)
5.4 µl	SDW
2 µl	Template (cDNA)
20 µl	Total volume

The primers had a final concentration of 2 pmol /µl in the reaction mixture.

All samples were run with the following parameters: 2 min at 50 °C and 10 min at 95 °C, followed by 1 min at 95 °C, 1min at 60 °C for 45 cycles followed by dissociations curve analysis.

3.1.13 Bestkeeper and Rest programs

The stability of the HKGs was analyzed in a program called Bestkeeper version 1 (Pfaffl et al. 2004). This is an excel-based program and does pair wise correlation analysis of the HKG pairs to determine the stability of the expression. This makes it possible to see if the HKGs are suitable to use as standardization for the amount of RNA in each sample. Based on Cq values obtained in the PCR assay, the program combined the HKG expression results in to a Bestkeeper index to be used in normalization and of the target gene Cq values in a relative expression software tool called REST CMS© version 2. This excel based program calculate relative expression values (R- value) of the target genes according to formula 3 (Pfaffl et al. 2002).

3) $$R = \frac{(E_{target})^{\Delta CPtarget\ (MEAN\ control - MEAN\ sample}}{(E_{ref})^{\Delta CPref\ (MEAN\ control - MEAN\ sample}}$$ (Pfaffl et al. 2002),

where E= Efficiency of the PCR reaction

The expression ratio of a target gene versus a reference gene is the basis of the relative expression. The expression of the *Sod* gene in a sample with only *F. langsethiae* (extracted, DNase treated and reverse transcribed by Monica Skogen) was used as reference for the other samples (*F. langsethiae* infected oat, at two different concentrations) in the REST SMS© software tool (Pfaffl et al. 2002).

3.2 Production of *Fusarium* inoculums

Fusarium isolates used were; *F. langsethiae* wild type (wt) strain 9821-61-1 (IBT9951) (Divon et al. 2012), *F. avenaceum* wt strain 6A, *F. culmorum* wt strain 17C, *F. graminearum* wt strain 140/08 and transgenic *F. langsethiae* strain 9821-16-1 (IBT9951) expressing green fluorescence protein (*Fl*-GFP). *Fl*-GFP was transformed with vector gGFP. The strains were used in the following experiments.

Fungal cultures were grown in petri dishes containing SNA medium for 3 days in RT. Agar with fungal growth was cut in small pieces and used as inoculums for conidial production in shaking cultures of 500 ml SNB medium in 1L flasks (one plate per flask). The flask with SNB and fungal growth was incubated for 3 days at 24 °C and 150 rpm. The transformed strain was kept on SNA plates with 25 µg/ml hygromycin B. Mycelial growth was removed by filtering through sterile cheese cloth, and the spore suspension was then centrifuged in 50 ml centrifuge tubes for 25 min at 3000 rpm at 10°C. The supernatant was removed and all pellets with fungal spores were pooled for each species, respectively. In order to remove hygromycin B originating from the agar where *Fl*-GFP was grown, the collected spores were washed with SDW by adding SDW to 50 ml in total and centrifuging as described above. The wet paste *Fusarium* inoculums were then stored at 4 °C until use (maximum four days). Conidial concentrations were determined for every study using a Bürker hemacytometer and adjusted to the working concentrations. The working concentrations used in the different studies are listed in Table 10.

Table 10: Spore concentrations of the *Fusarium* inoculums used in the different experiments.

Study	***Fusarium* specie**	**conidial concentrations (conidia /ml)**
In vitro co-inoculation	*F. langsethiae* wt	8×10^6
	Fl-GFP	1.2×10^7
	F. graminearum	1.7×10^6
	F. culmorum	1.6×10^5
	F. avenaceum	9.8×10^6
Detached leaf assay	*F. langsethiae* wt	5×10^5
	Fl-GFP	
Histological characterization	*F. langsethiae* wt	10^6
	Fl-GFP	

3.3 *In vitro* co-inoculation study

Inoculation

Interactions between *F. langsethiae* and the other *Fusarium* species were investigated *in vitro* using SNA medium on petri dishes. Conidial suspension of *Fl*-GFP (100µl of working concentration) was plated out with a sterile glass rod together with 100 µl of conidial suspension of *F. graminearum, F. avenaceum* and *F. culmorum,* respectively. Three replicates were made. In addition, one replicate of control plates were made by inoculating each fungus on a separate plate. Conidial density (conidial working concentrations) was determined empirically in advance (Table 10). The plates were incubated at RT and studied in the microscopy after approximately 14 hpi. New plates were inoculated the day of inspection and were studied in the microscope at 1 hr after inoculation.

Microscopy

Interactions between *Fl*-GFP and the other *Fusarium* species were investigated using confocal laser scanning microscopy (CLSM) at approximately every 3rd hour. One picture of the different fungi on the control plates was taken at every time-point and three pictures were taken when the species was co-inoculated at every time-point. Regarding measurements, the pictures taken were divided in four equal parts as shown in Figure 9. One part (¼) with containing representative spores were chosen to be used when measuring the spores. All spores in the ¼ were measured, and the total hyphal lengths (spore +germtube) were measured in µm.

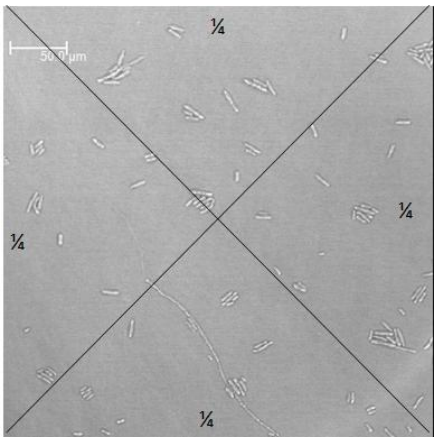


Figure 9: Total hyphal length (spore + germtube) was measured in µm using the pictures taken with CSLM during the co-inoculation study. ¼ was selected for measurements from the control picture since one picture was taken. When the species were co-inoculated three pictures was taken, and ¼ from every picture was chosen to be used in the measurements.

The CLSM permits collections of focused images of single planes within a thick specimen, and can be used to visualize fluorescent signals within a narrow plane of focus (Yoneda et al. 2007). Fluorescent cells can easily be traced and studied in high resolution with CLSM (Dhanoa et al. 2006). *Fl*-GFP was visualized with excitation by an Argon laser at 488nm and emission in the range of 500-550nm. The *Fusarium* wild types were visualized using bright field (BF) mode of the CLSM. The pictures taken and the measurements were used to illustrate the interactions between the species. A t- test was conducted in excel to test if the possible interactions were significant.

3.5 Histological characterization of *F. langsethiae* infection in oat grain

This study was a part of Hege Divons research and she performed the growth of plant material, the dehydration steps and the sputter-coating. I participated in the other parts.

Growth of plant material

Oat cultivar cv. Gere, was used, and plants were grown in a glasshouse using standard 1.5 l plant pots (LOG A/S, Norway) with perlite-added "P-jord", a mix of peat with 10 % soil (LOG A/S, Norway). Five plants were grown per pot, in white light (HPI) with 16 h photoperiod. Temperatures at sowing were 25 °C at day and 18 °C at night, with a relative humidity (RH) at 50 %. Within the first month temperatures were gradually lowered to 18 °C at day and 14 °C at night, and a RH at 75 % and these conditions was kept throughout the experiment. The plants were transferred to S3 growth chambers and watered upon demand

Plant infection

Plants were inoculated 1-2 weeks after flowering at Zadoks scale Z69-77. Inoculations were done by spraying inoculums on the oat panicles separately using; mock control (SDW + 0.1 % Tween20), *F. langsethiae* wt, and *Fl*-GFP separately. Five panicles were used for each inoculation and were then bagged to ensure humid conditions.

Sampling

The oat grain was sampled at 3 dpi and five panicles were collected from the pots with mock control, *F. langsethiae* wt and *Fl*-GFP respectively. The grains were sliced in two different ways

(Figure 10) to allow for efficient fixation and 4 % paraformaldehyde (PFA) in 1x PBS buffer (pH 7.2) for 3 days at 4 °C.

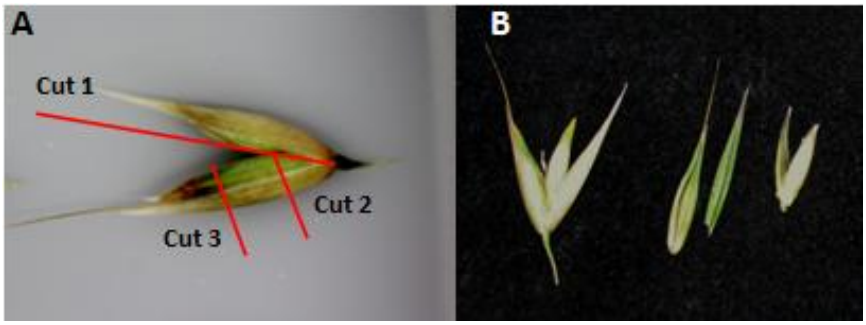


Figure 10: Oat grains sliced in two different ways. A: Oat grain cut in three small pieces. B: Oat grain layers separated from each other.

Successful infection of the oat grains was confirmed by re-isolation of the fungus from the grains (performed by Hege Divon).

Microscopy

The samples collected 3 dpi were investigated using scanning electron microscope (SEM). The samples that had been fixed in 4 % PFA in 1x PBS buffer were then rinsed in 1x PBS buffer. Dehydration was done in a gradient ethanol series as followed:

The first step was to use 30 % EtOH in PBS for 30 minutes, then 50 % EtOH i PBS for 30 minutes, 70 % EtOH in SDW for 30 minutes, 80 % EtOH for 2x3 minutes, 90 % EtOH for 2x3 minutes and at last 100 % EtOH for 2x5 minutes. This ensures complete exchange of water for ethanol. Thereafter samples were critical point dried in order to exchange ethanol for carbon dioxide (CO_2). Finally, samples were mounted on stubs, sputter-coated with gold-palladium and viewed in a scanning electron microscope operating at 25 kV.

3.6 Detached leaf assay

Production of oat leaves and inoculation

Oat seeds cv. Gere, were sown in pots with soil, and placed at RT with approximately 12 hrs of daylight during a period of 14 days and the plants were watered upon demand. After 14 days four cm long segments were cut from the tip of the second seedling leaf and placed with as axial side facing up on the surface of 0.5 % water agar (WA) amended with kinetin (10 mg/l). Kinetin is not soluble in water and was therefore dissolved in 1M NaOH, sterile filtrated and added to the autoclaved agar. To balance the pH value in the agar, 1M HCl was added as well. A volume of 10 µl of conidial suspension (see working concentration in Table 10) was placed in the center of each oat segment with a pipette tip. Control leaves were treated with 10 µl SDW. The plates were then incubated at RT with approximately 12 hrs of daylight and in high humidity.

Assessment of leaf colonization

Disease development and the aggressiveness of the fungi on oat leaves were investigated after 7 dpi using binoculars. Some oat leaves were stained with nitro blue tetrazolium to assess the presence of super oxide radicals (O_2^-). Oat leaves were incubated in 0.25 mM NBT in 10 mM sodium phosphate, pH 7.8 for 30-120 minutes in the dark. Other oat leaves were first incubated in 10 mM KCN to inhibit CuZn-SOD in 5-10 minutes. All oat leaves were stored in EtOH until visualization using binocular. SDW controls (leaves inoculated with SDW and stained as already described, and *Fusarium* infected oat leaves but not stained (put in water)) were included.

Chapter 4 Results

4.1 Quantification of *Fusarium* DNA in oat

The interactions between *Fusarium* species grown on oats were the focus in this study, with particular interest in *F. avenaceum* and *F. langsethiae*. The interactions between *F. avenaceum*, *F. langsethiae* and other *Fusarium* species were studied molecularly using qPCR to quantify the amount of fungal DNA in spray-inoculated oat samples.

4.1.1 Extraction of genomic DNA

Total DNA was extracted from oat kernels, and the quality and quantity were tested using agarose gel electrophoresis (Figure 11).

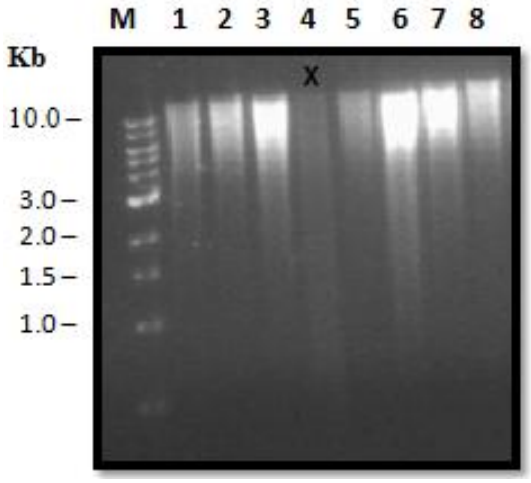


Figure 11: Total DNA extracted from 8 samples of *Fusarium* infected oat seeds. Lane no. 4 indicates a sample of poor quality. The molecular marker is located in the lane marked M in the gel.

Good quality DNA was observed as a clear high molecular band, or compact smear on the gel. If no clear band or a weak smear was shown after gel electrophoresis, a new DNA sample was extracted from those grains. The sample in lane no. 4 in Figure 11 shows such poor quality sample.

4.1.2 Quantification by qPCR

An efficient estimation of the biomass of individual *Fusarium* species and oats was provided by quantitative real-time PCR (qPCR). Five different qPCR assays were used and each assay was intended for quantification of individual *Fusarium* species in plant samples or quantification of

the oat itself. The primers used in each assay were specific to detect only one species. Amounts of DNA in the samples were calculated by a standard curve algorithm using known standards of *Fusarium* or oat DNA. Figure 12 shows examples of results from qPCR.

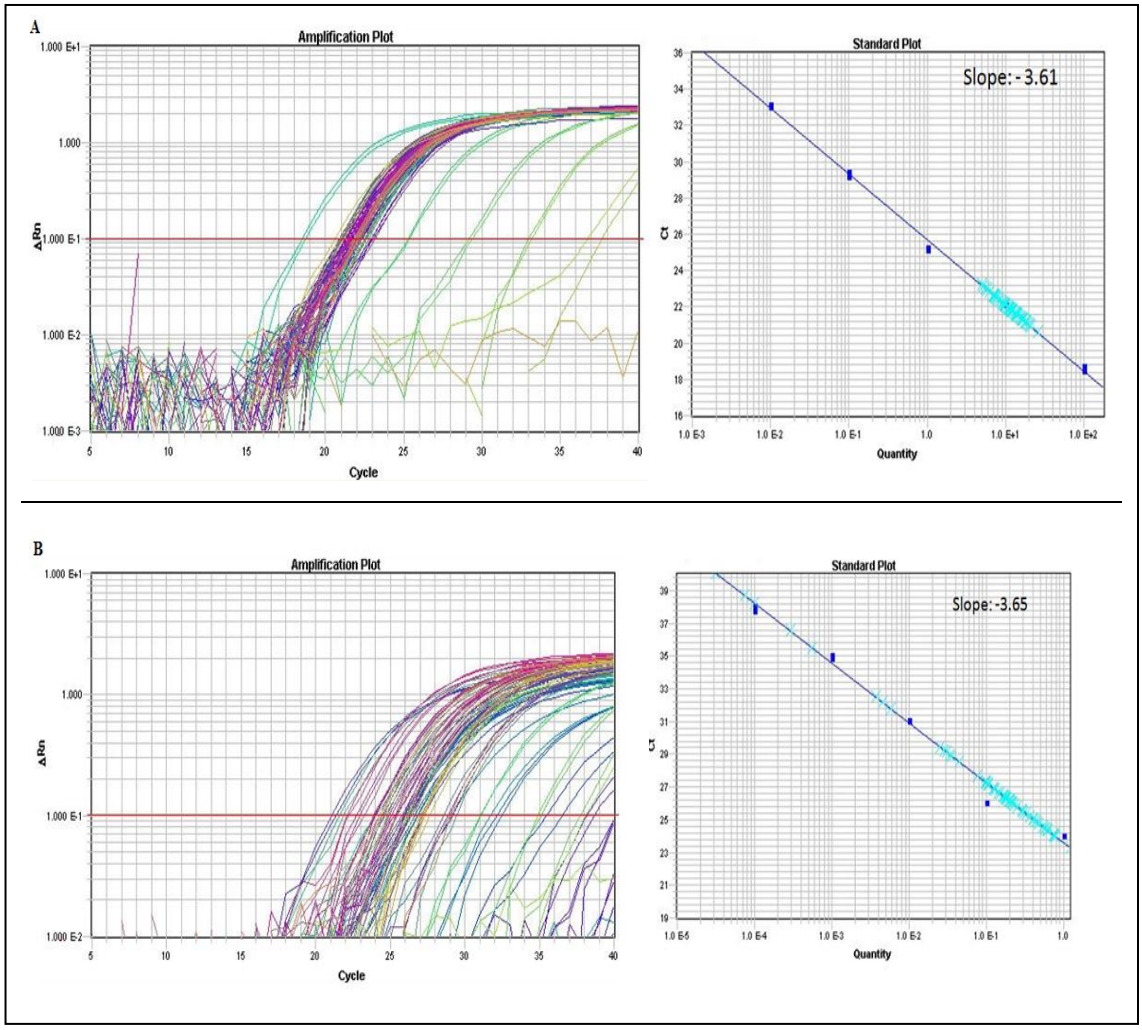


Figure 12: Amplification plots with corresponding standard curves from qPCR. A: Quantification of oat DNA using a qPCR reaction based on the *Cox* gene. B: Detection and quantification of *F. langsethiae* DNA using qPCR. Amount of DNA in the samples was quantified by a standard curve algorithm using five 10-fold dilutions of known amounts of DNA, starting with 1 ng per reaction for *Fusarium* DNA and 100 ng per reaction for oat DNA. In the standard curved DNA quantity (x-axis) is plotted against Ct (Cq) value (y-axis). In the amplification plot cycle (x-axis) is plotted against fluorescent signal (ΔRn) (y-axis).

Approximately equal amounts of oat DNA were present in all samples (Figure 12 A).

The samples infected with *F. langsethiae* or any of the other *Fusarium* species were expected to get widely spread Cq values ranging from approximately 20 to 36. Non infected samples were expected to have an undetermined value. This can be seen in Figure 12B. The curves are more spread than in Figure 12A and indicate different amounts of *Fusarium* DNA in the samples.

All samples were tested in two technical PCR replicates, and replicates with values differing by $C_q \leq 1$ were accepted. Figure 13 shows examples of curves with technical replicates.

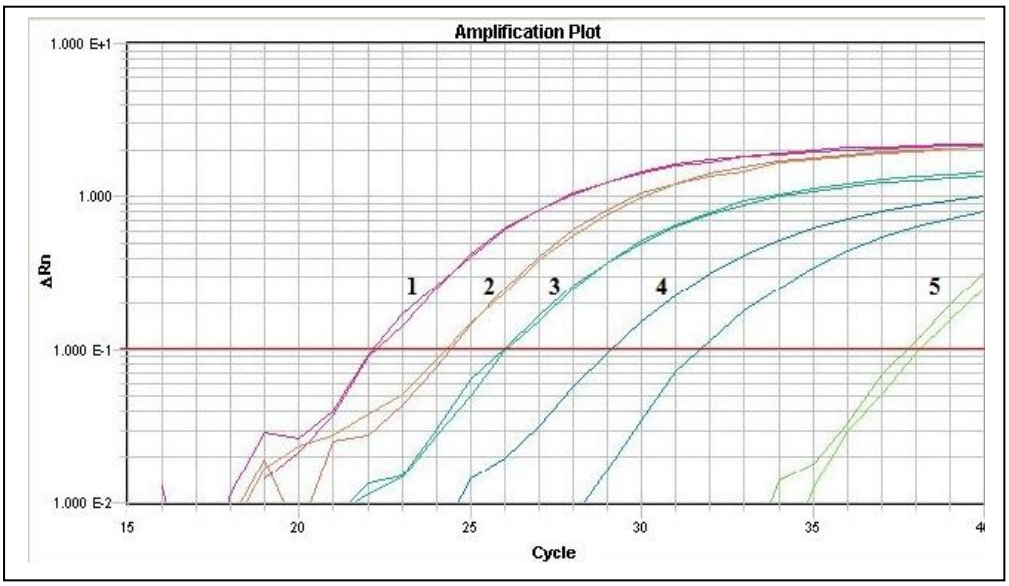


Figure 13: Examples of technical replicates using the qPCR. Curve 1, 2 and 3 represent replicates differing by $C_q < 1$. Curve 4 represents technical replicates differing by $C_q > 1$. Curve 5 is an example of two technical replicates with DNA amount under the limit of quantification (LOQ. Cq=36). In the amplification plot cycle number (x-axis) is plotted against fluorescent signal (ΔRn) (y-axis).

All samples with technical replicates differing by $C_q > 1$ were run again. The limit of quantification (LOQ) was set to $C_q = 36$. In samples with $C_q \geq 36$, the *Fusarium* species were detected, but the amount of DNA was under the LOQ.

Amount of *Fusarium* DNA in inoculated oat grains was calculated relative to plant DNA as described in methods, section 3.1.5.

Only oat DNA was quantified in the SDW sprayed control samples. Fungal DNA was either not detected or got C_q values above the LOQ (data not shown).

4.1.3 Interactions between the *Fusarium* species

To investigate the interactions between *F. avenaceum* and other *Fusarium* species when grown on oats, the fungus was inoculated pair wise with *F. graminearum*, *F. culmorum* and *F. langsethiae*. The amounts of *F. avenaceum* DNA when grown alone on oat and in combinations with the other species on oat are given in Figure 14.

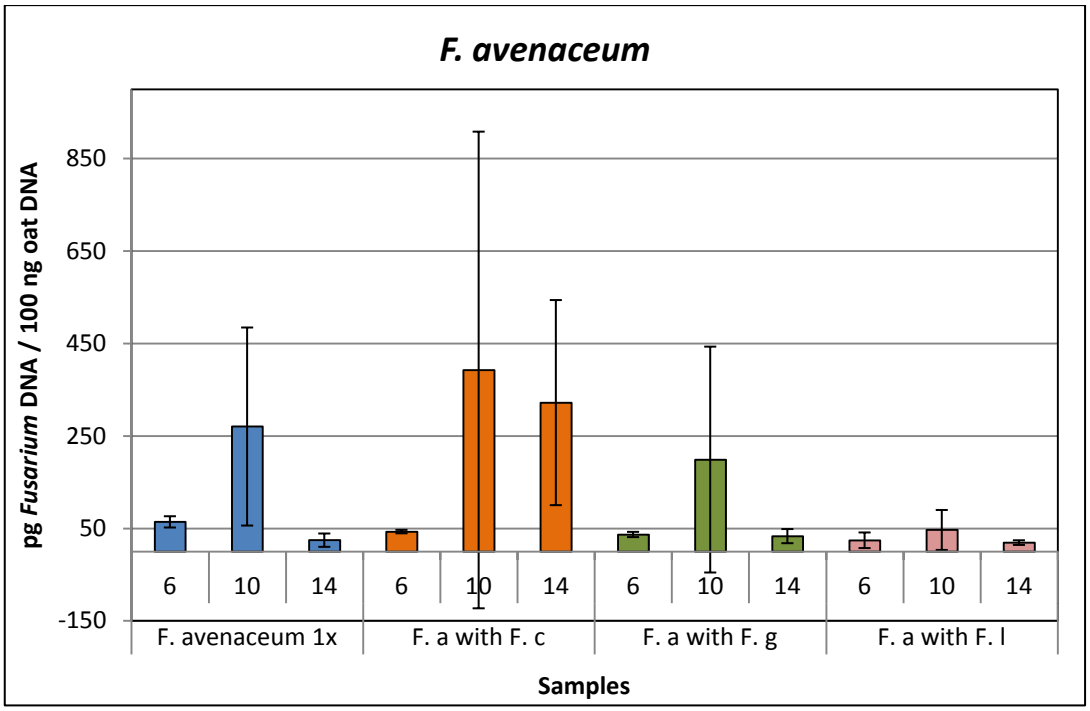


Figure 14: Amounts of *F. avenaceum* DNA when grown alone on oat (1x spore suspension), and in combinations with other *Fusarium* species on oat at 6, 10 and 14 dpi. The combinations were *F. avenaceum* with *F. culmorum* (*F. a* with *F. c*), *F. avenaceum* with *F. graminearum* (*F. a* with *F. g*) and *F. avenaceum* with *F. langsethiae* (*F. a* with *F. l*). Each bar represents the mean of three biological replicates with the exception of the bar marked *F. a* with *F. g* at 6 dpi, which is based on two biological replicates. Every biological replicate was based on two technical replicates. Error bars show standard deviations between the biological replicates. The samples (x-axis) are plotted against DNA amount (pg *Fusarium* DNA / 100 ng oat DNA) (y-axis).

The amount of *F. avenaceum* DNA did not seem to change when grown together with *F. culmorum* compared to when grown alone at 6 dpi (Figure 14). Regarding 10 and 14 dpi on the other hand, the DNA amount of *F. avenaceum* had increased when grown together with *F. culmorum* compared to when grown alone at the same time points (Figure 14). Nevertheless, the observed differences were not significant ($P > 0.05$).

When *F. avenaceum* was grown together with *F. graminearum*, the DNA amount did not seem to change from when grown alone (Figure 14). This was observed regarding all time points (6, 10 and 14 dpi).

Minimal changes in the amounts of *F. avenaceum* DNA was observed at 6 and 14 dpi when grown together with *F. langsethiae* compared to when grown alone at the same time points (Figure 14). At 10 dpi on the other hand, a decrease in DNA amount of *F. avenaceum* was

observed when grown together with *F. langsethiae* compared to when grown alone at 10 dpi (Figure 14). This observed decrease was however not significant (P > 0.05).

To investigate the interactions between *F. langsethiae* and other *Fusarium* species when grown on oats, the fungus was inoculated pair wise with *F. avenaceum*, *F. culmorum* and *F. graminearum*. The amounts of *F. langsethiae* DNA in the oat samples, co-inoculated and grown alone are illustrated in Figure 15.

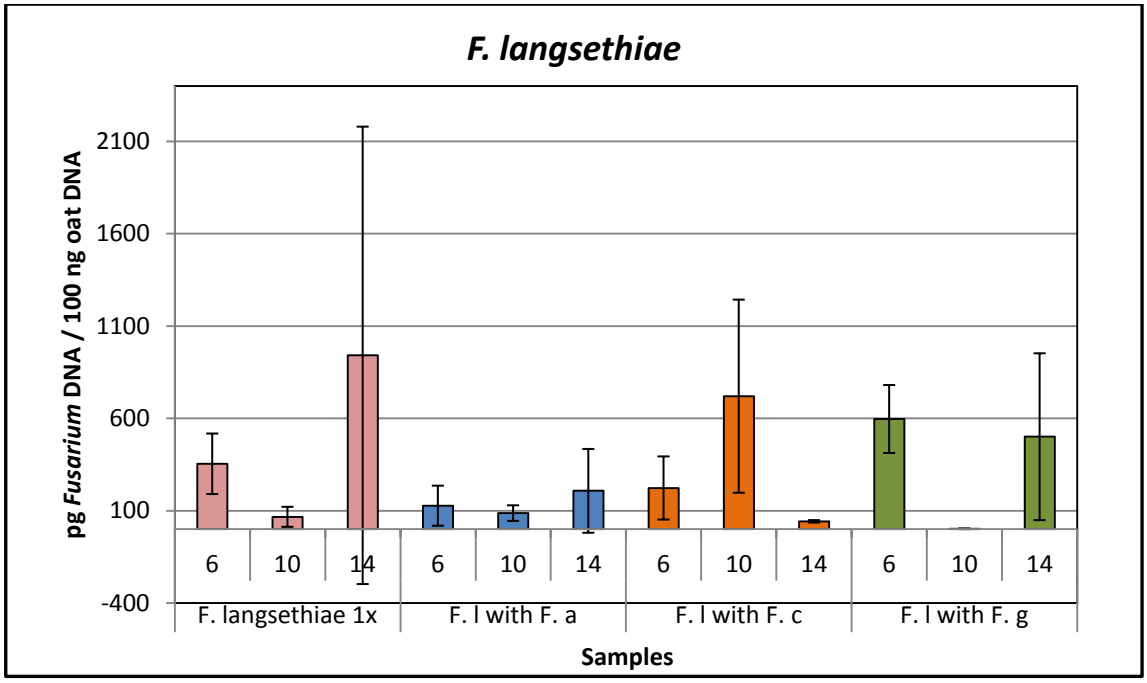


Figure 15: Amounts of *F. langsethiae* DNA when grown alone on oat (1x spore suspension), and in combinations with other *Fusarium* species on oat at 6, 10 and 14 dpi. The combinations were *F. langsethiae* with *F. avenaceum* (*F. l* with *F. a*), *F. langsethiae* with *F. culmorum* (*F. l* with *F. c*) and *F. langsethiae* with *F. graminearum* (*F. l* with *F. g*). Each bar represents the mean of three biological replicates with the exception of the bar marked *F. a* with *F. g* at 6 dpi, which is based on two biological replicates. All biological replicates are based on two technical replicates. Error bars show standard deviations between the biological replicates. The samples (x-axis) are plotted against DNA amount (pg *Fusarium* DNA / 100 ng oat DNA) (y-axis).

The amount of *F. langsethiae* DNA did not seem to change when grown together with *F. avenaceum* at 10 dpi compared to when grown alone the same time point (Figure 15). Regarding 6 and 14 dpi on the other hand, the DNA amount of *F. langsethiae* had decreased (Figure 15). However, the observed differences were not significant (P > 0.05).

When *F. langsethiae* was grown together with *F. culmorum*, the DNA amount decreased at 6 and 14 dpi and increased at 10 dpi compared to the DNA amount when grown alone at the respective time points (Figure 15). The observed differences were however not significant ($P > 0.05$).
The DNA amount of *F. langsethiae* had increased at 6 dpi when grown together with *F. graminearum* compared to when grown alone at the same time point (Figure 15). When it comes to 10 and 14 dpi on the other hand, a decrease in DNA amount of *F. langsethiae* was observed (Figure 15). The observed differences in DNA amounts were however not significant ($P > 0.05$).

The effect of *F. langsethiae* and *F. avenaceum* on *F. culmorum* and *F. graminearum* was investigated. Oat samples spray inoculated with only *F. culmorum* and only *F. graminearum* were not included in this study. Only the fungal growth of *F. culmorum* and *F. graminearum* when grown together with the two other *Fusarium* species was therefore investigated. The amounts of *F. graminearum* and *F. culmorum* DNA in the oat samples where it had grown together with other *Fusarium* species are illustrated in Figure 16.

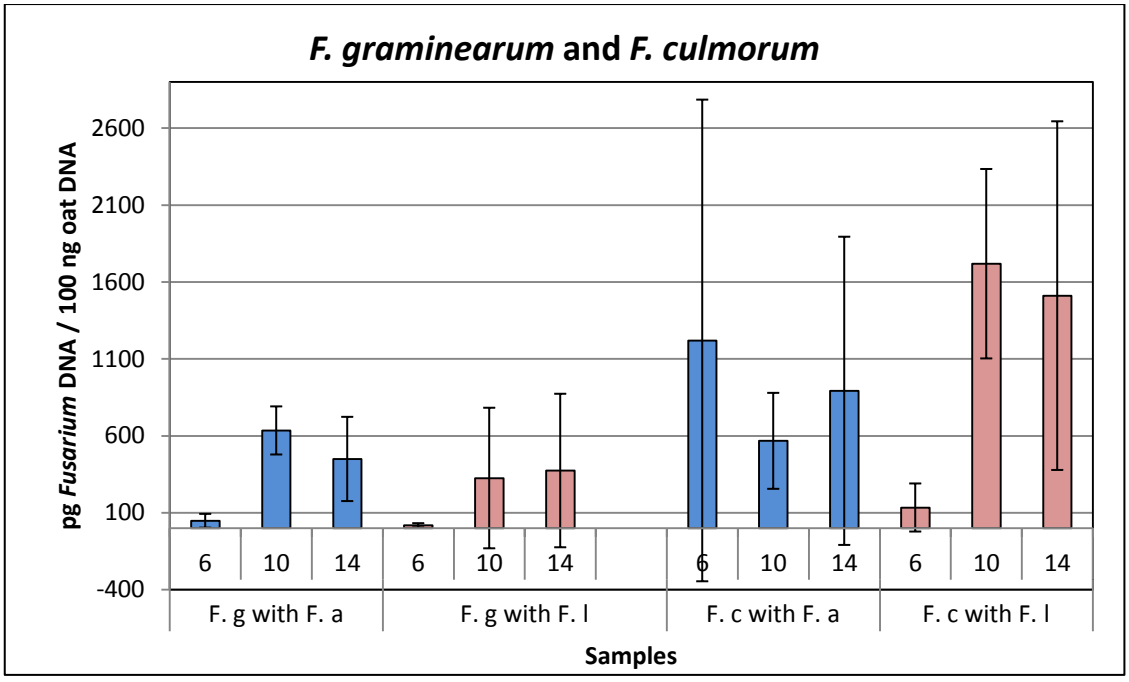


Figure 16: Amounts of *F. graminearum* and *F. culmorum* DNA when grown in combinations with other *Fusarium* species on oat at 6, 10 and 14 dpi. The combinations were *F. graminearum* with *F. avenaceum* (*F. g* with *F. a*), *F. graminearum* with *F. langsethiae* (*F. g* with *F. l*), *F. culmorum* with *F. avenaceum* (*F. c* with *F. a*) and *F. culmorum* with *F. langsethiae* (*F. c* with *F. l*). Each bar represents the mean of three biological replicates, with the exception of the bar marked F. g with F. a at 6 dpi which is based on two biological replicates. All biological replicates were based on two technical replicates. Error bars show

standard deviations between the biological replicates. The samples (x-axis) are plotted against DNA amount (pg *Fusarium* DNA / 100 ng oat DNA) (y-axis).

Regarding the amount of *F. graminearum* DNA at 10 and 14 dpi compared to 6 dpi, the DNA amount increased both when grown with *F. avenaceum* and with *F. langsethiae* (Figure 16). Only the increase from 6 to 10 dpi when grown with *F. avenaceum* was found significant ($P < 0.05$). The increase was highest when *F. graminearum* was grown together with *F. avenaceum* compared to the increase when grown together with *F. langsethiae*, but these differences were not significant ($P > 0.05$).
When it comes to *F. culmorum*, a decrease in DNA amount at 10 and 14 dpi compared to 6 dpi was observed when *F. culmorum* was grown pair wise with *F. avenaceum* (Figure 16). The amount of *F. culmorum* increased on the other hand when grown together with *F. langsethiae*. These observed differences from 6 dpi to 10 and 14 dpi were however not significant ($P > 0.05$).

4.2 Gene expression studies

Oat samples spray inoculated with *F. langsethiae* from the greenhouse experiment were used to investigate the expression of various genes in *F. langsethiae* during oat infection. Non-infected samples were used as control. The genes of interest in this study were the *Sod* gene encoding Superoxide Dismutase, the *Nps4* gene encoding a Nonribosomal Peptide Synthetase, a gene with unknown function (*Fl-est17042*) and *Dep5 pks* encoding Depudecin Polyketide Synthetase. These putative genes were all identified previously in a transcriptome study (Lysøe et al. unpublished data), as potentially up-regulated during *in planta* growth. The aim in this study was to verify the expression patterns of infected oat, which will support a possible role for these genes during plant infection.

4.2.1 RNA extraction and analysis of RNA quantity and quality

RNA was extracted from collected oat grains. To get rid of residual DNA contamination, the samples were treated with DNase. To confirm successful DNase treatment, all RNA samples were tested with real-time qPCR without the presence of reverse transcriptase. In most samples there were no detectable DNA remaining, with the exceptions of a few samples (data not shown). On these samples, a second DNase treatment was performed, and when the samples were retested with real-time qPCR, DNA was either not detectable, or the level was over the LOQ (($C_q > 37$). RNA concentration was measured both with Agilent Bioanalyzer 2100 and with

Nanodrop. Examples of some typical electropherograms from the bioanalyzer are given in Figure 17.

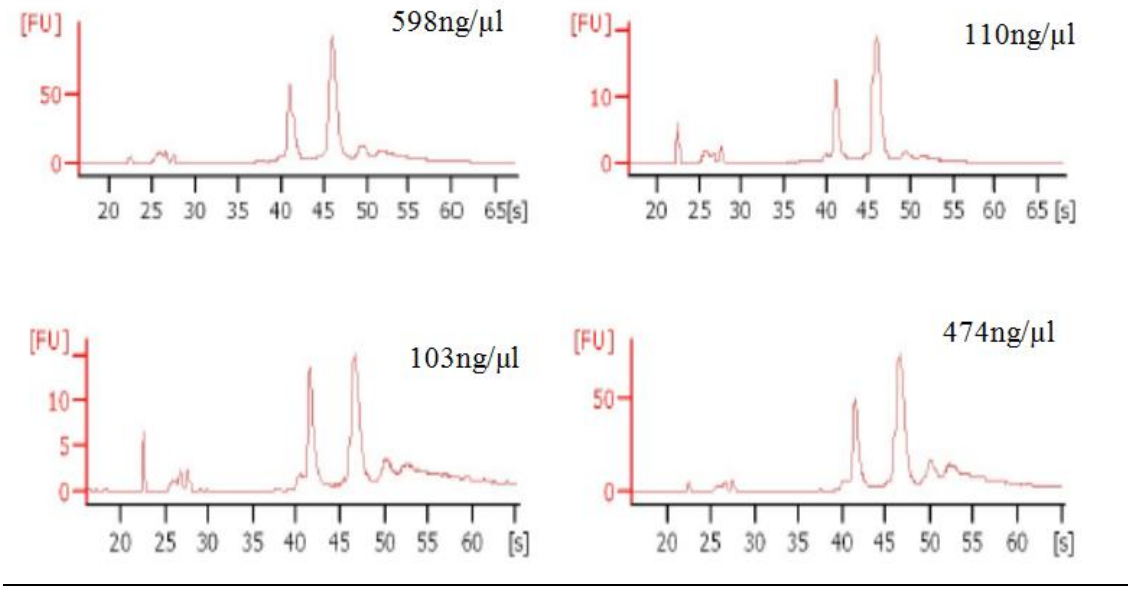


Figure 17: Examples of electropherograms for RNA extracted from oat samples (10.6.II, 10.6.III, 10.10.I and 10.10.II). The Agilent Bioanalyzer 2100 provides RNA concentrations but is not shown in the electropherograms. The RNA is therefore supplemented and given in ng /µl The fluorescence (FU) (y-axis) is plotted against seconds (x-axis)

The electropherograms in Figure 17 shows two big peaks. 18s and 28s is found in fungi and other eukaryotic ribosomes (Deacon 2006), but the amount of plant RNA is so much higher than the amount of *Fusarium* RNA, meaning that the two peaks indicates 18s and 25 s representing plant RNA (Polanco & Vega 1994). Mean concentration of two Nanodrop measurements and value from the Agilent Bioanalyzer 2100 were calculated for every sample. After inspecting the electropherograms from Agilent Bioanalyzer 2100, all RNA samples were found to be of good quality with RIN- values between 7 and 10 (data not shown), and the RNA concentration ranging from 114.9 ng/µl to 1198,6 ng/µl (data not shown).

4.2.2 Reverse transcription

Complementary DNA from all samples was synthesized with SuperScript® VILO™ cDNA Synthesis Kit. The cDNA was then analyzed on agarose gel and the results are shown in Figure 18.

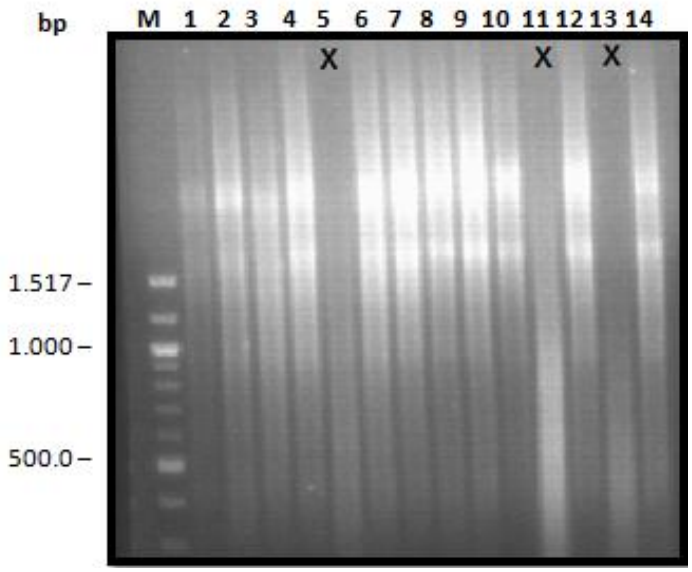


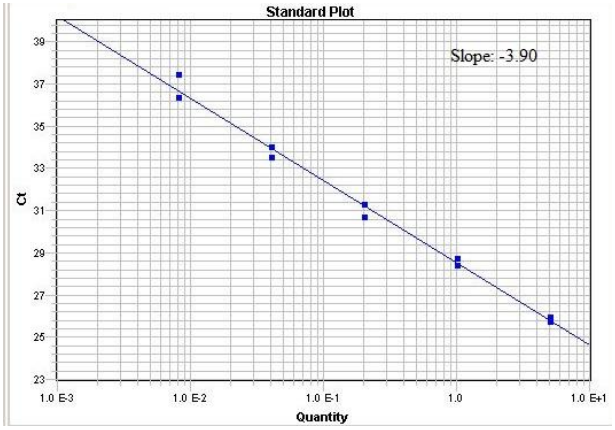
Figure 18: Agarose gel electrophoresis of 14 samples containing cDNA synthesized from *F. langsethiae* infected oat. The molecular marker is placed in the lane marked M.

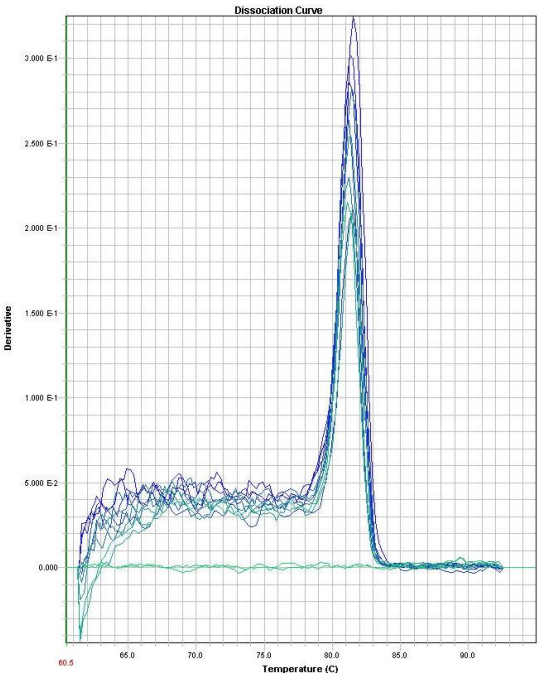
The more or less even distribution of high molecular cDNA smear was seen for almost all samples (Figure 18). There might be two bands visible in the smear of cDNA. The cDNA in lane no 5, 11 and 13 in Figure 18 looked degraded and were not considered in further work.

4.2.3 Testing of primer pairs

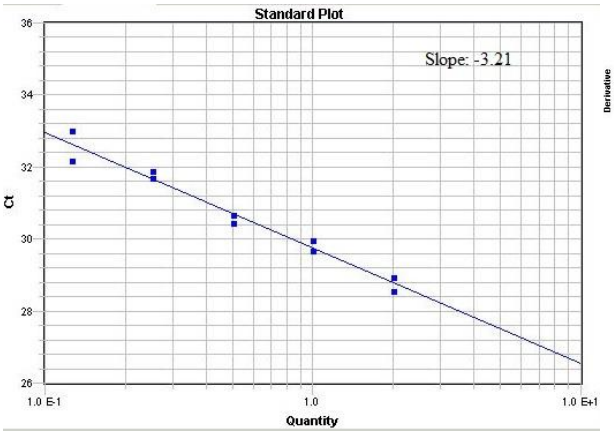
Primer pairs for the target gene *Dep5 pks* and the two HKGs *Ubc* and *β-Tub* were already tested and optimized by Christin Anstensrud (unpublished data). I tested the remaining primer pairs (one primer pair detecting the *Sod* gene, two primer pairs detecting the *Nps4* gene and one primer pair for the *Fl-est17042* gene) using cDNA from *F. langsethiae* and oat as template.
All primer pairs detected its representative gene in *F. langsethiae*. Specific binding of the primer to the target gene was analyzed by a melting curve, which would detect putative unspecific binding and /or primer dimers. All primers tested gave only one melting curve and indicated that no unspecific binding had occurred.
The efficiency of the PCR reaction was calculated using formula 2. Standard curves with slope and dissociation curves for primer pairs tested are given in Figure 19.

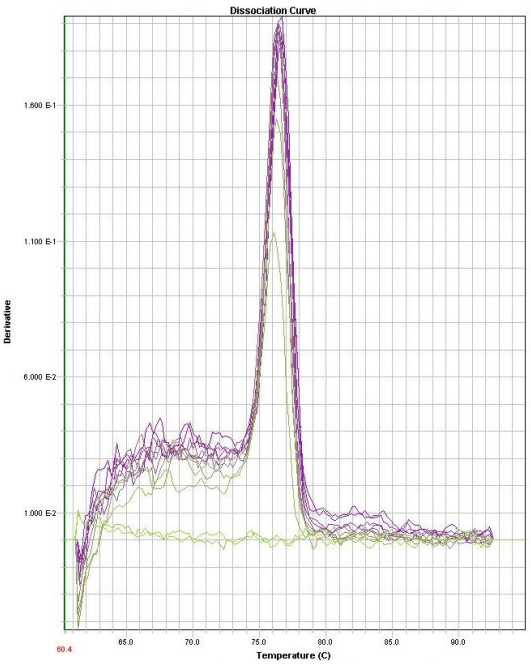
A


Standard Plot
Slope: -3.90
Ct
Quantity
1.0 E-3
1.0 E-2
1.0 E-1
1.0
1.0 E+1


Dissociation Curve
Derivative
Temperature (C)

B


Standard Plot
Slope: -3.21
Ct
Quantity
1.0 E-1
1.0
1.0 E+1


Dissociation Curve
Derivative
Temperature (C)

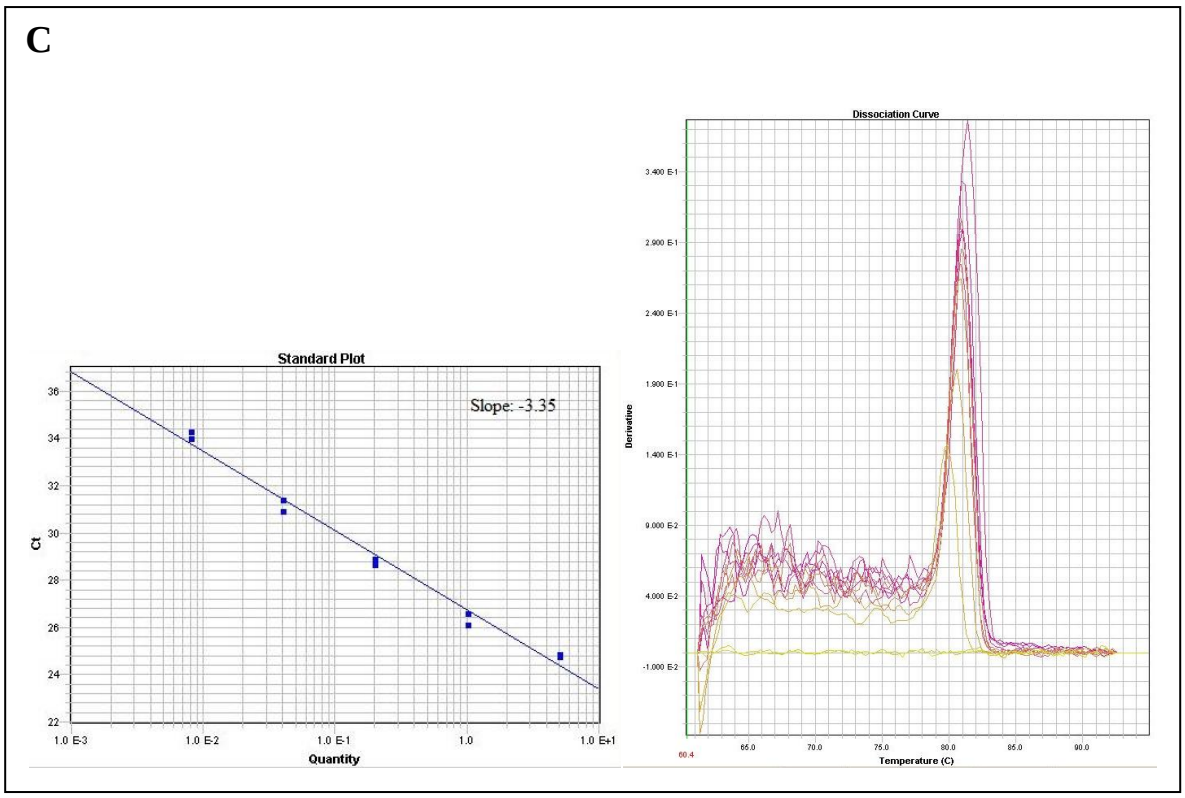


Figure 19: Standard curves and dissociation curves for primer pairs for (A) the *Sod* gene (Sudis f1+r1), (B) the *Nps4* gene (NRPS f1 + r1) and (C) the *Fl-est17042* gene (17042 f1+r1). Known standards of *F. langsethiae* cDNA were used in the standard curve. Primer pairs for the *Nps4* gene were tested using a 2 folds dilution series and primer pairs for the gene *Sod* and the gene *Fl-est17042* were tested using a 5 folds dilution series with a starting concentration of 1991.5 ng /µl. In the standard curve cDNA quantity (x-axis) is plotted against Ct (Cq) 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 dissociation curves for all three primer pairs (Figure 19 A, B and C) showed a single peak and confirmed the binding specificity. Calculations using the slopes gave the PCR reactions an efficiency of 80% for the *Sod* gene, 105 % for the *Nps4* and 99% for the gene *Fl-est17042*.
Standard curve and dissociation curve for the second primer pair for *Nps4* (NRPS2) were not used due to a PCR reaction efficiency of 122 %. The primer pair giving the best results was chosen, i.e. NRPS1.
The standard curves and dissociation curves for primer pairs for the genes *Dep5 Pks, Ubc* and *β-Tub* were performed by Christin Anstensrud and the PCR reaction efficiencies were 86 %, 105 % and 108 %, respectively (data not shown).

The two HKGs (*Ubc and β-Tub*) were analyzed in Best keeper software for determination of their expression stability under the experimental conditions. The results are shown in Figure 20.

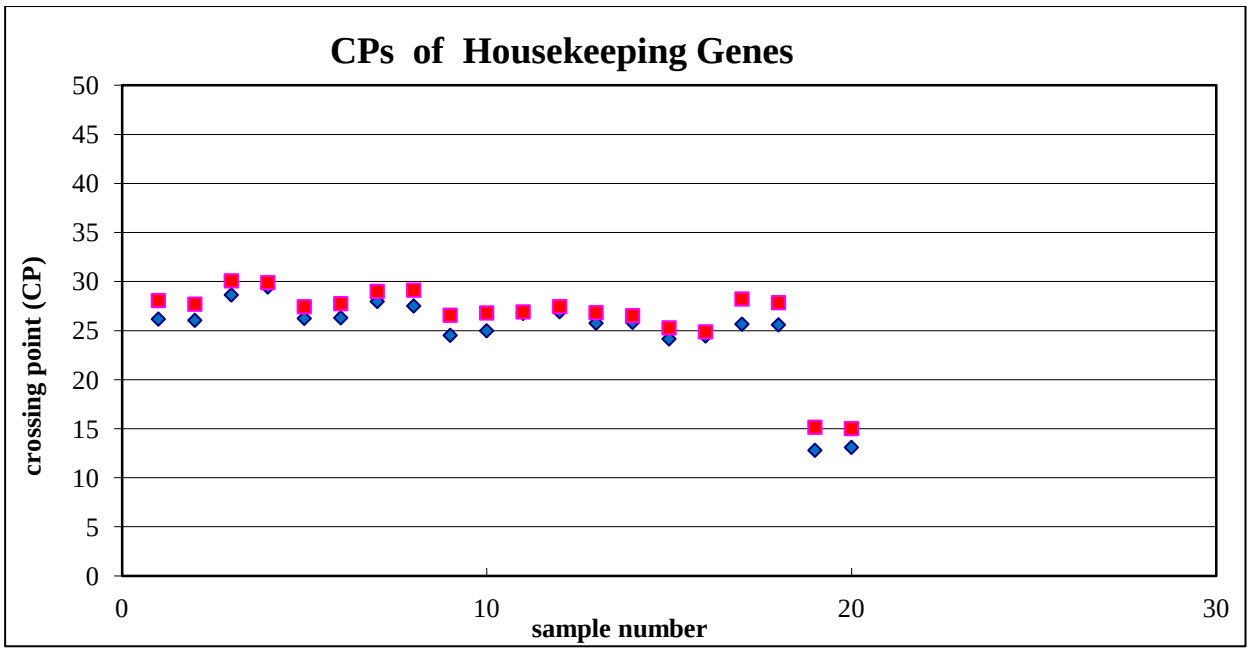


Figure 20: Cq values of the HKGs *Ubc* (◆) and *β-Tub* (■) generated with Best keeper (Pfaffl et al. 2004). The sample number (x-axis) is plotted against crossing point (CP) (y-axis).

Figure 20 is an illustration of the HKGs stable relative expression using HKGs Cq values for all samples. This was done to test if they were suitable for standardization of target gene expression data. The two HKGs were found to be acceptably stable for this application. *Ubc* and *β-Tub* are often used as reference genes in gene expression studies, and have been found to be stable in other similar biological systems as well (Lysoe et al. 2006).

4.2.4 Real-time qPCR

Relative expression levels of the target genes in every sample were calculated based on the Cq-values for the target genes and the HKGs from the qPCR with the software tool REST MCS© version 2 (Pfaffl et al. 2002). The expression of the *Sod* gene in mycelia of *F. langsethiae* grown on agar was used as the reference condition. The expression of all genes was normalized according to this reference. The expression of the *Sod* gene in *F. langsethiae* mycelia and in the *F. langsethiae* infected oat samples can be seen in Figure 21.

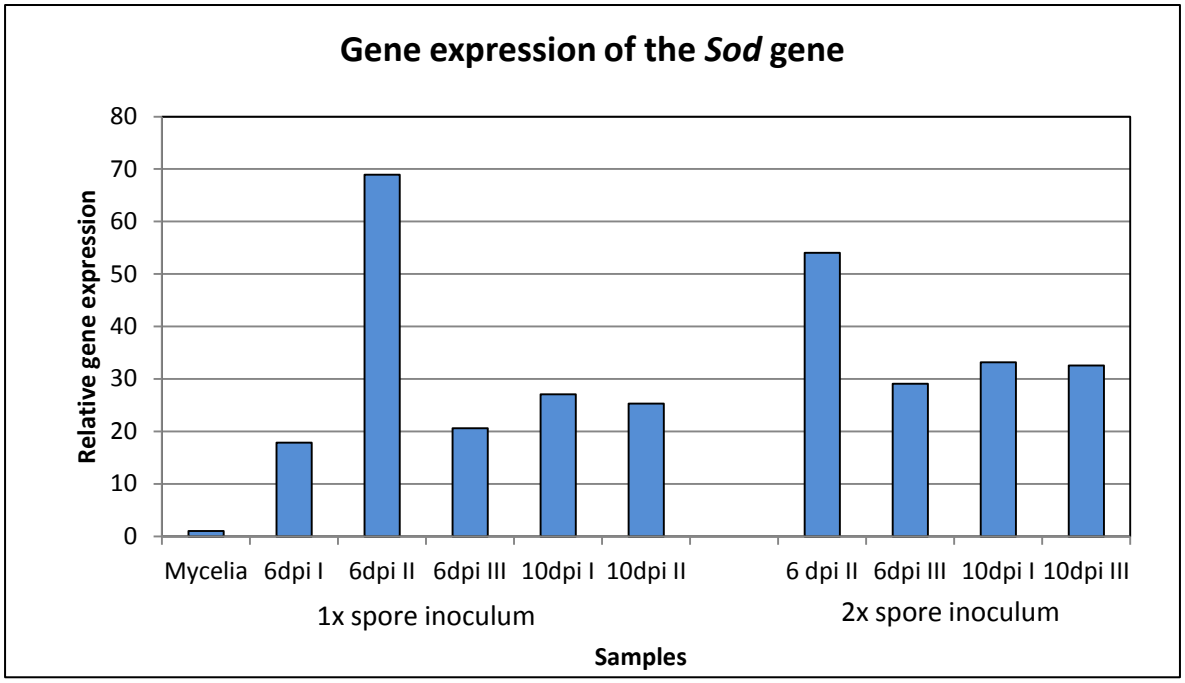


Figure 21: Relative gene expression of *Sod* in *F. langsethiae*. The sample with mycelia indicates expression of Sod in *F. langsethiae* mycelia alone. The other samples infected with 1x and 2 x spore inoculums (1x = 2 x 10^6 and 2x = 4 x 10^6) were collected at 6 and 10 dpi. I, II and III represent the biological replicates which are based on two technical replicates. The samples (x-axis) are plotted against relative gene expression (y-axis).

The expression of *Sod* in mycelia was used as a reference condition and values > 1 indicate up-regulation and values < 1 indicate down-regulation compared to the reference condition. *Sod* was expressed in mycelia (Cq = 20.39). Because it was used as reference condition, the value is shown as value 1 in Figure 21. The expression of *Sod* in *F. langsethiae* during oat infection was clearly up regulated (Figure 21). There was no clear difference in expression between 1x spore and 2x spore inoculums, as well as no clear difference between samples collected at 6 dpi and samples at 10 dpi (Figure 21).

The gene expression of *Nps4* in *F. langsethiae* mycelia and in oat samples can be seen in Figure 22. *Nps4* expression was un-detected in *F. langsethiae* mycelia in the RT qPCR reaction. All samples of *F. langsethiae* infected oat showed clear expression during infection compared to no expression of the gene in mycelia alone(Figure 22), and most samples showed 5-fold or more expression compared to *Sod* in mycelia. Double inoculums did seem to result in slightly higher expression, but no difference between samples collected at 6dpi and 10dpi was observed (Figure 22).

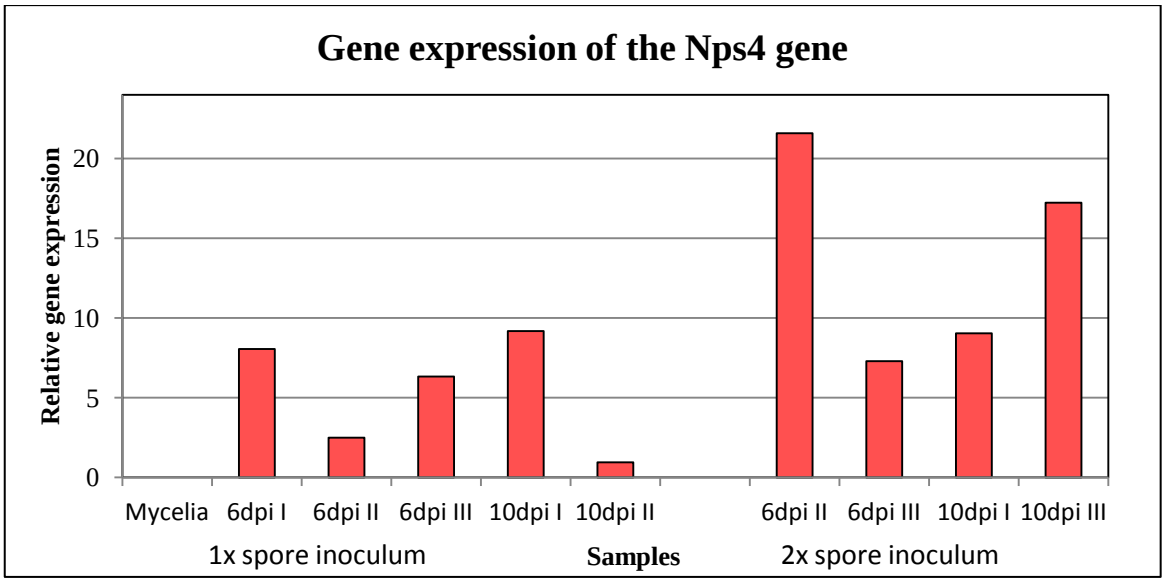


Figure 22: Relative gene expression of *Nps4* in *F. langsethiae.* Samples infected with 1x and 2 x spore inoculums (1x = 2 x 10^6 and 2x = 4 x 10^6) were collected at 6 and 10 dpi. I, II and III represent the biological replicates which are based on two technical replicates. The samples (x-axis) are plotted against gene expression relative to *Sod* in mycelia (y-axis).

The expression of the gene with unknown function (*Fl-est17042*) in the mycelia and in the *F. langsethiae* infected oat samples can be seen in Figure 23. The expression of this gene was un-detected in *F. langsethiae* mycelia in the RT qPCR reaction.

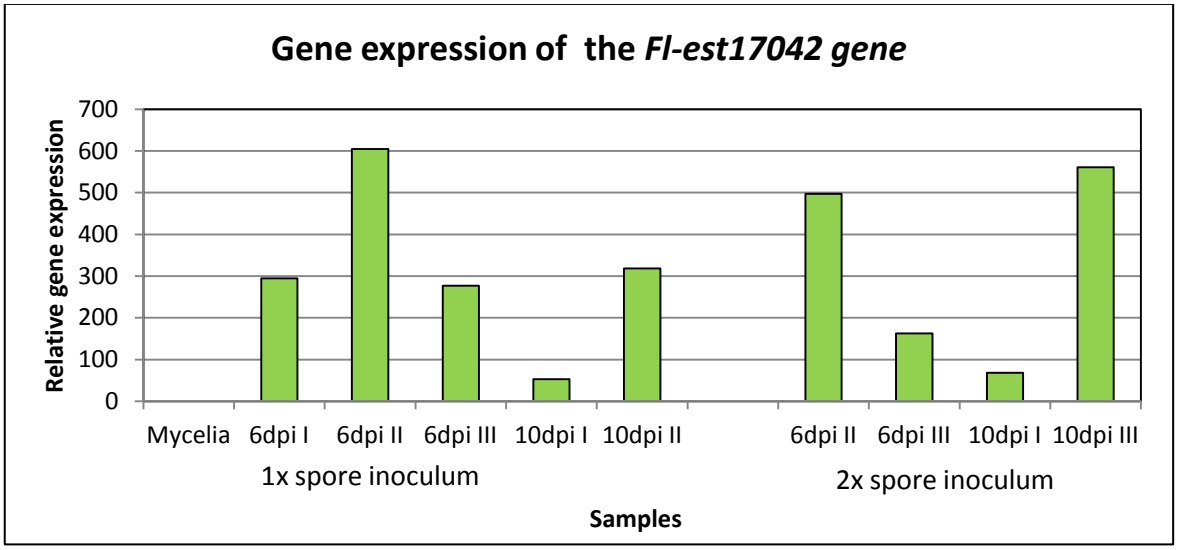


Figure 23: Relative gene expression of the gene *Fl-est17042* in *F. langsethiae.* Samples infected with 1x and 2 x spore inoculums (1x = 2 x 10^6 and 2x = 4 x 10^6) were collected at 6 and 10 dpi. I, II and III represent the biological replicates which are based on two technical replicates. No expression was detected in *F. langsethiae* mycelia. The samples (x-axis) are plotted against gene expression relative to *Sod* in mycelia alone (y-axis).

The *Fl-est17042* gene was highly expressed in the oat samples infected with *F. langsethiae* at 6 and 10 dpi compared to no expression in mycelia (Figure 23). There were no clear differences between 1x and 2x spore inoculum, nor a difference between samples collected at 6 and 10 dpi.

The gene expression of *Dep5 Pks* in mycelia and in the oat samples can be seen in Figure 24.

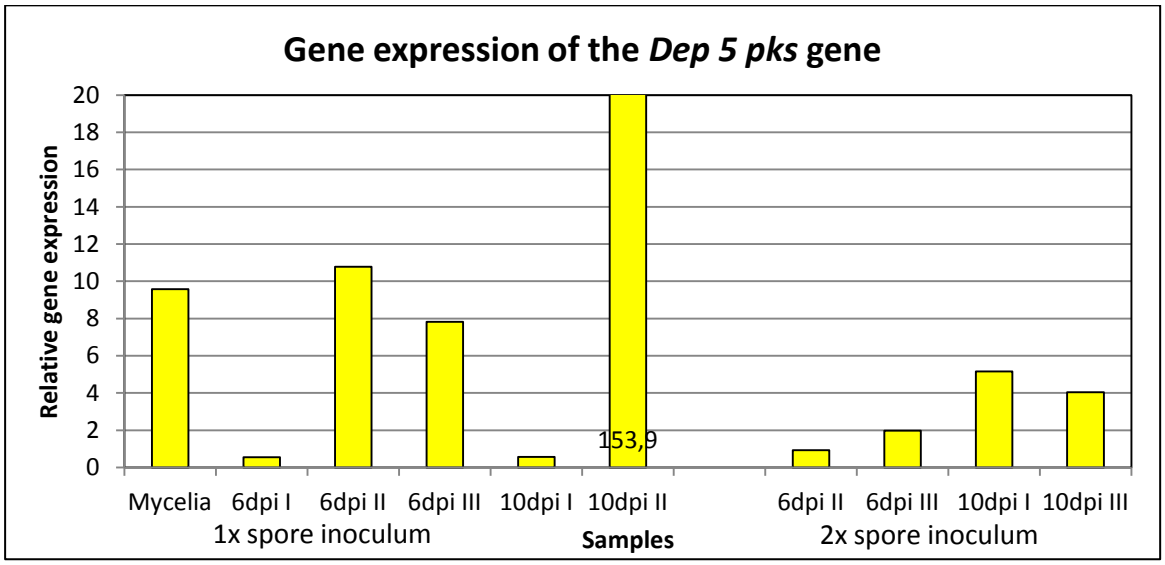


Figure 24: Relative gene expression of the gene *Dep5 Pks* in *F. langsethiae*. Samples infected with 1x and 2 x spore inoculums (1x = 2 x 10^6 and 2x = 4 x 10^6) were collected at 6 and 10 dpi. I, II and III represent the biological replicates which are based on two technical replicates. The samples (x-axis) are plotted against gene expression relative to *Sod* in mycelia alone (y-axis).

Dep5 pks was expressed in mycelia, but no clear trend showing lower or higher expression of *Dep5 pks* in the oat samples infected with *F. langsethiae* could be seen (Figure 24). There might look like a lower expression in the samples containing 2x spore inoculum compared to 1x spore inoculum and the expression in mycelia.

To be able to easily compare the expression of the four target genes used in this study, a summary of the expression of all target genes is shown in Figure 25. The mean of the biological replicates and the standard deviations were calculated.

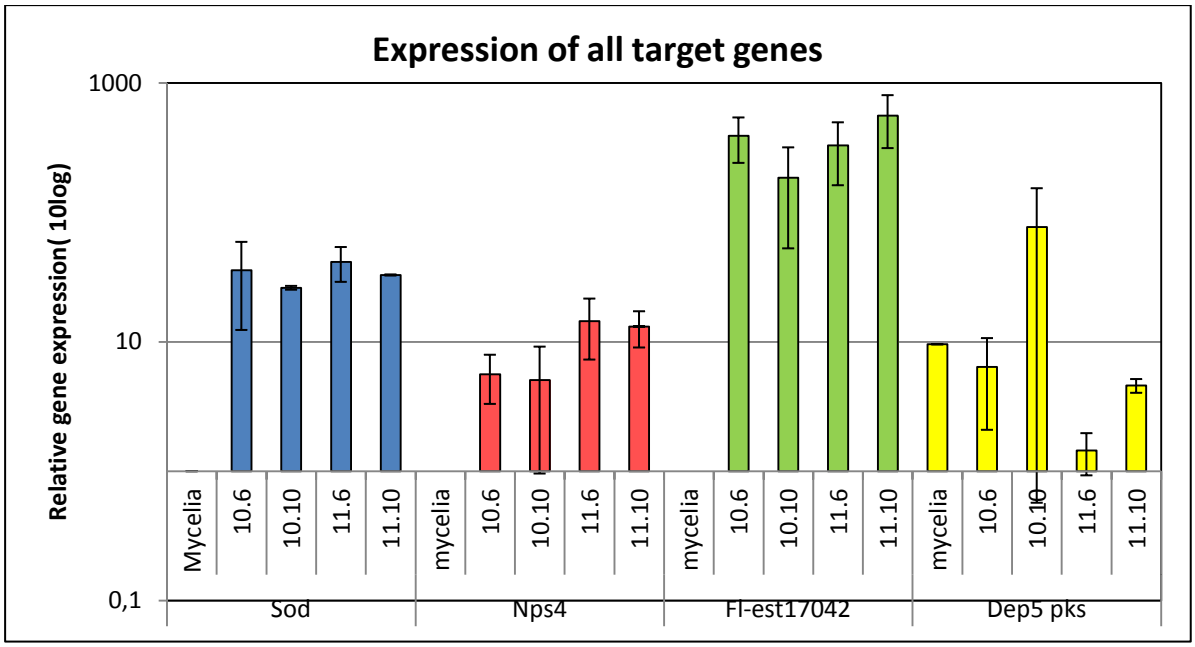


Figure 25: Gene expression of *F. langsethiae* target genes relative to *Sod* in mycelia. Two concentrations were used of inoculums (10 = 2 x 10^6 and 11 = 4 x 10^6), and the oat material was collected at 6 and 10 dpi. The bars marked 10.6 are the mean of three biological replicates, whereas the bars marked 10.10, 11.6 and 11.10 are means of two biological replicates. Each biological replicate was always a mean of two technical replicates. *Sod* (blue bars), *Nps4* (red bars), *Fl-est17042* (green bars) and *Dep 5 pks* (yellow bars). Error bars show standard deviations between the biological replicates. The samples (x-axis) are plotted against relative gene expression normalized to *Sod* in mycelia alone (y-axis).

Figure 25 shows that among the four target genes tested in this study, the gene with the highest expression was the one with unknown function (*Fl-est17042*). However, all genes were confirmed higly expressed *in planta*. There seems to be relatively equal expression of *Sod*, *Nps4* and *Dep5 pks* in samples containing *F. langsethiae* growing on oat. The expression of *Dep5* in mycelia was quite similar to the expression in the oat samples, but there were no detectable expression of Nps4 or *Fl-est17042* in the *F. langsethiae* mycelia.

4.3 Comparison of the *Fl*-GFP strain with *F. langsethiae* wt

To investigate the interactions between *Fl*-GFP and three other *Fusarium* species at the microscopic level, an *in vitro* co-inoculation study on SNA plates was done. The three other Fusarium species used were *F. avenaceum*, *F. culmorum* and *F. graminearum*. CLSM allowed for the differentiation of *Fl*-GFP from the other fungi, and was used to detect potential differences in growth behavior between the species when grown alone and when co-inoculated in

pairs. Fluorescent cells can easily be traced and studied in high resolution with CLSM, and the *Fl*-GFP was visualized with excitation by an Argon laser at 488nm and emission in the range of 500-550nm. The *Fusarium* wt was visualized using bright field (BF) mode in the CLSM. It was important to test if *Fl*-GFP behaved approximately the same as *F. langsethiae* wt.

Spore size and growth structures looked approximately the same for both *Fl*-GFP and *F. langsethiae* wt, and these similar observations were a trend, and seen at all time points (Figure 26 and 27.)

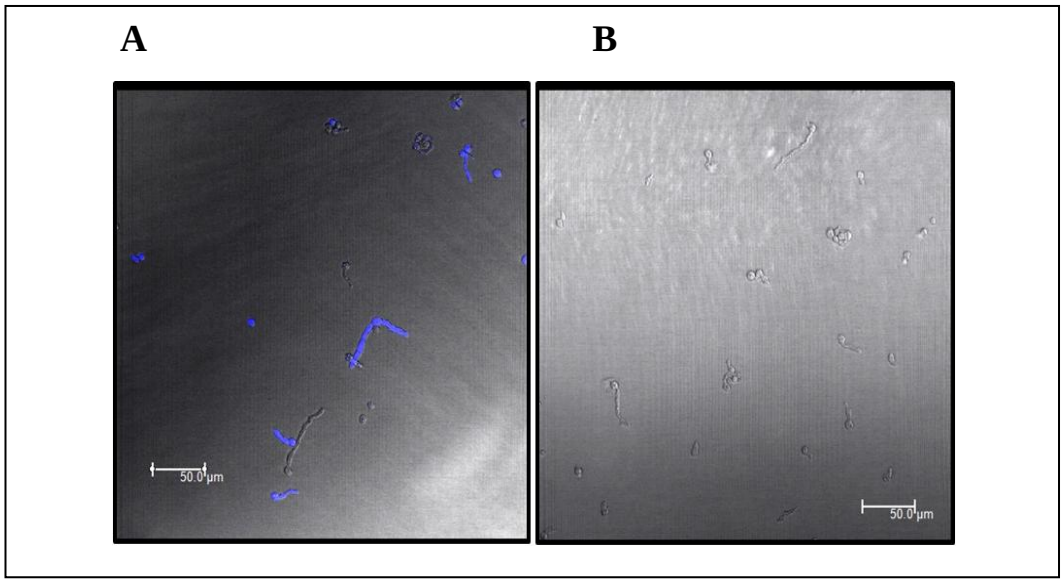


Figure 26: Fungal growth of *F. langsethiae* viewed with CLSM at 6 hpi. A: Fl-GFP (emission at 500-550nm). B: *F. langsethiae* wt (bright field). Bars = 50 µm.

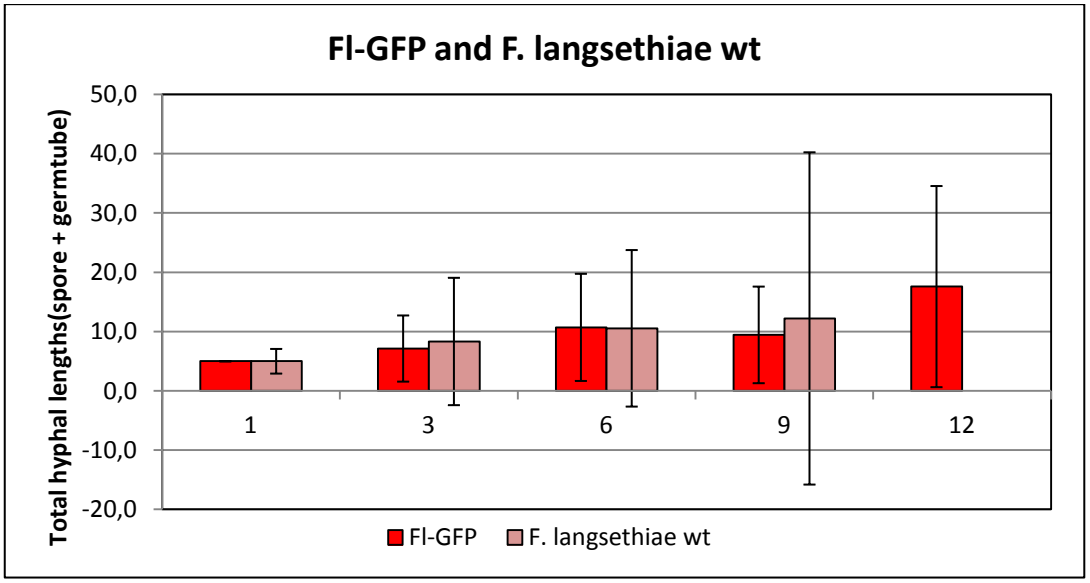


Figure 27: Total hyphal lengths (spore + germtube) of *Fl*-GFP and *F. langsethiae* wt grown alone on SNA plates. The hpi (x-axis) is plotted against total hyphal length (spore + germtube) (µm) (y-axis).

The total hyphal length (spore + germtube) of *Fl*-GFP and *F. langsethiae* wt grown separately on SNA plates showed no clear difference over time (Figure 27) and the T- test confirmed no significant differences ($P > 0.05$). Total hyphal length (spore + germtube) for *F. langsethiae* wt were measured until 9 hpi, whereas *Fl*-GFP was measured until 12 hpi. The reason for this was the differential onset of invasive growth in the two strains, the only observation with some deviation between *Fl*-GFP and wt. *Fl*-GFP started invasive growth at 14 hpi (Figure 28 A) while *F. langsethiae* wt started invasive growth at 12 hpi (Figure 28 B).
Invasive growth is when the fungus grows in the agar instead of on top of the agar, and the hyphal tips can no longer be seen. In the CLSM, this was seen as germtubes being more and more diffuse until it disappears into the agar.

The fungal growth of *Fl*-GFP at 14 hpi and the growth of *F. langsethiae* wt at 12 hpi are shown in Figure 28.

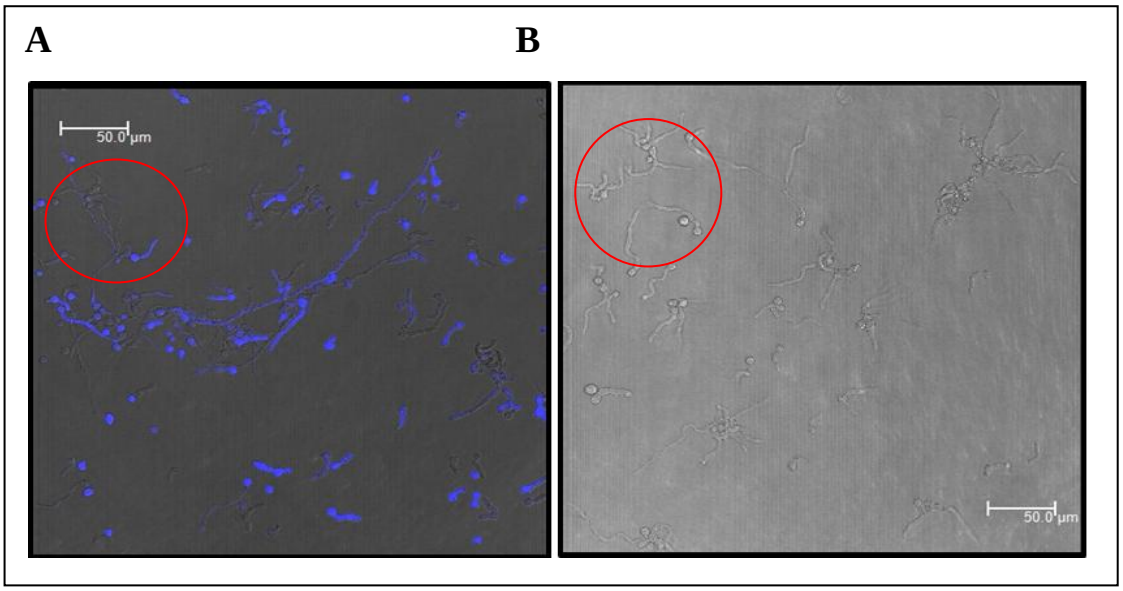


Figure 28: Observed invasive growth of *F. langsethiae* in the co-inoculation study using CLSM. A: *Fl-GFP* at 14 hpi (emission at 500-550 nm). B: *F. langsethiae* wt at 12 hpi (bright field). Encircled areas indicate examples of observed invasive growth. Bars = 50 µm.

F. langsethiae wt and *Fl*-GFP were both used as inoculums for the histological *in planta* characterization. Any obvious differences between the inoculated oats were looked for during the experiment. Figure 29 shows oats inoculated with the two strains.

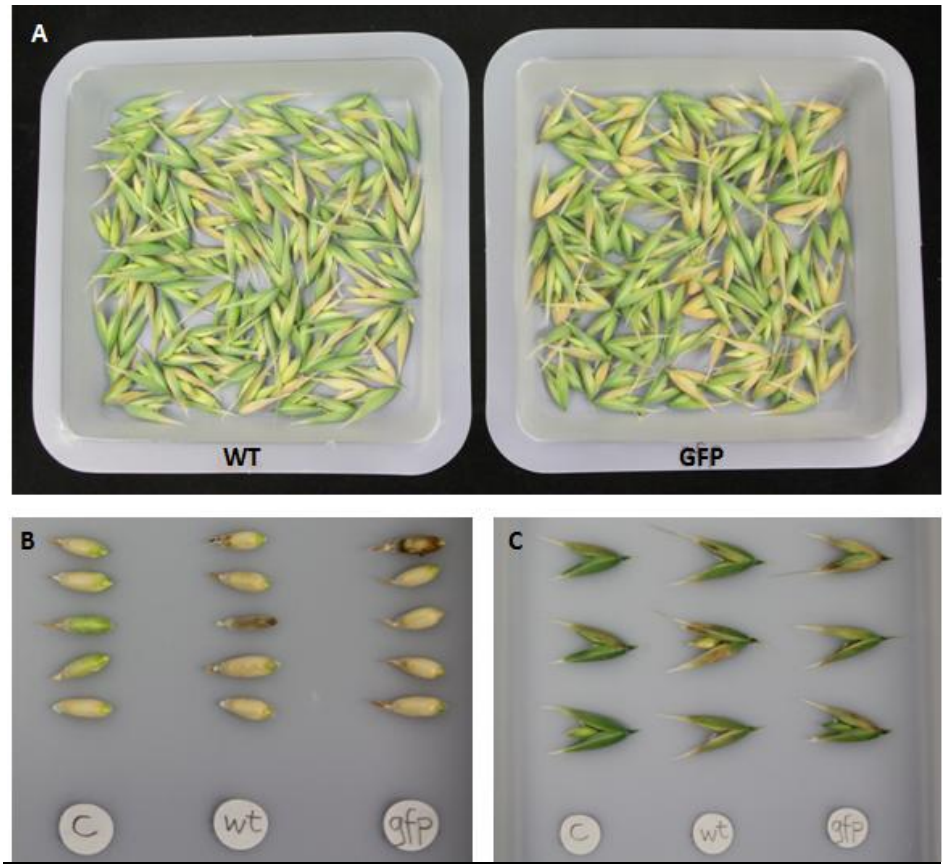


Figure 29: Oats inoculated with *F. langsethiae* wt and *Fl*-GFP at 14 dpi. A: Oat spikelets, B: Dehulled grain of oat C: Oat spikelets. C, control spray inoculated with SDW; wt, *F. langsethiae* wt used as inoculums; GFP, Fl-GFP used as inoculums.

There was no visible difference between the oat spikelets infected with *Fl*-GFP and *F. langsethiae* wt (Figure 29 A). When comparing infected dehulled grains with the control dehulled there was, however, a clear difference, indicating that the infection was successful (Figure 29 B). The infected dehulled grains were less green and had brown spots. A few of them were totally brown and shrunken. No clear difference was observed between the *F. langsethiae* wt infected dehulled grain and *Fl*-GFP infected dehulled grain (Figure 29 B). Figure 29 C shows oat spikelets infected with *F. langsethiae* wt, *Fl*-GFP and non-infected oat spikelets. The ones that were infected with *Fusarium* had a more brownish color than the control spikelets. It did not look like *Fl*- GFP differed from *F. langsethiae* wt in the ability to colonize oat regarding visual symptoms on the oat.

4.4 Co-inoculation study

In the co- inoculation study total hyphal length (spore + germtube) of each species alone (*F. culmorum, F. avenaceum, F. graminearum* and *Fl*-GFP) was used as controls. One representative picture of the different fungi was taken at every time point. Measurements of the spores + germtubes were done as described in methods, section 3.3 The total hyphal length (spore + germtube) of the respective species on control plates are given in Figure 30.

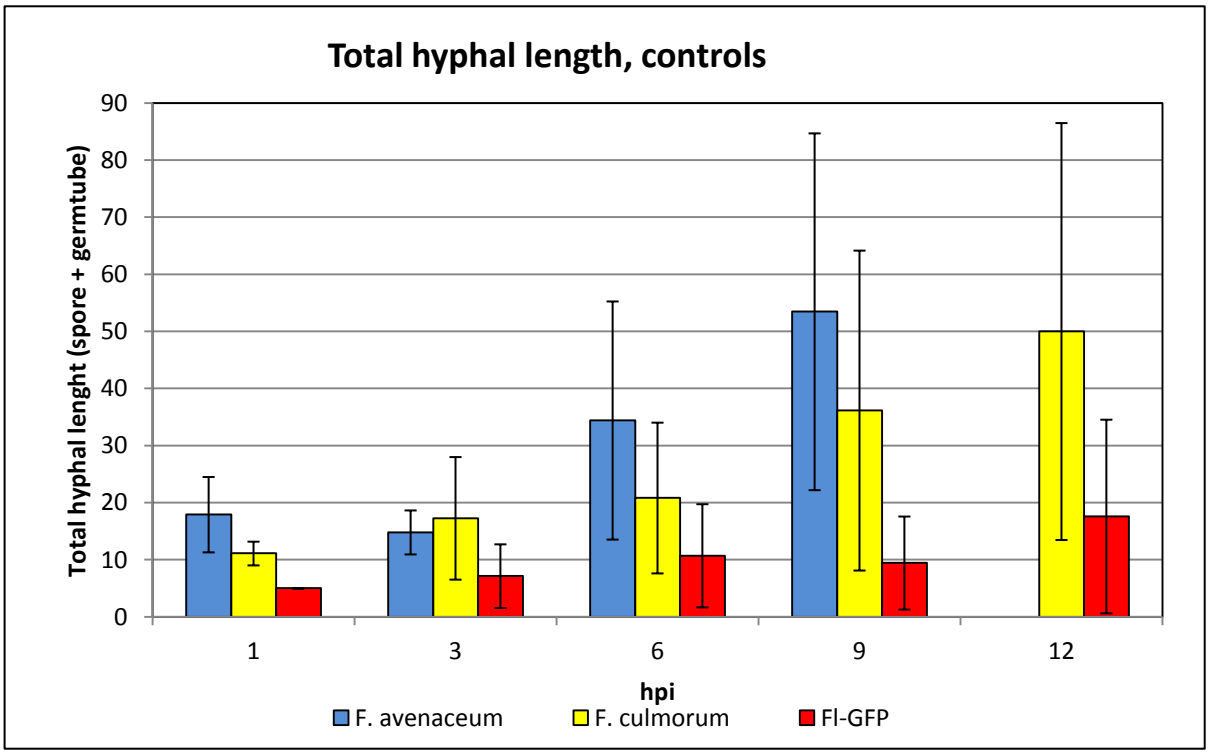


Figure 30: Total hyphal length of *F. avenaceum, F. culmorum* and *Fl*-GFP grown alone on SNA medium in the co-inoculation study. The hpi (x-axis) is plotted against total hyphal length in µm (spore + germtube) (y-axis).

The growth of *Fl*-GFP was considerably slower than the fungal growth of *F. avenaceum* and *F. culmorum* (Figure 30). Invasive growth and hyphal ends not visible in the pictures made it difficult to include data from all time points, and the growth of *F. graminearum* was not measured at all due to invasive growth already at 3 hpi.

Fl-GFP was co-inoculated in pair-wise combinations with *F. avenaceum, F. culmorum* and *F. graminearum.* Three representative pictures of the growth were taken at every time- point and were used to study any possible trends in growth behavior when two species were co-inoculated.

It was of interest to see if there were some difference in total hyphal lengths (spore + germtube) between the controls and when the *Fusarium* species were co-inoculated. Measurements were done as described in methods, section 3.3. The growth of *Fl*-GFP and *F. avenaceum*, growing alone and together, are shown in Figure 31.

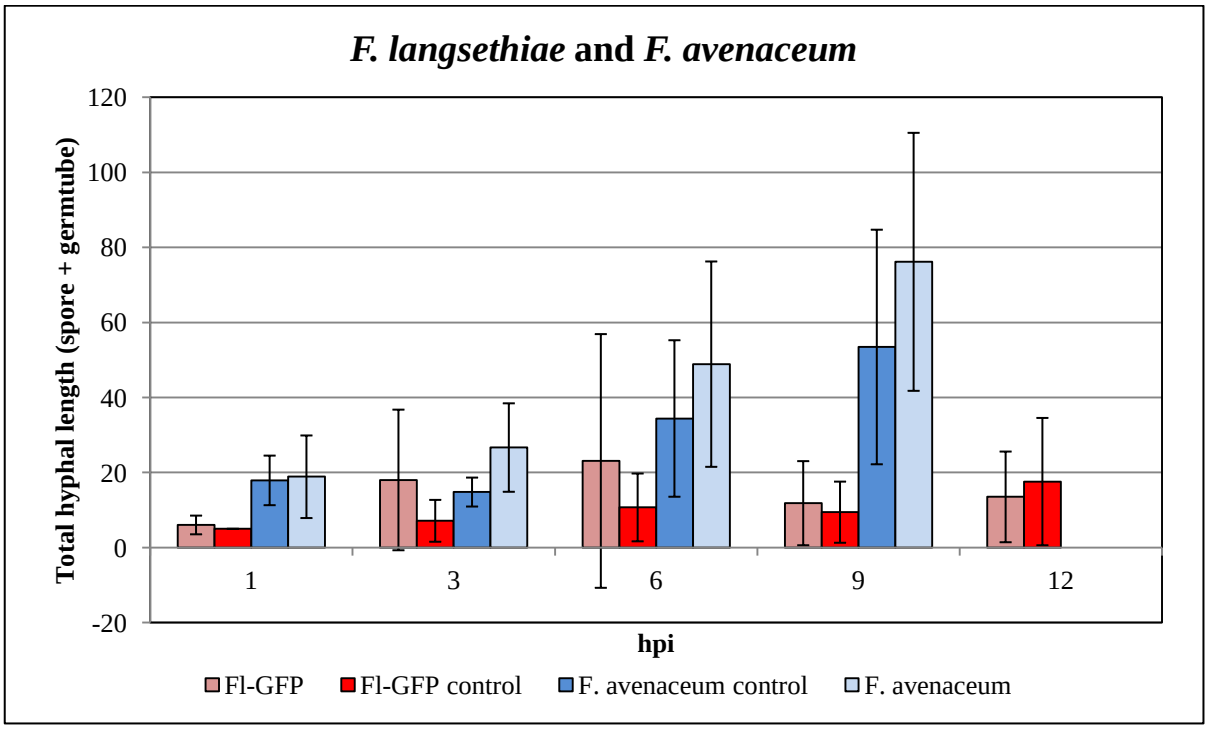


Figure 31: Total hyphal length (spore + germtube) of *Fl*-GFP and *F. avenaceum* on SNA medium over time in the co-inoculation study. The controls indicate the growth when grown alone. The hpi (x-axis) is plotted against total hyphal length (µm) (y-axis).

Similar total hyphal length (spore + germtube) were observed at 1 hpi when *F. langsethiae* and *F. avenaceum* were co-inoculated compared to when grown alone (controls). At 3 and 6 hpi, greater hyphal lengths were observed when the species were co-inoculated compared to when grown alone at the same time-points. This was also observed at 9 hpi regarding *F. avenaceum*, but not for *F. langsethiae*. No difference was observed at 12 hpi.

A T-test confirmed significant difference at 3 hpi ($P<0.05$) for both species, whereas the other time-points did not give any significant difference. Data from time-points 12 to 26 hpi for *F. avenaceum* are not shown in Figure 31 due to rapid growth, and the hyphal ends were not visible in the pictures taken. Data of *F. langsethiae* from time-point 14 to 26 hpi are not shown due to invasive growth.

Fl-GFP was also co-inoculated with *F. culmorum* and the total hyphal length (spore + germtube) of *Fl*-GFP and *F. culmorum*, both when grown alone and in co-inoculation, can be seen in Figure 32.

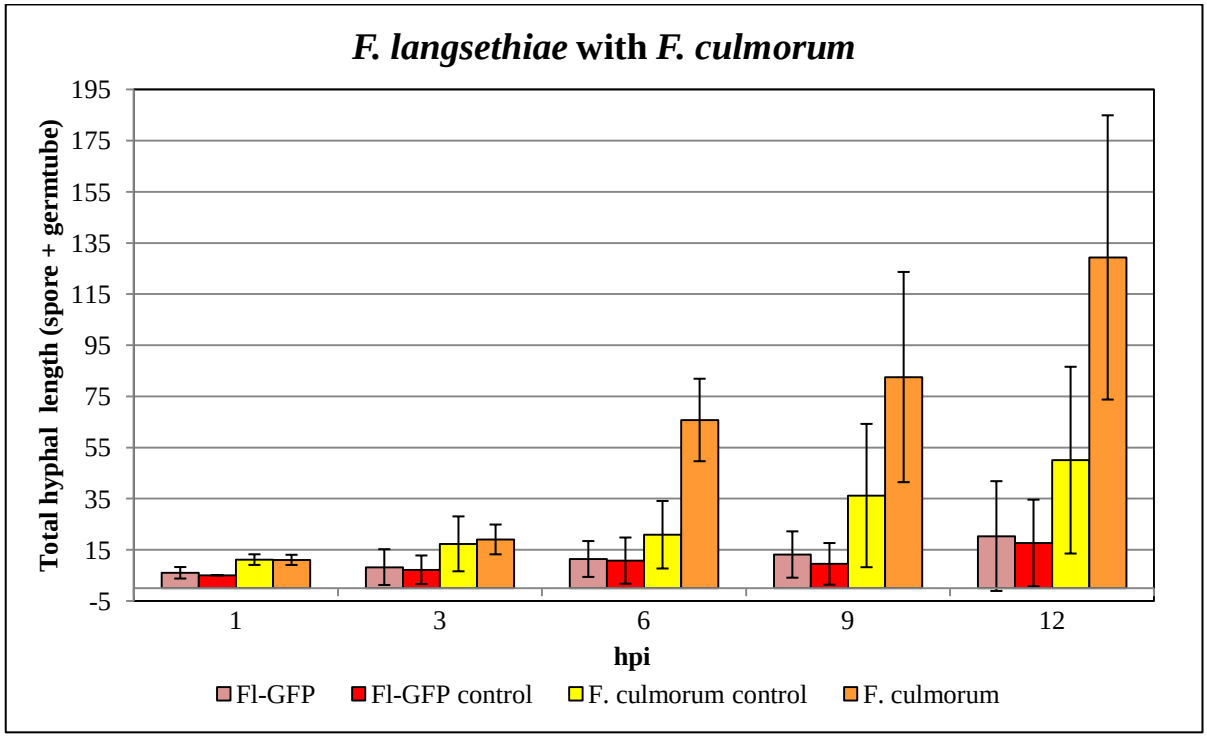


Figure 32: Total hyphal length (spore + germtube) of *Fl*-GFP and *F. culmorum* in co-inoculation on SNA medium over time. The hpi (x-axis) is plotted against total hyphal length (µm) (y-axis).

The total hyphal length (spore + germtube) of *Fl*-GFP was unaffected by the presence of *F. culmorum* at all time points (Figure 32). However, from 6 hpi to 12 hpi the total hyphal length of *F. culmorum* increased in the presence of *Fl*- GFP. The T-test gave P-values that indicated significant difference in total hyphal length (spore + germtube) at 6 hpi and 12 hpi.

F. graminearum started invasive growth already at 3 hpi regardless of the presence of *Fl*-GFP. It was still interesting to look at possible differences between the total hypal length (spore + germtube) of *Fl-GFP* alone and when it was grown together with *F. graminearum* (Figure 33).

A greater total hyphal length (spore + germtube) of *Fl*-GFP was observed from 1 hpi to 9 hpi when it was co-inoculated with *F. graminearum* compared to when it was grown alone. A T-test was conducted to test if there were any significant differences. The P-values indicated significant difference at 1hpi and 9hpi.

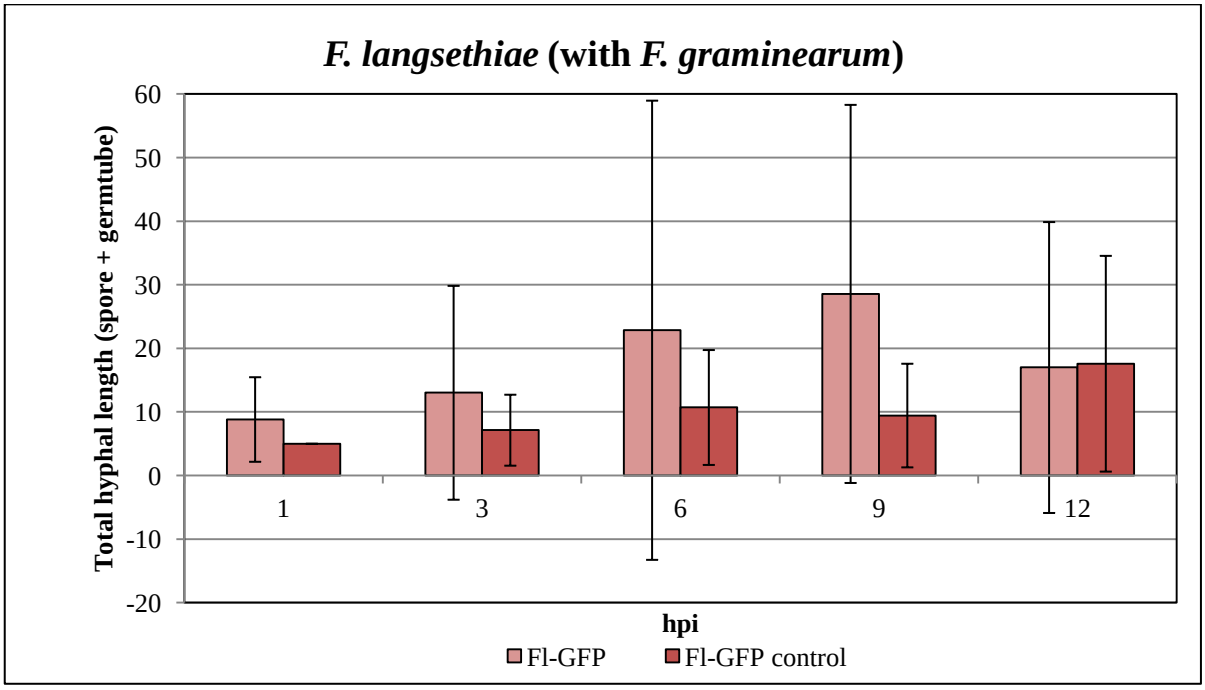


Figure 33: Total hypal length (spore + germtube) of *Fl*-GFP alone and in co-inoculation with *F. graminearum* on SNA medium over time. The hpi (x-axis) is plotted against total hyphal length (µm) (y-axis).

No clear trend whether the fungi were growing towards or away from each other was observed. It looked like a random growth regarding growth directions, and no signs of stress were observed (data not shown).

The pictures taken during the experiment were used to further investigate at which time point the fungi started the invasive growth when the *Fusarium* species grew alone compared to when they were co-inoculated. Examples of invasive growth of *Fl*-GFP and *F. avenaceum* are shown in Figure 34.

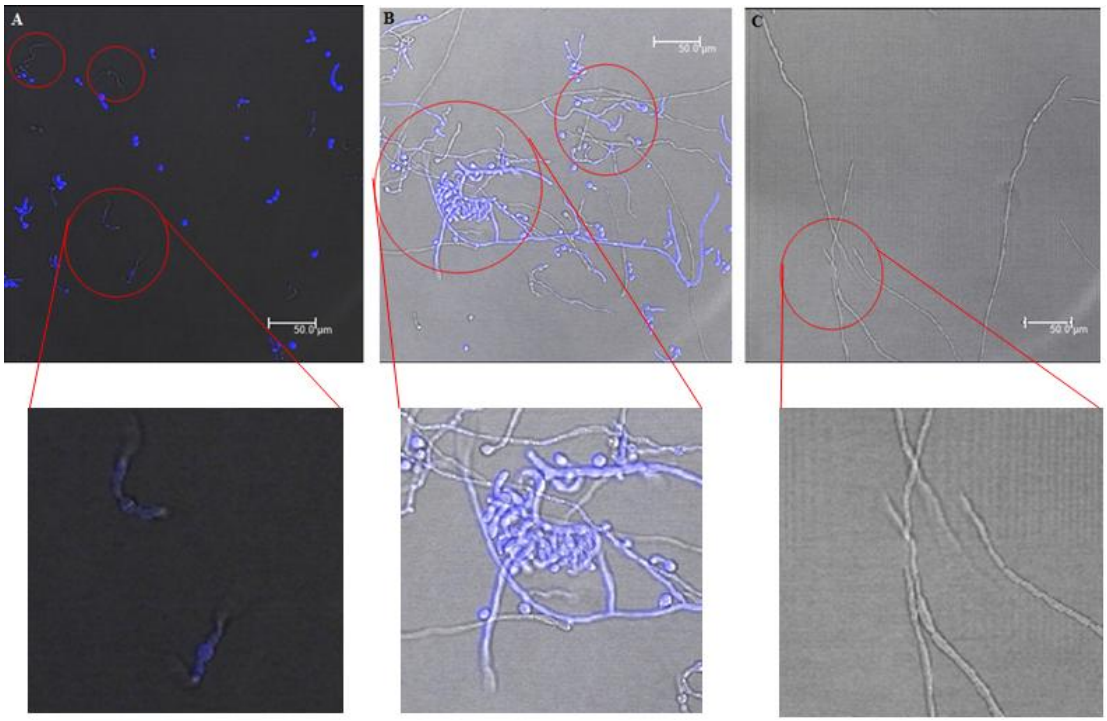


Figure 34: Observed invasive growth of *Fusarium* species in the co-inoculation study using the CLSM. A: *Fl*-GFP growing alone at 14 hpi (emission at 500-550 nm), B: *Fl*-GFP in co-inoculation with *F. avenaceum* after 17 hpi (emission at 500-550nm) and C: *F. avenaceum* growing alone after 20 hpi (bright field). Bars = 50 µm.

F. avenaceum started invasive growth at 20 hpi when it was growing alone (Figure 34 C), but no clear invasive growth was observed when it was co-inoculated with *Fl*-GFP.
Fl-GFP started invasive growth at 14 hpi when growing alone (Figure 34 A), and at 17 hpi when growing together with *F. avenaceum* (Figure 34 B). When *Fl*-GFP was co-inoculated with *F. culmorum*, the invasive growth was first observed after 20hpi, and at 17 hpi when co-inoculated with *F. graminearum*. When it comes to *F. culmorum*, invasive growth was observed after 14 hpi when grown alone and after 17 hpi when it was grown with *Fl-GFP*. *F. graminearum* had the earliest onset of invasive growth, already at 3 hpi, shortly after germination, both when grown alone and grown together with Fl-GFP.

4.5 Histological characterization

To investigate infection routes of *F. langsethiae* during colonizationof oat, a greenhouse experiment was conducted. Oats spray inoculated with *F. langsethiae* wt was sampled at 3 dpi, and used to investigate the fungal growth routes using SEM.

There was not observed a lot of fungal growth on the oat grains, but *F. langsethiae* had overgrown a pollen grain and was therefore easily found. Figure 35 shows growth of *F. langsethiae* on glume ab-axial side of an oat-sample collected at 3 dpi.

The pollen grain on glume ab-axial side in the middle of Figure 35 is overgrown by *F. langsethiae.* On both sides of the parallel veins of the oat, stomata can be found. Stomata are natural openings in the plant and are showed in Figure 35 by arrows. Figure 35 shows that the fungus has started growing in all directions.

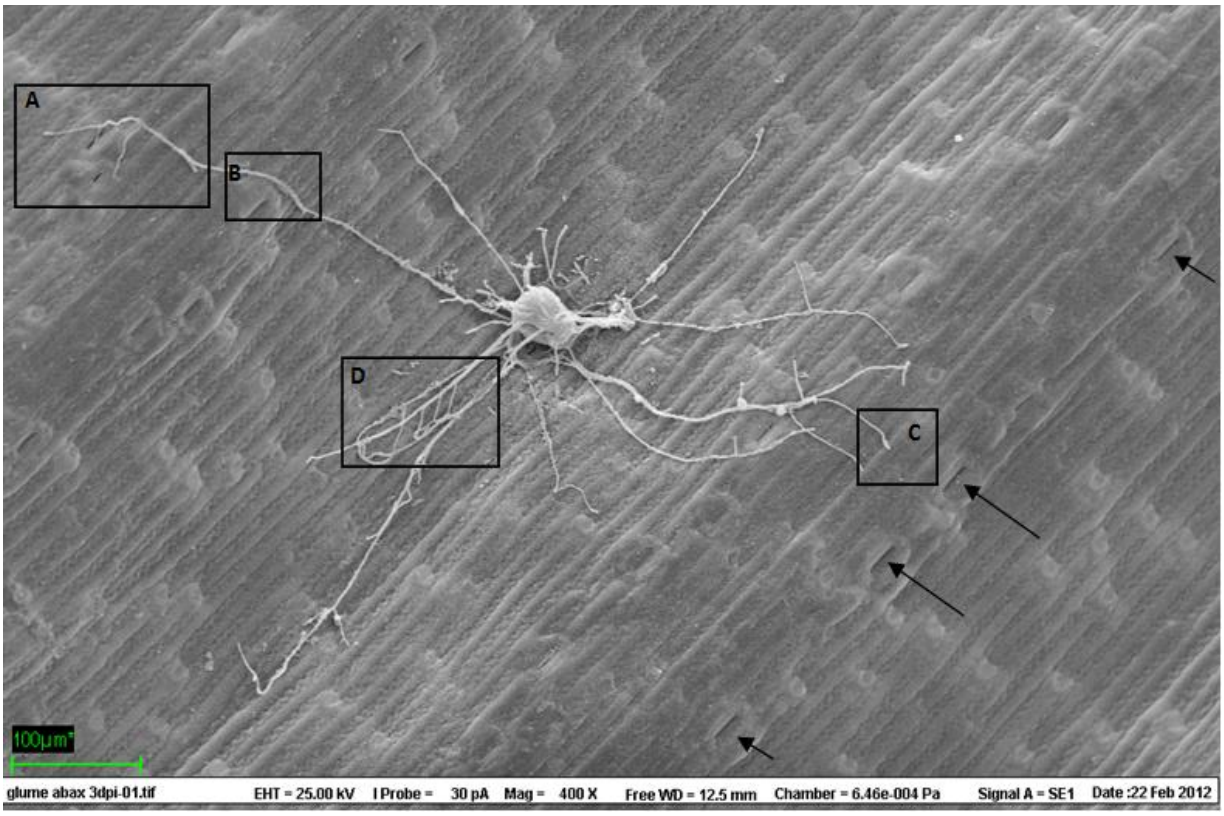


Figure 35: Growth of *F. langsethiae* on a pollen grain on glume ab-axial side of an oat seed collected 3dpi using SEM. Bar = 100 µm. The arrows shows stomata, and the squares indicate close ups shown in Figure 36.

A closer look into the structures of *F. langsethiae* hyphae on the oat is shown in Figure 36.

Figure 36 A shows the growth of *F. langsethiae* towards the natural openings in oat (stomata). This might indicate that *F. langsethiae* is able to infect the inside of the oat by growing through stomata. On the other hand, this was not a trend seen at all hyphal ends.

In many occasions the growth went straight by the stomata. Figure 36 B shows fungal hyphae with a secreted layer around it. This layer might be secreted proteins or sugars (polysaccharides) that might come from the fungus itself. Figure 36 C shows the end of a fungal hyphea.

A curling structure was observed at almost all hyphal ends. This structure might be the starting buildup of an infection cushion, however, may also be a result of the treatment during the preparation for SEM. Figure 36 D shows random growth of *F. langsethiae* on oat. The fungus first grows straight forward, then suddenly back and forth or in a curly pattern.

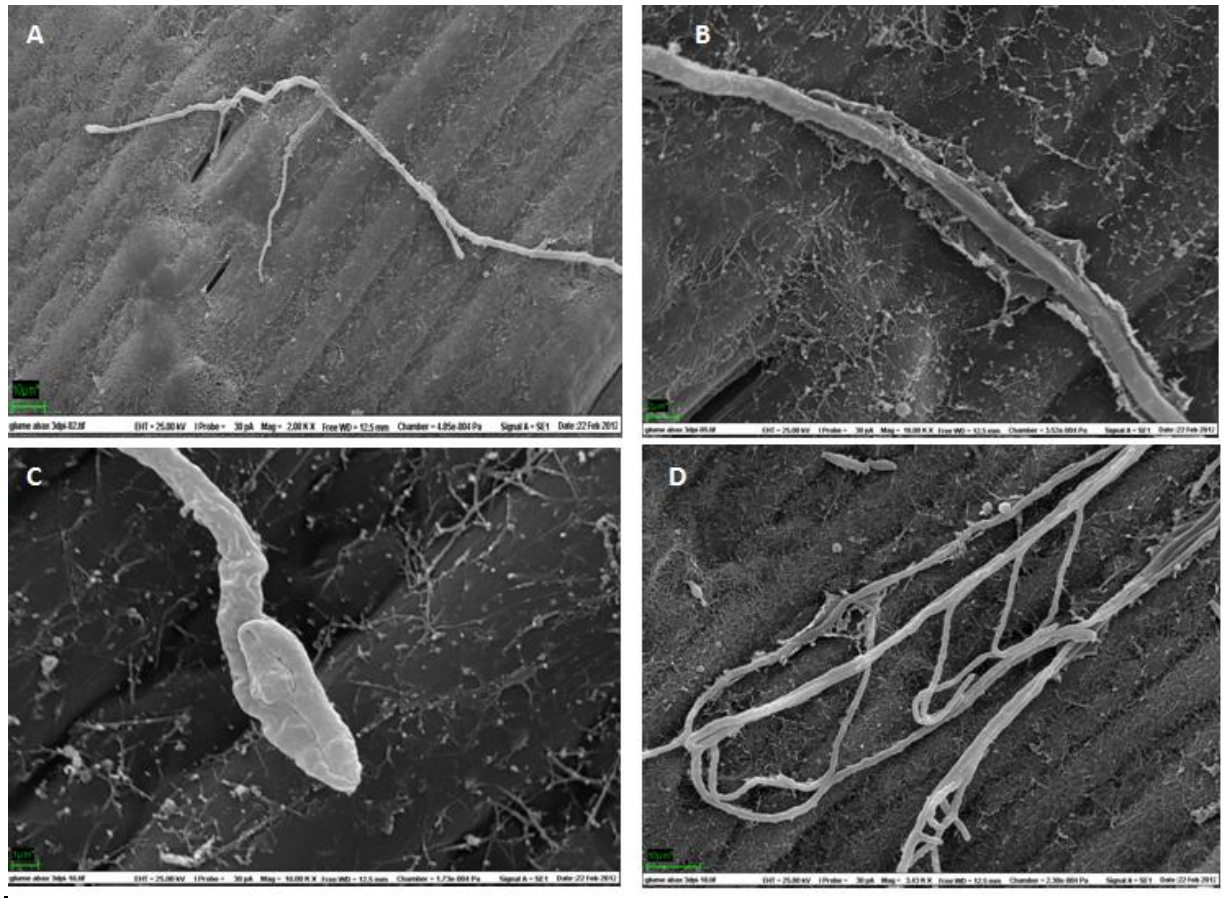


Figure 36: Observation of growth of *F. langsethiae* on the pollen grain on glume ab-axial side of the oat. A: Growth towards natural opening in the oat (bar = 10 µm), B: Secretion of a layer around the fungal hyphae (bar = 2 µm), C: Curling at the end of the fungal hyphae (bar =1µm), D: Random growth of the hyphae (bar = 10 µm).

4.6 Detached leaf assay

To see if *F. langsethiae* can infect unwounded oat leaves and if the oat leaves were affected by the presence of the fungus, a detached leaf assay was performed.

Oat leaves were infected with *F. langsethiae* wt, *Fl*-GFP and SDW as control, and the results were scored at 7 dpi (Figure 37).

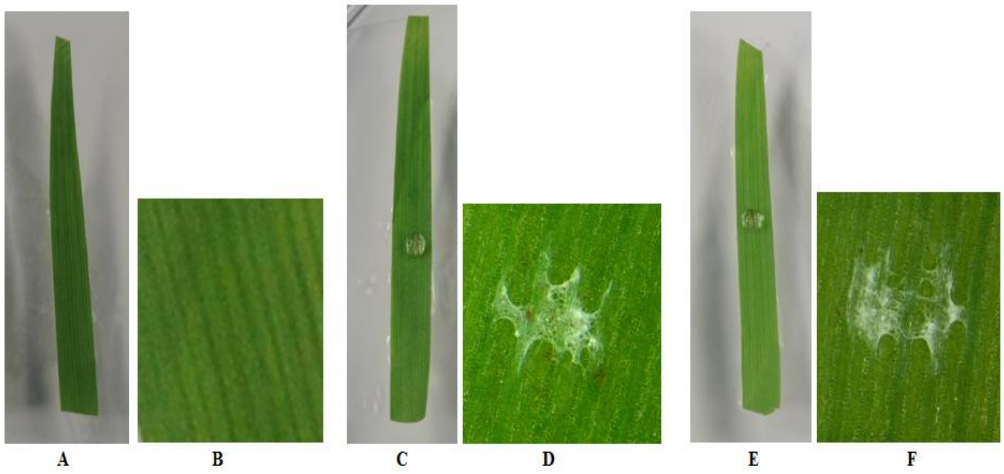


Figure 37: Observations on oat leaves 7 days after inoculating with mock (A and B), *F. langsethiae* wt (C and D) and *Fl*-GFP (E and F).

Fungal growth was observed on the oat leaves inoculated with *Fusarium* Figure 37 C shows an oat leaf with a droplet of inoculum containing *F. langsethiae* wt. *Fusarium* mycelia and brown spots were observed when the droplet was removed (Figure 37 D). This was also observed on the oat leaves inoculated with *Fl*-GFP (Figure 37 E and F). Both *Fusarium* mycelia and a few brown spots were seen when the inoculums droplet was removed. No fungal growth was observed on the oat leaf treated with SDW (Figure 37 A and B).

Oat leaves inoculated with SDW, *Fl*-GFP and *F. langsethiae* wt s were stained with nitro blue tetrazolium in order to assess the presence of superoxide radicals ($O_2^{-)}$ on the leaf. This might give some information about the interactions between oat and *F. langsethiae*. Other oat leaves were first treated with superoxide dismutase inhibitor before staining with nitro blue tetrazolium as described in section 3.6. Controls were infected and "stained" with SDW. The results are shown in Figure 38.

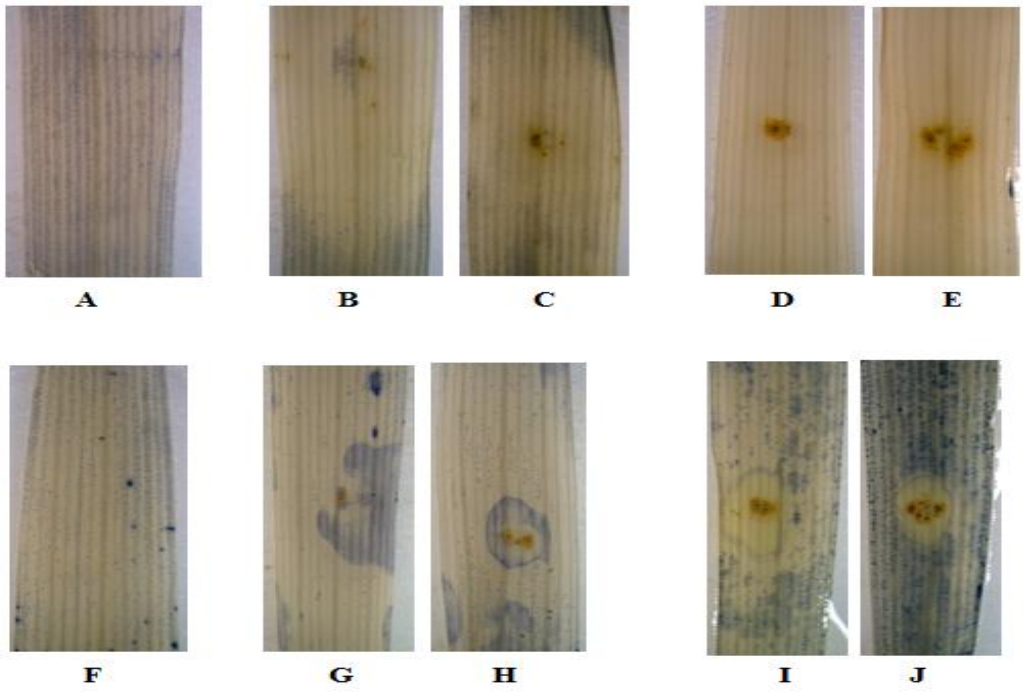


Figure 38: Oat leaves stained with nitro blue tetrazolium to detect superoxide radicals (O_2^-). A: Oat leaf treated with SDW and stained with NBT, B and C: oat-leaves infected with *F. langsethiae* wt and stained with NBT, D and E: Oat-leaves infected with *Fl*-GFP and stained with NBT, F: Oat-leaf infected with SDW and stained with NBT/inhibitor, G and H: Oat-leaves infected with *F. langsethiae* wt and stained with NBT/inhibitor, I and J: Oat-leaves infected with *Fl*-GFP and stained with NBT/inhibitor.

Some stained cells were observed on the oat leaf treated with SDW and stained with NBT (Figure 38A). There were less stained cells around the wounding area on the oat leaves infected with *F. langsethiae* wt stained with NBT (Figure 38B and C). No stained cells were observed on the oat leaves infected with *Fl*-GFP and stained with NBT (Figure 38 D and E).

Treatment with SOD inhibitor prior to NBT staining enhanced the blue stain (Figure 38 F, G H, I and J). The oat leaf infected with SDW and stained with inhibitor and NBT looked approximately the same as the one stained with only NBT (Figure 38 F). Patchy stained cells were observed on the oat leaves infected with *F. langsethiae* wt (Figure 38 G and H). More stained cells were observed on the oat leaves infected with *Fl*-GFP (Figure 38 I and J). Importantly, there was a visible zone around the brown infection areas with no stained cells. Stained cells were found further away from the infection area.

As technical controls, oat leaves treated with SDW, *F. langsethiae* wt and *Fl*-GFP respectively were also treated with water. No staining was visible (data not shown).

Chapter 5 Discussion

5. 1 Interaction between different *Fusarium* species

Interactions between different *Fusarium* species were investigated using an *in planta* study and an *in vitro* co-inoculating study. The *in planta* study was based on a premade greenhouse experiment, and samples were chosen on the basis of results from oat at ripening stage from the same experiment. In this study, both the effect of interactions between species and within species during early infection of oats was provided, due to pair-wise co-inoculations of the fungal species *F. avenaceum*, *F. graminearum, F. culmorum* and *F. langsethiae*, and the use of three isolates for each specie when inoculums were prepared. This will more truly reflect the situation in the field where both several isolates and several fungal species co-exist on the same plant, and potentially compete with each other. The interactions between the *Fusarium* species at even earlier time points (1 – 26 hpi) were investigated on SNA plates in the co-inoculation study. These two interaction studies gave insight to possible interactions between the *Fusarium* species.

5.1.1 Fungal interactions during early infection of oats (*in planta*)

The greenhouse experiment was conducted with mixtures of equal amount of three different isolates to make sure that the inoculums were functional. If only one isolate of each species had been used, it would have had to be characterized according to its aggressiveness on the plant, toxin production, and the ability to produce spores to know that the strain was representative for the species. Such knowledge was not available. The extraction kit used in DNA extraction was the FastDNA®SPIN Kit for Soil and MP Biomedicals states that the kit gives rapid isolation of genomic DNA from soil samples. The fact that only approximately 5 % of the samples in this study had to be extracted again, supports that the kit also applies for oat samples infected with *Fusarium*.

The oat samples not actively infected with *Fusarium* conidia contained no *Fusarium* DNA, indicating that there have probably not been any contaminations during the incubation period, and that the oat did not contain *Fusarium* DNA before the active infection started.

The greenhouse experiment was conducted using three biologically independent repeats for all treatments, and large variation between the biological replicates was observed regarding the amount of fungal DNA at early stage, even though less variation was observed in the samples at ripening stage (Aamot, unpublished data. To reduce this variation, one solution would have been to use more than three biological replicates, or to have selected a higher number of oat kernels

and performed several DNA extractions for one sample. High level of deviation is not unusual regarding *Fusarium* experiments, and to minimize the high level of deviation, many researchers pool the biological replicates together before the quantification by qPCR. In the present study, this was not done, and the results are therefore giving a more nuanced picture than if the pooling was performed prior to the quantification. The variation in the data shows that there are multiple unknown factors influencing fungal growth and metabolism. Variation was also observed in the study of Xu et al. (2007a) where wheat was inoculated with *Fusarium* species. They stated that the variation might be due to differences in the quality of the inoculums, and differences in the condition of the plant (i.e. temperature and light). Factors like temperature and light may have contributed to the high variation observed in this study as well, due to the fact that the plants were grown at different time periods.

The results of this study showed that no change in DNA amount of *F. avenaceum* was observed when *F. avenaceum* was grown together with *F. culmorum* in oat at 6 dpi. An increase in DNA amount at 10 and 14 dpi was, however observed. This indicates that the interaction between *F. avenaceum* and *F. culmorum* enhance the development of *F. avenaceum* on oat at early stage. The trends showed that *F. graminearum* had no effect on *F. avenaceum* at any time point. *F. langsethiae* had minor effect on *F. avenaceum*, but a decrease in DNA amount was observed at 10 dpi when co-inoculated, compared to when grown alone at the same time-point. The results regarding oat at ripening stage showed a reduction of *F. avenaceum* DNA in combinations with all the other species (Aamot, unpublished data). The results indicate that *F. avenaceum* is slightly more competitive in oat at early stages compared to its less competitive ability on oat towards ripening stage. The observed differences in the present study were not found significant, but the results, nevertheless, show trends in possible interactions between *F. avenaceum* and the other species on oat at an early stage.

Lower DNA amount of *F. langsethiae* was observed when grown together with either *F. avenaceum, F. graminearum* or *F. culmorum* with some exceptions of increased amount.

This is in agreement with findings from oat at ripening stage, when all combinations gave approximately equal reduction in DNA amount of *F. langsethiae* (Aamot, unpublished data). The observed differences in the present study were not significant, but the results showed however trends in possible interactions between *F. langsethiae* and the other species during early oat infection.

Oat samples inoculated with *F. graminearum* and *F. culmorum* alone were not included in the present study. It was therefore not possible to say if the growth of these species had been influenced by the presence of *F. langsethiae* and *F. avenaceum*. It was on the other hand possible

to say something about the differences in the DNA amounts between interactions with *F. avenaceum* and *F. langsethiae* and differences over time.

Both the interactions with *F. avenaceum* and *F. langsethiae* gave an increase in the amount of *F. graminearum* DNA from 6 dpi to 10 and 14 dpi. The increase was highest when *F. graminearum* was grown with *F. avenaceum*, and the increase from 6 to 10 dpi was significant. This result differs from what seen at ripening stage, where the combination with *F. avenaceum* resulted in lower DNA amount of *F. graminearum* than the amount in combination with *F. langsethiae* (Aamodt, unpublished data). Regarding *F. culmorum*, interaction with *F. langsethiae* resulted in higher amount of DNA from 6 to 10 and 14 dpi, and interaction with *F. avenaceum* resulted in lower DNA amount from 6 to 10 and 14 dpi during early oat infection. At ripening stage both combinations gave similar amounts of *F. culmorum*. The observed differences in the present study from 6 dpi to 10 and 14 dpi were however not significant.

Even though some of the observed differences did not turn out to be significant, the results showed trends in possible interactions between the *Fusarium* species at early infection of oat.

Xu et al. (2007b) reviewed that competitive interactions are the rule rather than the exception in almost all published research when fungal and disease development is considered. This was probably the case in the present study as well. This means that the interactions observed in the present study was an outcome of competition between the species. Both Velluti et al (2000) and Simpson et al. (2004) indicated that the dominant species gained no advantage from the presence of the weaker fungi, but the development of the weaker fungi was reduced in the presence of a stronger fungi. Both *F. graminearum* and *F. culmorum* have been shown to be competitive species, while *F. langsethiae* and *F. avenaceum* was less competitive species during infection of wheat among the species tested (Klemsdal et al. 2009). Similar interactions have been observed in oat, but *F. culmorum* was shown to be slightly more competitive than *F. graminearum* (Klemsdal et al. 2009). Because of this, it was expected to see a reduction in amount of *F avenaceum* and *F. langsethiae* during competitive interaction with *F. graminearum* and *F. culmorum* on oats. This was observed for *F. langsethiae* only at early stage, but for both at ripening stage. This indicates that *F. avenaceum* is slightly more competitive during infection of oat at early stage compared to *F. langsethiae*.

F. langsethiae is a producer of T-2 and HT-2, while *F. graminearum* and *F. culmorum* are producers of DON and NIV (Placinta et al. 1999; Torp & Nirenberg 2004). Several studies have reported a mutual exclusion between HT-2/T-2 (combined) and DON (when one is high, the other one is low) (van der Fels-Klerx 2010), and the production of mycotoxins may have been a factor in the interactions observed in this study.

5.1.2 Interactions in a co-inoculation study (*in vitro*)

The co-inoculation study provided a fast and easy method for studying fungal interactions under *in vitro* conditions, and made it possible to study the fungi in an efficient way using CLSM. The *Fl*-GFP strain made it possible to distinguish between the species using emission filters for green fluorescence as well as bright field.

The results in this study showed that *F. langsethiae* had the shortest hyphal length (spore + germtube) of the *Fusarium* species tested, and this corresponds with the findings of Torp and Nirenberg (2004), that showed that *F. langsethiae* has a slower growth rate than the other *Fusarium* species tested. Detectable differences as an effect of co-inoculation were however found. The species with the most positive effect on *F. langsethiae* was *F. avenaceum*. Both *F. langsethiae* and *F. avenaceum* had greater total hyphal length (spore + germtube) when co-inoculated compared to when grown alone. This indicates that *F. avenaceum* and *F. langsethiae* affect each other's growth positively instead of negatively as an effect of competition. *F. langsethiae* was on the other hand not notably affected by *F. culmorum.* *F. culmorum* had greater total hyphal length (spore + germtube) when co-inoculated with *F. langsethiae,* which indicates that the growth of *F. culmorum* had been positively affected by *F. langsethiae*. Interestingly, the total hyphal length (spore + germtube) of *F. langsethiae* was higher when co-inoculated with *F. graminearum* compared to when grown alone at 1-9 hpi. The observed increase in total hyphal length was unexpected, especially regarding when co-inoculated together with *F. graminearum*, a dominant and competitive specie (Xu et al. 2007a). *F. langsethiae* produces microconidia (Torp & Nirenberg 2004), while *F. graminearum* produces larger macroconidia (Nelson et al. 1994) giving *F. graminearum* an advantage regarding nutrient storage. The fact that SNA is a nutrient poor medium should probably have limited both species, and especially *F. langsethiae* due to its small size microspores. It is possible that the presence of *F. graminearum* did not affect *F. langsethiae* negatively due to its invasive growth already at 3 dpi, resulting in nutrient uptake at different places. However, invasive growth seemed to be delayed in all co-inoculated samples as compared to the control plates with each fungus growing separately, thus not supporting this hypothesis.

The present study indicates that the species can take advantage of each other at early time points. One of the challenges in the experiment was the time limitation due to the time laps series. Only one picture of the different fungi on the control plates was taken at every time-point. Three pictures were taken when the species were co-inoculated. All combinations and controls were studied and photographed every 3rd hour. If the pictures had been taken every 4th or 5th hour, a

higher number of pictures could have been analyzed, however, in this case we might have missed important changes. Other challenges were the spore concentration, synchronization of spore germination, and differences between the agar pieces prepared for CLSM. The spore suspensions were not held on ice during the inoculation, and inoculation of a high number of plates did cause some delay and may account for some of the differences observed. The logistics created problems due to observations at 0 hpi. The first observations in the CLSM were therefore at 1 hpi. Nevertheless, several time points in the present study showed a significant difference between the species, and the experiment gives interesting indications about the first interactions between the various fungi. The choice of medium and incubation temperature in this kind of interaction will affect the growth rate, the competition between the pathogens and mycelia interactions. Nevertheless, given the limitations of the in vitro method used here, the results indicate that potential early interactions between *Fusarium* species may indeed occur.

5.2 Infection processes of *F. langsethiae* on oat

The infection process of *F. langsethiae* is poorly understood, and to gain more knowledge about this, different experiments were done. The gene expression study was conducted to investigate whether selected genes might be involved during early infection on oats, the histological characterization was done to try to understand how the fungus grows, infect and enter the oat, and the detached leaf assay was used to look closer into the interactions between the plant and the fungus.

5.2.1 *F. langsethiae* genes involved during early infection of oats

The samples used in the gene expression study originated from the same greenhouse experiment already discussed. Samples inoculated with *F. langsethiae* (1x and 2x spore inoculums) and control samples collected at 6 and 10 dpi were included. This was based on observations regarding the ability of *F. langsethiae* to enter the oat grain between 6 and 10 dpi (Divon, unpublished data). The choice of using spectrum™ Plant Total RNA Kit for RNA extraction was based on former experience (Anstensrud, unpublished data), and it was shown that this kit supplemented with the DNA-free™ Kit resulted in high quality RNA. Based on the transcriptome analysis previously done (Lysøe et al. unpublished data) genes were selected for the gene expression study. The genes chosen were higher expressed *in vitro* on oat based media and on oat flower media than on rich media without any oat residues (transcriptome analysis,

data not shown). This indicated that these genes might be involved during infection on oats. Thus it was desirable to test this hypothesis using the same genes on infected oat. Expression of the *Sod* gene in *F. langsethiae* mycelia was chosen as reference due to its relatively high expression. This way the expression of all genes was comparable with each other and the expression of each gene could also be compared between the different samples.

The gene expression study showed higher expression of the *Sod* gene *in planta* than in *F. langsethiae* mycelia alone, indicating that SOD activity might be required during the infection process. Plants have a defense against pathogenic microbes, and one of the first lines of defense is production of ROS. The enzyme superoxide dismutase (SOD) catalyzes the dismutation of O_2^- to O_2 and H_2O_2. The higher expression of the *Sod* gene in *F. langsethiae* when grown on oat indicates that the pathogenic fungus have avoided the plant defense mechanism and might enhance the infection by this mechanism.

There was no expression of the gene *Nps4* in *F. langsethiae* mycelia, but *Nps4* was on the other hand expressed when the fungus was grown on oat. This means that the *Nps4* gene might be involved in the *F. langsethiae* infection process, and this corresponds with other research on other *Nps* genes. For one, Lee et al. (2005) suggested in their study that the *Nps6* gene might be involved in defense of fungi against a variety of stresses (Lee et al. 2005). They also observed that *Nps6* mutants had enhanced sensitivity to hydrogen peroxide. This indicated that the small peptides might be involved in the protection of the fungus from oxidative stress, and would be consistent with the observation that an oxidative burst commonly occurs when plants are attacked by microbes (Lee et al. 2005). A second example is the study of Oide et al. (2006) that demonstrated that the NRPS encoded by *Nps6* was responsible for the biosynthesis of extracellular siderophores, thus proposing that extracellular siderophores play a role in fungal infection of plant. Deletion of *Nps6* orthologs in for example *F. graminearum*, resulted in a reduction of virulence towards the host, as well as increased sensitivity to ROS and to low iron concentration (Oide et al. 2006).

One of the target genes tested in this gene expression study have an unknown function, *Fl-est17042*. Several *Fusarium* species have had their whole genome sequenced, and *F. graminearum* was one of the first plant pathogens to be selected for full genome sequencing (Brown et al. 2012). Nevertheless, this putative gene has not been found in any other microorganism other than *F. langsethiae*. Moreover, this gene does not share resemblance to any other known gene in the databases. No expression of the gene *Fl-est17042* was detected in mycelia of *F. langsethiae*, but was highly expressed *in planta*. This indicates that this gene probably have a function in the infection process of *F. langsethiae* in oats. The *Fl-est17042* gene

was also the transcript with the highest expression in the oat samples by far. Further work is necessary to verify the role of this putative gene product and to fully understand its function.

The last putative gene tested in this study was the *Dep5 pks* gene coding for Depudecin Polyketide Synthetase. There was no clear difference between the expression of the *Dep5 pks* gene in *F. langsethiae* grown on oat compared to the expression in *F. langsethiae* mycelia alone. Depudecin is an inhibitor of histone deacetylase (HDAC) which can alter the compaction of the cromatine and potentially control the expression of numerous genes in the target. HDACs are involved in the regulation of many processes such as embryo and flower development, abiotic stress responses and root-hair density (Wight et al. 2009). The transcriptome analysis showed that Depudecin was specifically expressed on oat based agar, however the present study indicated that the interactions between *F. langsethiae* and oat might not contribute to higher expression of *Dep5 pks* and that this protein might not be regulated at transcription level

The results in the present study support the data from the transcriptome analysis regarding the *Sod* gene, the *Nps4* gene and the *Fl-est17042* gene, but not the *Dep5 pks* gene. As such, this study indicated that these genes have a function *in planta* during *F. langsethiae* infection on oats, with the exception of the *Dep5 pks* gene.

A plant infection study with fungal knock-outs of the genes would be necessary to prove their roles during plant infection.

5.2.2 Early stage of colonization of oats

To learn more about *F. langsethiae* early stages of colonization on oats, the infection process was studied histologically using SEM.

Despite a relative high amount of inoculum, fungal growth on the surface of the grain was scarce. Vigorous growth of hyphae was observed in association with pollen grains, however, and this might indicate that establishment of *F. langsethiae* on oat is dependet on nutrient source outside the fungal spore, such as pollen grains. This finding corresponds to other research. Two components (choline and glycine betaine) are found in pollen and anthers, and are highly stimulating hyphal growth and branching in *Fusarium* species (Strange et al. 1974). Research by Strange and Smith (1971) showed that removal of anthers by physical emasculation resulted in reduced infection of *F. graminearum* in certain wheat cultivars. Another study using plants like tobacco, tomato, Arabidopsis and wheat showed that flowers lacking functional anthers were highly resistant to *Fusarium* attack (Urban et al. 2002).

The present study showed that some of the hyphae seemed to grow towards and perhaps penetrate through stomata. These natural openings can therefore be one way for *F. langsethiae* to

enter the oat grain. This corresponds to the findings of *F. graminearum* on wheat where one of the early entry points were glume stomata (Pritsch et al. 2000). Penetration through stomata, however, did not seem to be an essential factor for successful infection.

Fungi produce secreted cell wall -degrading enzymes that enable the pathogen to penetrate, grow and infect through the plant tissue (Kikot et al. 2009). Fungal hyphae were often observed surrounded by an extra layer. This layer might be secreted proteins or polysaccharides produced by the fungus during the infection process or perhaps produced as protection.

It also seemed like *F. langsethiae* grew randomly with growth straight forward, then suddenly back and forth or in curly patterns. A curling structure was observed at almost all hyphal ends and this structure might indicate early attempts to form penetration structure. Penetration abilities have been detected in other *Fusarium* species, and *F. graminearum* is able to penetrate and invade a host with the help of secreted (Kikot et al. 2009). It has also been shown that *F. graminearum* forms foot structures, infection cushions and compound appressoria from runner hyphae on inoculated wheat caryopsis, palea, lemmea and glume (Boenisch & Schafer 2011). Whether *F. langsethiae* have the same abilities is still unknown, but this study indicates some of the possible early infection processes of *F. langsethiae* on oat.

5.2.3 Infection of oat leaves and interaction between *F. langsethiae* and the plant

The initial stages of infection were also studied using detached leaf assay. Both *Fl*-GFP and *F. langsethiae* wt were used as inoculums, and even though similarities between the two strains were observed in earlier experiments, the detached leaf assay revealed some slight differences.

The results in the present study showed that fungal mycelia were found at 7 dpi, but only minor necrosis or chlorosis was observed. It is known that *F. langsethiae* hardly causes any symptoms of disease on oat (Divon et al. 2012; Torp & Nirenberg 2004), however, in a detached leaf study conducted by Imathiu et al. (2009), some more wounding was observed. One known factor to influence the fungal virulence and aggressiveness is the temperature. The infected oat leaves were in the present study incubated at room temperature (21 °C) for 7 days. Imathiu et al. (2009) used both 10 °C and 20 °C in their experiments and saw that the greatest lesions on the plants occurred at 20 °C. The study by Imathiu et al. (2009) also showed necrosos and chlorosis on both wounded and non-wounded oat leaves, supporting the choice of non-wounded leaves and temperature used in the present study.

F. langsethiae expressed one transcript encoding Superoxide Dismutase (SOD) during infection, and the detached leaf assay gave the opportunity to study further the interaction between the fungus and the plant.

The infected oat leaves were stained with nitro blue tetrazolium (NBT) that reacts with the O_2 and gives a blue color. Production of reactive oxygen species (ROS) is a key role in plant defense against attack by pathogens and it is often one of the first responses of the plant (HammondKosack & Jones 1996). Because of the toxic effect of O_2^-, plants and fungi produce SOD that catalyzes the dismutation of O_2^- to O_2 and H_2O_2 as a defense. (Cadenas 1995). The infected oat leaves in the present study clearly showed more blue staining than the control. Treatment with inhibitor of SOD prior to NBT staining resulted in stronger staining. This indicates that the staining was caused by production of O_2^-. Interestingly, a clear area with no stained cells was observed at the spot where the inoculums were placed, indicating that a more efficient reduction of O_2^- had taken place prior to inhibition of SOD in this area. This strengthens the results from the expression study, and suggests that SOD is actively used by *F. langsethiae* in protection against plant produced ROS during infection.

More studies are needed to fully understand the pathogenicity of *F. langsethiae* and the interactions between the plant and the fungus.

5.3 Conclusive remarks and future aspects

The two experiments conducted to give insight to the interactions between four plant pathogenic *Fusarium* species give somewhat varying results. The experiment quantifying *Fusarium* DNA in oat, showed the interactions between the species during early infection of oat (6, 10 and 14 dpi). Competitive interactions were observed among the species, and *F. avenaceum* was found to be somewhat more competitive than *F. langsethiae* which in general were negatively affected in the interactions with the other *Fusarium* species. The co-inoculation study showed the interaction *in vitro* at 1-26 hpi and indicated on the other hand that the interactions between the species resulted greater hyphal length (spore + germtube) and that the initiation of invasive growth was delayed when co-inoculated compared to when grown alone,

The gene expression study, the histological characterization and the detached leaf assay gave useful information in understanding the infection process of *F. langsethiae* on oat, and the possible interaction between the plant and the fungus. The present study showed that the genes *Sod*, *Nps4* and *Fl-est17042* are actively transcribed in *F. langsethiae* during oat infection.

It has been elucidated in this study that *F. langsethiae* can grow through the natural opening of the plant and possibly be able to penetrate, but it might be dependent on an energy source outside itself, such as pollen grains to infect and establish disease in oats. This study also provided insight to the interactions between *F. langsethiae* and oats. The known interaction between plants and pathogens regarding ROS and SOD production seemed to have occurred in the present study when *F. langsethiae* was infecting oat leaves.

All in all, the infection process by *F. langsethiae* on oats carries many similarities to the infection of cereals by other FHB causing fungi. As such, the results in the present study supports the finding reported by Divon et al. (2012), suggesting that *F. langsethiae* can be characterized as a weak pathogen.

Even though the present study have indicated some of the possible interactions between pathogenic *Fusarium* species during early infection of oat and the infection process of *F. langsethiae* on oats, further analyses are required. There is still a long way to go towards a better understanding and hopefully more work will promote the discovery of potential interactions and infection routes that is still unknown.

Chapter 6 Literature

Adams, P. S. (2006). Data analysis and reporting. In Dorak, M. T. (ed.) *Real-time PCR*, pp. 39-62. New York, Taylor & Francis.

Boenisch, M. J. & Schafer, W. (2011). Fusarium graminearum forms mycotoxin producing infection structures on wheat. *Bmc Plant Biology*, 11.

Brodal, G., Elen, O. & Hofgaard, I. S. (2012). Fusarium og mykotoksiner i norsk korn før og nå. *Bioforsk Fokus*, 7: 57-59.

Brodhagen, M. & Keller, N. P. (2006). Signalling pathways connecting mycotoxin production and sporulation. *Molecular Plant Pathology*, 7 (4): 285-301.

Brown, N. A., Antoniw, J. & Hammond-Kosack, K. E. (2012). The Predicted Secretome of the Plant Pathogenic Fungus Fusarium graminearum: A Refined Comparative Analysis. *Plos One*, 7 (4).

Cadenas, E. (1995). Mechanisms of oxygen activation and reactive oxygen species detoxification. In Sami, A. (ed.) *Oxidative stress and antioxidant defenses in biology*, pp. 1-46. New York, Chapman & Hall.

Commision, E. (2006). *Commission Regulation (EC) No 1881/2006 Setting maximum levels of certain contaminants in foodstuff*. Official Journal of the European Union. L364: 5-24 p.

D'Mello, J. P. F., Placinta, C. M. & Macdonald, A. M. C. (1999). Fusarium mycotoxins: a review of global implications for animal health, welfare and productivity. *Animal Feed Science and Technology*, 80 (3-4): 183-205.

Dangl, J. L. & Jones, J. D. G. (2001). Plant pathogens and integrated defence responses to infection. *Nature*, 411 (6839): 826-833.

Deacon, J. W. (2006). Fungal interactions: mechanisms and pratical explotation. In Deacon, J. W. (ed.) vol. 4th edition *Fungal biology*, pp. 237-254. Malden, Mass., Blackwell.

Desjardins, A. E., Hohn, T. M. & McCormick, S. P. (1993). Trichothecene Biosynthesis in *Fusarium* Species - Chemistry, Genetics, and Significanse. *Microbiological Reviews*, 57 (3): 595-604.

Desjardins, A. E. (2006). Trichothecenes. In Desjardins, A. E. (ed.) *Fusarium mycotoxins : chemistry, genetics, and biology*, pp. 13-64. St. Paul, Minn., APS Press.

Dhanoa, P. K., Sinclair, A. M., Mullen, R. T. & Mathur, J. (2006). Illuminating subcellular structures and dynamics in plants: a fluorescent protein toolbox. *Canadian Journal of Botany-Revue Canadienne De Botanique*, 84 (4): 515-522.

Divon, H. H., Razzaghian, J., Udnes-Aamot, H. & Klemsdal, S. S. (2012). Fusarium langsethiae (Torp and Nirenberg), investigation of alternative infection routes in oats. *European Journal of Plant Pathology*, 132 (1): 147-161.

Doohan, F. M., Brennan, J. & Cooke, B. M. (2003). Influence of climatic factors on Fusarium species pathogenic to cereals. *European Journal of Plant Pathology*, 109 (7): 755-768.

Foroud, N. A. & Eudes, F. (2009). Trichothecenes in Cereal Grains. *International Journal of Molecular Sciences*, 10 (1): 147-173.

Goswami, R. S. & Kistler, H. C. (2004). Heading for disaster: Fusarium graminearum on cereal crops. *Molecular Plant Pathology*, 5 (6): 515-525.

Guenther, J. C. & Trail, F. (2005). The development and differentiation of Gibberella zeae (anamorph : Fusarium graminearum) during colonization of wheat. *Mycologia*, 97 (1): 229-237.

Halstensen, A. S., Nordby, K. C., Eduard, W. & Klemsdal, S. S. (2006). Real-time PCR detection of toxigenic Fusarium in airborne and settled grain dust and associations with trichothecene mycotoxins. *Journal of Environmental Monitoring*, 8 (12): 1235-1241.

HammondKosack, K. E. & Jones, J. D. G. (1996). Resistance gene-dependent plant defense responses. *Plant Cell*, 8 (10): 1773-1791.

Hofgaard, I. S., Brodal, G., Elen, O., Aamot, H. U. & Klemsdal, S. S. (2009). Fusarium graminearum i Norge. *Bioforsk Fokus*, 4: 96-97.

Ilgen, P., Hadeler, B., Maier, F. J. & Schafer, W. (2009). Developing Kernel and Rachis Node Induce the Trichothecene Pathway of Fusarium graminearum During Wheat Head Infection. *Molecular Plant-Microbe Interactions*, 22 (8): 899-908.

Imathiu, S. M., Ray, R. V., Back, M., Hare, M. C. & Edwards, S. G. (2009). Fusarium langsethiae pathogenicity and aggressiveness towards oats and wheat in wounded and unwounded in vitro detached leaf assays. *European Journal of Plant Pathology*, 124 (1): 117-126.

Imathiu, S. M., Hare, M. C., Ray, R. V., Back, M. & Edwards, S. G. (2010). Evaluation of pathogenicity and aggressiveness of F. langsethiae on oat and wheat seedlings relative to known seedling blight pathogens. *European Journal of Plant Pathology*, 126 (2): 203-216.

Kikot, G. E., Hours, R. A. & Alconada, T. M. (2009). Contribution of cell wall degrading enzymes to pathogenesis of Fusarium graminearum: a review. *Journal of Basic Microbiology*, 49 (3): 231-241.

Klemsdal, S. S., Aamot, H. U., Elen, O., Hofgaard, I. S. & Brodal, G. (2009). Samspill mellom ulike Fusariumarter- betydningen for soppens vekst og mycotoksinproduksjon. *Bioforsk Fokus*, 4: 202-203.

Kosiak, B., Torp, M., Skjerve, E. & Thrane, U. (2003). The prevalence and distribution of Fusarium species in Norwegian cereals: a survey. *Acta Agriculturae Scandinavica Section B-Soil and Plant Science*, 53 (4): 168-176.

Kristensen, R., Torp, M., Kosiak, B. & Holst-Jensen, A. (2005). Phylogeny and toxigenic potential is correlated in Fusarium species as revealed by partial translation elongation factor 1 alpha gene sequences. *Mycological Research*, 109: 173-186.

Krska, R., Schubert-Ullrich, P., Josephs, R. D., Emteborg, H., Buttinger, G., Pettersson, H., van Egmond, H. P., Schothorst, R. C., MacDonald, S. & Chan, D. (2007). Determination of molar absorptivity coefficients for major type-B trichothecenes and certification of calibrators for deoxynivalenol and nivalenol. *Analytical and Bioanalytical Chemistry*, 388 (5-6): 1215-1226.

Kulik, T., Pszczolkowska, A. & Lojko, M. (2011). Multilocus Phylogenetics Show High Intraspecific Variability within Fusarium avenaceum. *International Journal of Molecular Sciences*, 12 (9): 5626-5640.

Lacey, J., Bateman, G. L. & Mirocha, C. J. (1999). Effects of infection time and moisture on development of ear blight and deoxynivalenol production by Fusarium spp. in wheat. *Annals of Applied Biology*, 134 (3): 277-283.

Langseth, W. & Rundberget, T. (1999). The occurrence of HT-2 toxin and other trichothecenes in Norwegian cereals. *Mycopathologia*, 147 (3): 157-165.

Lee, B. N., Kroken, S., Chou, D. Y. T., Robbertse, B., Yoder, O. C. & Turgeon, B. G. (2005). Functional analysis of all nonribosomal peptide synthetases in Cochliobolus heterostrophus reveals a factor, NPS6, involved in virulence and resistance to oxidative stress. *Eukaryotic Cell*, 4 (3): 545-555.

Lemmens, M., Buerstmayr, H., Krska, R., Schuhmacher, R., Grausgruber, H. & Ruckenbauer, P. (2004). The effect of inoculation treatment and long-term application of moisture on Fusarium head blight symptoms and deoxynivalenol contamination in wheat grains. *European Journal of Plant Pathology*, 110 (3): 299-308.

Lysoe, E., Klemsdal, S. S., Bone, K. R., Frandsen, R. J. N., Johansen, T., Thrane, U. & Giese, H. (2006). The PKS4 gene of Fusarium graminearum is essential for zearalenone production. *Applied and Environmental Microbiology*, 72 (6): 3924-3932.

Medina, A., Valle-Algarra, F. M., Jimenez, M. & Magan, N. (2010). Different sample treatment approaches for the analysis of T-2 and HT-2 toxins from oats-based media. *Journal of Chromatography B-Analytical Technologies in the Biomedical and Life Sciences*, 878 (23): 2145-2149.

Medina, A. & Magan, N. (2011). Temperature and water activity effects on production of T-2 and HT-2 by Fusarium langsethiae strains from north European countries. *Food Microbiology*, 28 (3): 392-398.

Miedaner, T., Cumagun, C. J. R. & Chakraborty, S. (2008). Population genetics of three important head blight pathogens Fusarium graminearum, F-pseudograminearum and F-culmorum. *Journal of Phytopathology*, 156 (3): 129-139.

Munkvold, G. P. (2003). Epidemiology of Fusarium diseases and their mycotoxins in maize ears. *European Journal of Plant Pathology*, 109 (7): 705-713.

Nalim, F. A., Elmer, W. H., McGovern, R. J. & Geiser, D. M. (2009). Multilocus Phylogenetic Diversity of Fusarium avenaceum Pathogenic on Lisianthus. *Phytopathology*, 99 (4): 462-468.

Nelson, P. E., Dignani, M. C. & Anaissie, E. J. (1994). Taxonomy, biology, and clinical aspects of Fusarium species. *Clinical Microbiology Reviews*, 7 (4): 479-504.

Oide, S., Moeder, W., Krasnoff, S., Gibson, D., Haas, H., Yoshioka, K. & Turgeon, B. G. (2006). NPS6, encoding a nonribosomal peptide synthetase involved in siderophore-mediated iron metabolism, is a conserved virulence determinant of plant pathogenic ascomycetes. *Plant Cell*, 18 (10): 2836-2853.

Osbourn, A., Carter, J., Daniels, M., Dufresne, M., Hugouvieux, V., Leggett, M., Martin-Hernandez, A., Melton, R., Morrissey, J. & Papadopoulou, K. (2000). Preformed Antifungal Compounds and Plant Defence. In De Wit, P. J. M., Bisseling, T. & Stiekema, W. J. (eds) vol. 2 *Biology of plant-microbe interactions*, pp. 170-172. St. Paul, Minnesota, USA, International Society for Molecular Plant-Microbe Interactions.

Osbourn, A. E. (1996). Preformed antimicrobial compounds and plant defense against fungal attack. *Plant Cell*, 8 (10): 1821-1831.

Parry, D. W., Jenkinson, P. & McLeod, L. (1995). Fusarium ear blight (scab) in small-grain cereals- A review. *Plant Pathology*, 44 (2): 207-238.

Pfaffl, M. W., Horgan, G. W. & Dempfle, L. (2002). Relative expression software tool (REST (c)) for group-wise comparison and statistical analysis of relative expression results in real-time PCR. *Nucleic Acids Research*, 30 (9).

Pfaffl, M. W., Tichopad, A., Prgomet, C. & Neuvians, T. P. (2004). Determination of stable housekeeping genes, differentially regulated target genes and sample integrity: BestKeeper - Excel-based tool using pair-wise correlations. *Biotechnology Letters*, 26 (6): 509-515.

Placinta, C. M., D'Mello, J. P. F. & Macdonald, A. M. C. (1999). A review of worldwide contamination of cereal grains and animal feed with Fusarium mycotoxins. *Animal Feed Science and Technology*, 78 (1-2): 21-37.

Polanco, C. & Vega, M. P. (1994). The structure of the rDNA intergenic spacer of Avena sativa L.: a comparative study. *Plant Molecular Biology*, 25 (4): 751-756.

Pritsch, C., Muehlbauer, G. J., Bushnell, W. R., Somers, D. A. & Vance, C. P. (2000). Fungal development and induction of defense response genes during early infection of wheat spikes by Fusarium graminearum. *Molecular Plant-Microbe Interactions*, 13 (2): 159-169.

Reischer, G. H., Lemmens, M., Farnleitner, A., Adler, A. & Mach, R. L. (2004). Quantification of Fusarium graminearum in infected wheat by species specific real-time PCR applying a TaqMan Probe. *Journal of Microbiological Methods*, 59 (1): 141-146.

Rocha, O., Ansari, K. & Doohan, F. M. (2005). Effects of trichothecene mycotoxins on eukaryotic cells: A review. *Food Additives and Contaminants*, 22 (4): 369-378.

Ryu, J. C., Yang, J. S., Song, Y. S., Kwon, O. S., Park, J. & Chang, I. M. (1996). Survey of natural occurrence of trichothecene mycotoxins and zearalenone in Korean cereals harvested in 1992 using gas chromatography mass spectrometry. *Food Additives and Contaminants*, 13 (3): 333-341.

Sarkisov, E. S. (1954). Energiya kristallicheskoi reshethi i stroenie atoma. *Zhurnal Fizicheskoi Khimii*, 28 (4): 627-636.

Schmidt, H., Adler, A., Holst-Jensen, A., Klemsdal, S. S., Logrieco, A., Mach, R. L., Nirenberg, H. I., Thrane, U., Torp, M., Vogel, R. F., Yli-Mattila, T. & Niessen, L. (2004). An integrated taxonomic study of Fusarium langsethiae, Fusarium poae and Fusarium sporotrichioides based on the use of composite datasets. *International Journal of Food Microbiology*, 95 (3): 341-349.

Simpson, D. R., Thomsett, M. A. & Nicholson, P. (2004). Competitive interactions between Microdochium nivale var. majus, M-nivale var. nivale and Fusarium culmorum in planta and in vitro. *Environmental Microbiology*, 6 (1): 79-87.

Stakheev, A. A., Ryazantsev, D. Y. & Zavriev, S. K. (2011). Novel DNA markers for taxonomic characterization and identification of Fusarium species. *Russian Journal of Bioorganic Chemistry*, 37 (5): 593-601.

Strange, R. N. & Smith, H. (1971). Fungal growth stimulant in anthers which predisposes wheat to attack by by Fusarium- graminaerum *Physiological Plant Pathology*, 1 (2): 141-144.

Strange, R. N., Majer, J. R. & Smith, H. (1974). Isolation and identification of choline and betaine as 2 major components in anthers and wheat-germ that stimulate Fusarium- graminearum invitro *Physiological Plant Pathology*, 4 (2): 277-290.

Thrane, U., Adler, A., Clasen, P. E., Galvano, F., Langseth, W., Logrieco, A., Nielsen, K. F. & Ritieni, A. (2004). Diversity in metabolite production by Fusarium langsethiae, Fusarium poae, and Fusarium sporotrichioides. *International Journal of Food Microbiology*, 95 (3): 257-266.

Torp, M. & Langseth, W. (1999). Production of T-2 toxin by a Fusarium resembling Fusarium poae. *Mycopathologia*, 147 (2): 89-96.

Torp, M. & Adler, A. (2004). The European Sporotrichiella project: a polyphasic approach to the biology of a new Fusarium species. *International Journal of Food Microbiology*, 95 (3): 241-245.

Torp, M. & Nirenberg, H. I. (2004). Fusarium langsethiae sp nov on cereals in Europe. *International Journal of Food Microbiology*, 95 (3): 247-256.

Trail, F. & Common, R. (2000). Perithecial development by Gibberella zeae: a light microscopy study. *Mycologia*, 92 (1): 130-138.

Trail, F. (2009). For Blighted Waves of Grain: Fusarium graminearum in the Postgenomics Era. *Plant Physiology*, 149 (1): 103-110.

Urban, M., Daniels, S., Mott, E. & Hammond-Kosack, K. (2002). Arabidopsis is susceptible to the cereal ear blight fungal pathogens Fusarium graminearum and Fusarium culmorum. *Plant Journal*, 32 (6): 961-973.

van der Fels-Klerx, H. J. (2010). Occurrence data if trichothecene mycotoxins T-2 toxin and HT-2 toxin in food and feed. EFSA. 1-43 p.

van Egmond, H. P., Schothorst, R. C. & Jonker, M. A. (2007). Regulations relating to mycotoxins in food. *Analytical and Bioanalytical Chemistry*, 389 (1): 147-157.

Velluti, A., Marin, S., Bettucci, L., Ramos, A. J. & Sanchis, V. (2000). The effect of fungal competition on colonization of maize grain by Fusarium moniliforme, F-proliferatum and F-graminearum and on fumonisin B-1 and zearalenone formation. *International Journal of Food Microbiology*, 59 (1-2): 59-66.

Waalwijk, C., Kastelein, P., de Vries, I., Kerenyi, Z., van der Lee, T., Hesselink, T., Kohl, J. & Kema, G. (2003). Major changes in Fusarium spp. in wheat in the Netherlands. *European Journal of Plant Pathology*, 109 (7): 743-754.

Waalwijk, C., van der Heide, R., de Vries, I., van der Lee, T., Schoen, C., Costrel-de Corainville, G., Hauser-Hahn, I., Kastelein, P., Kohl, J., Lonnet, P., Demarquet, T. & Kema, G. H. J. (2004). Quantitative detection of Fusarium species in wheat using TaqMan. *European Journal of Plant Pathology*, 110 (5-6): 481-494.

Wagacha, J. M. & Muthomi, J. W. (2007). Fusarium culmorum: Infection process, mechanisms of mycotoxin production and their role in pathogenesis in wheat. *Crop Protection*, 26 (7): 877-885.

Walter, S., Nicholson, P. & Doohan, F. M. (2010). Action and reaction of host and pathogen during Fusarium head blight disease. *New Phytologist*, 185 (1): 54-66.

Wight, W. D., Kim, K. H., Lawrence, C. B. & Walton, J. D. (2009). Biosynthesis and Role in Virulence of the Histone Deacetylase Inhibitor Depudecin from Alternaria brassicicola. *Molecular Plant-Microbe Interactions*, 22 (10): 1258-1267.

Xu, X. M., Monger, W., Ritieni, A. & Nicholson, P. (2007a). Effect of temperature and duration of wetness during initial infection periods on disease development, fungal biomass and mycotoxin concentrations on wheat inoculated with single, or combinations of, Fusarium species. *Plant Pathology*, 56 (6): 943-956.

Xu, X. M., Nicholson, P. & Ritieni, A. (2007b). Effects of fungal interactions among Fusarium head blight pathogens on disease development and mycotoxin accumulation. *International Journal of Food Microbiology*, 119 (1-2): 67-71.

Yli-Mattila, T., Paavanen-Huhtala, S., Bulat, S. A., Alekhina, I. A. & Nirenberg, H. I. (2002). Molecular, morphological and phylogenetic analysis of the Fusarium avenaceum/F-arthrosporioides/F-tricinctum species complex - a polyphasic approach. *Mycological Research*, 106: 655-669.

Yoneda, A., Kutsuna, N., Higaki, T., Oda, Y., Sano, T. & Hasezawa, S. (2007). Recent progress in living cell imaging of plant cytoskeleton and vacuole using fluorescent-protein transgenic lines and three-dimensional imaging. *Protoplasma*, 230 (3-4): 129-139.

Zadoks, J. C., Chang, T. T. & Konzak, C. F. (1974). Decimal code for growth stages of cereals. *Weed Research*, 14 (6): 415-421.

Websites:

http://www.gov.mb.ca/agriculture/crops/insects/fad64s00.html (Assessed: 14.06.2012)

http://www.ag.ndsu.nodak.edu/aginfo/barleypath/fhbdisease.html (Assessed: 14.06.2012)

Chapter 7 Attachments

7.1 Overview of amount of *Fusarium* DNA (pg/ng oat DNA) in all the samples using qPCR

Samples	*F. avenaceum*	*F. culmorum*	F. graminearum	*F. langsethiae*
	pg *Fusarium* DNA / 100 ng oat DNA			
K.6.I	0,0	0,0	0,0	0,0
K.6.II	0,0	0,0	0,0	0,0
K.6.III	0,0	0,0	0,0	0,0
K.10.I	0,0	0,0	0,0	0,0
K.10.II	0,0	0,0	0,0	0,0
K.10.III	0,0	0,0	0,0	0,0
K.14.I	0,0	0,0	0,0	0,0
K.14.II	0,0	0,0	0,0	0,0
K.14.III	0,0	0,0	0,0	0,0
1.6.I	52,8	0,0	0,0	0,0
1.6.II	58,4	0,0	0,0	0,0
1.6.III	81,0	0,0	0,0	0,0
1.10.I	158,8	0,0	0,0	0,0
1.10.II	570,0	0,0	0,0	0,0
1.10.III	82,2	0,0	0,0	4265,8
1.14.I	36,3	0,0	0,0	484,4
1.14.II	4,2	0,0	0,0	13,9
1.14.III	33,0	0,0	0,0	0,0
2.6.I	167,0	0,0	0,0	0,0
2.6.II	9159,9	0,0	0,0	0,0
2.6.III	1180,4	0,0	0,0	0,0
2.10.I	132,3	0,0	0,0	0,0
2.10.II	47,0	0,0	0,0	0,5
2.10.III	75,7	0,0	0,0	0,0
2.14.I	4,4	0,0	0,0	0,0
2.14.II	38,2	0,0	0,0	0,0
2.14.III	63,0	0,0	0,0	0,0
7.6.I	37,8	110,2	0,0	0,0
7.6.II	45,3	115,8	0,0	0,0
7.6.III	46,1	3432,8	49,6	0,0
7.10.I	47,5	134,1	1,6	0,0
7.10.II	1121,4	855,5	4,5	0,0
7.10.III	9,1	711,9	4,5	0,0
7.14.I	16,6	11,4	0,0	0,0
7.14.II	413,0	2293,1	30,5	0,0
7.14.III	536,7	372,3	16,6	0,0
8.6.II	31,2	0,0	3,1	0,0
8.6.III	42,5	0,0	92,6	0,0
8.10.I	19,8	0,0	713,7	0,3
8.10.II	32,4	0,0	774,3	0,0
8.10.III	544,3	0,0	416,6	0,0
8.14.I	30,0	0,0	806,3	0,0
8.14.II	16,5	0,0	141,5	0,0
8.14.III	53,4	0,0	400,5	0,0
10.6.I	0,0	0,0	0,0	512,3
10.6.II	0,0	0,0	0,0	128,6
10.6.III	0,0	1,9	0,0	422,6
10.10.I	0,0	0,0	0,0	132,9
10.10.II	0,0	0,0	0,0	0,3
10.10.III	0,0	0,3	0,0	68,8
10.14.I	0,0	0,0	0,0	2693,2
10.14.II	0,0	0,0	0,0	77,9
10.14.III	0,0	0,0	0,0	54,1

Continues on the next page

Continuation from the previous page

	F. avenaceum	F. culmorum	F. graminearum	F. langsethiae
Samples	pg *Fusarium* DNA / 100 ng oat DNA			
11.6.I	0,0	0,0	0,0	222,7
11.6.II	0,0	0,0	0,0	679,4
11.6.III	0,0	2,6	0,0	803,0
11.10.I	0,0	0,0	0,0	1526,3
11.10.II	0,0	0,0	0,0	4,2
11.10.III	0,0	1,4	0,0	111,7
11.14.I	0,0	0,0	0,0	183,1
11.14.II	0,0	0,0	0,0	77,2
11.14.III	0,0	0,0	0,0	351,2
12.6.I	13,4	0,0	0,0	80,5
12.6.II	48,2	0,0	0,0	277,7
12.6.III	11,4	0,0	0,0	24,6
12.10.I	10,8	0,0	0,0	92,2
12.10.II	107,4	0,0	0,0	136,6
12.10.III	21,7	0,0	0,0	33,4
12.14.I	26,0	0,0	0,0	527,8
12.14.II	18,0	0,0	0,0	69,9
12.14.III	13,8	0,0	0,0	26,3
13.6.I	41,8	0,0	0,0	415,5
13.6.II	0,0	49,1	0,0	0,1
13.6.III	0,0	353,1	3,1	254,9
13.10.I	0,0	1906,9	8,5	1460,0
13.10.II	0,0	2360,0	14,1	363,6
13.10.III	0,0	888,0	1,1	338,2
13.14.I	0,0	2737,9	14,8	52,1
13.14.II	0,0	5,1	0,0	36,2
13.14.III	0,0	1789,8	7,2	40,5
14.6.I	0,0	0,0	36,6	850,5
14.6.II	0,0	0,0	3,4	419,4
14.6.III	0,0	0,0	15,3	521,4
14.10.I	0,0	0,0	1,2	0,5
14.10.II	0,0	0,0	971,5	4,9
14.10.III	0,0	0,0	3,9	2,4
14.14.I	0,0	0,0	1079,7	3,7
14.14.II	0,0	0,0	13,3	402,8
14.14.III	0,0	0,4	30,0	1096,9