



Sveriges lantbruksuniversitet
Swedish University of Agricultural Sciences

Faculty of Natural Resources and
Agricultural Sciences (NJ)

Late infection of *Fusarium graminearum* in winter wheat grown under different nitrogen regimes

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Department of Crop Production Ecology
Master's thesis • 30 HEC • Advanced level, 2AE
Uppsala 2017

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Credits: 30 ects

Level: Advanced level, A2E

Course title: Independent project in Biology - Master's thesis

Course Code: EX0565

Place of publication: Uppsala

Year of publication: 2018

Cover picture: Bara'a Alkhaled

Online publication: <http://stud.epsilon.slu.se>

Keywords: *F. graminearum*; winter wheat; N fertilizer; late infection; DON; protein content

Abstract

Fusarium spp. infection is a serious risk in wheat cultivation worldwide. In addition of reduce grain yield and quality, it also leads to grain and chaff contamination by toxic metabolites (mycotoxins). The mycotoxin deoxynivalenol (DON) is among the most important mycotoxins in Swedish wheat production and is mainly produced by *F. graminearum*. *F. graminearum* infects wheat crops causing Fusarium head blight (FHB) disease. Nitrogen (N) fertilizers are very important in wheat production. In order to reach high yields and protein level goals, additional N fertilizers are commonly applied. But negative effects of extra N fertilization on *F. graminearum* and other FHB-causing species have been reported.

The objective of this project was to study the impact of different N contents of winter wheat spikes, realized by different N application rates, on *F. graminearum* late infection close to the harvest time. To achieve this, winter wheat spikes around harvesting time were collected from a field experiment near Uppsala fertilized with different amounts of N treatments. The N treatments comprised 0, 160 and 324 kg N/ha application rates. The spikes were inoculated with *F. graminearum* and incubated in a greenhouse chamber with climate conditions similar to those of Uppsala in August; the month of normal commercial harvest time for winter wheat in Uppsala region. For quantification of DNA of *F. graminearum* and quantification of DON, spikes tissues (grain and chaff) were analyzed by real-time PCR technique and ELISA assay, respectively.

The results showed that grain samples contained very little or undetectable amounts of *F. graminearum* DNA in both control and *F. graminearum* treated spikes for the three N application rates. However, chaff samples of the *F. graminearum* treated spikes yielded considerable amount of DNA of *F. graminearum*, but without significant differences between the N application rates. DON was only detectable in the chaff. Although chaff samples of control spikes were not infected by *F. graminearum*, but they were contaminated by DON as shown by ELISA results. Inoculation of spikes by *F. graminearum* significantly increased DON contents in *F. graminearum* treated spikes compared with control spikes, but without significant difference between the N application rates in the former. Based on these results, and since grain were not infected by *F. graminearum* in both control and fungal treated spikes, a direct calculation of the correlation between N grain content and fungal biomass was not possible.

Keywords: *F. graminearum*; winter wheat; N fertilizer; late infection; DON; protein content

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1 Introduction

Fusarium Head Blight (FHB), also called head scab or scab, is ranked among the most damaging diseases of wheat (*Triticum aestivum*). It is distributed worldwide and caused by several species of the fungal genus *Fusarium*, such as *F. graminearum*, *F. avenaceum*, *F. culmorum* and *F. poae* (Pirgozliev et al. 2003). However, *F. graminearum* (teleomorph *Gibberella zeae*) is the main species responsible for FHB of wheat worldwide causing yield and grain quality reduction (Bai and Shaner 1994). This fungus survives on alternative hosts and crop residues. In terms of cereal crops, *F. graminearum* can infect other small grain cereals such as barley and oats (Pereyra and Dill-Macky 2008) and can also infect maize causing ear and stalk rots (Payne 1999).

The life cycle of *F. graminearum*, causing FHB on wheat, is somewhat complicated. It has both sexual and asexual stages in its life cycle. The fungus overwinters as a saprotroph in the soil or in the crop residues from previous growing seasons. In spring, spores (asexual macroconidia and sexual ascospores) are produced on residues as temperature warm up. Ascospores are formed in reproductive organs called perithecia (sexual fruiting structures) that are forcibly discharged into the air. Once produced, macroconidia and/or ascospores are released from crop residues and transferred by wind or rain onto wheat spikes. During wet and warm weather conditions, the spores germinate and infect glumes, flower parts, or other parts of the spike. Infected wheat spikes may show symptoms within a few days of this infection course in favorable environmental conditions (Wegulo 2012).

In the field, spore production and release may occur at any time during the growing season as long as the climatic conditions are favorable (Xu 2003). Production of spores is favored by moist and relatively warm conditions (Parry et al. 1995). In a Swedish study, perithecia started to develop when the temperature exceeds 12 °C (Persson 2016). However, ascospores production requires higher temperature and humidity. Optimum temperatures for ascospores production are 15–20 °C (Xu 2003). The effect of environmental conditions on abundance of ascospores in the field could either be due to effects on ascospore production in perithecia, or on ascospore transport from these perithecia (Manstretta and Rossi 2015). Ascospores have the potential to travel over long distances by wind (>100 m) and may impact fields in neighboring regions (Francl et al. 1999; Prussin et al. 2014).

Wheats plants are susceptible to infection by *F. graminearum* from flowering up to hard dough stage (late kernel development stage), as long as environmental conditions are conducive for infection (Reis et al. 2016). The general conditions favorable for FHB are warm conditions coupled to high humidity, e.g. heavy dew or frequent rain fall events (McMullen et al. 2012). Optimum temperatures for FHB development are 25-30 °C (Wegulo 2012).

Infections that occur during anthesis or shortly afterwards are the most damaging because the fungus will colonize and kill the florets, and kernels will not develop (Cowger and Arrellano 2010). However, the infections which occur late in the season, during late kernel development,

should also be considered. Those infections may give healthy appearance grain, which in fact are contaminated with the fungus (Wegulo 2012; Cowger and Arrellano 2010).

Symptoms of FHB on wheat are manifested as bleached or white spikes with pink or salmon-colored sporodochia (mass of mycelium and macroconidia) and/or purple-black perithecia on the glumes of individual spikelets (Schmale and Bergstrom 2003; Osborne and Stein 2007). Spikes can be partially (one or a few spikelets) or completely infected. As the infection progresses, the fungus colonises the grain causing them to shrink and wrinkle. The infected kernels are generally light-weight and smaller than normal, and have a scabby appearance with a color ranging from light-brown to pink (Bai and Shaner 1994). The infected kernels are known as *Fusarium*-damaged kernels (FDK) or “tombstones”.

However, the most significant for a *Fusarium* infection is the toxins that these fungi produce in kernels. The most important mycotoxins produced by *F. graminearum* are deoxynivalenol (DON), nivalenol (NIV) and zearalenone (ZEA) (Bottalico and Perrone 2002; Snijders and Perkowski 1990). Those mycotoxins pose deleterious health problems to animals and humans upon ingestion in large quantities due to inhibition of protein synthesis and suppression of the immune system (Rocha et al. 2005).

Mycotoxins produced by *Fusarium* species are fungal secondary metabolites. Different roles for different mycotoxins have been suggested such as virulence factors (Jansen et al. 2005) a fungal response to microbial competition stress (Xu et al. 2007) in response to oxidative stress or to environmental stress (Reverberi et al. 2010). Mycotoxins are, chemically and thermally, stable and they are resistant to the classical transformation procedures of raw ingredients into food by physical or chemical means (Champeil et al. 2004). Therefore, they may be found in both raw material and processed food and feed products.

The mycotoxin DON (also called vomitoxin) is the least toxic mycotoxin among those produced by *F. graminearum* (Wegulo 2012). However, it is the most predominant and encountered *Fusarium* mycotoxin in wheat throughout Europe (Bottalico and Perrone 2002) and it has the most important economically for the farmer and for the grain trade. The contaminated grain are rejected or downgraded at marketing leading to a big economic loss. In the European Union, a maximum threshold of 750 µg/kg of DON in processed wheat products such as flour and semolina and a threshold of 1250 µg/kg for whole unprocessed wheat grain, were set to protect a human health (Anonymous 2006b).

DON accumulation in grain is not only affected by the factors of host resistance, *Fusarium* species and isolate (Ward et al. 2008) but is also influenced by climatic conditions occurring during kernel development and prior to harvest (Ma et al. 2013). An understanding of the factors that favor FHB incidence and DON production is very important in devising management strategies controlling the disease and minimizing the mycotoxin production (Wegulo 2012).

Many surveys and field or greenhouse experiments have shown a positive, linear relationship between *F. graminearum* contamination in the grain and DON (Wegulo et al. 2011; Fredlund

et al. 2013). Thus, control options that reduce *Fusarium* infection will also reduce DON accumulation in grain. Some field and greenhouse studies could not find this linear relationship studying DON and FHB intensity. Instead, they found very high levels of DON in grain while FHB intensity was relatively low or the symptoms were absent, especially in late infections after flowering growth stage (Siou et al. 2014; Schmale and Bergstrom 2003).

Consequently, it is not important only to control FHB in the field, but also to reduce the amount of mycotoxins in the grain. To minimize the potential risk of mycotoxin contamination, some preventive measures can be applied to reduce the incidence of FHB. In that context, agronomic management practices such as cultivar selection, crop rotation and tillage, and chemical or biological control have been used to manage FHB and DON (reviewed in Wegulo et al. 2015; Pirgozliev et al. 2003).

Optimizing nitrogen (N) fertilization might contribute to the control of *Fusarium* and hence DON contamination in grain. High N fertilization may increase wheat plants density (tillering) resulting in favorable microclimatic conditions (high humidity) that promote inoculum production and spike infection (Lemmens et al. 2004b). Also, N fertilization may prolong the flowering period during which wheat plants are more susceptible to *Fusarium* infection (Lemmens et al. 2004b). Moreover, N fertilization influences the N concentrations in plant tissues which in turn affect plant defense and pathogen virulence (Fagard et al. 2014).

However, the studies on effects of different amounts of N fertilization have given inconsistent results. Some studies found an increasing level of *Fusarium* infection and DON contamination in wheat associated with an increased level of N fertilization (Lemmens et al. 2004b; Heier et al. 2005). Conversely, other studies found no significant effects of N fertilization on FHB or DON (Fauzi and Paulitz 1994; Yoshida et al. 2008). Thus, the effect of N application on FHB and mycotoxin accumulation remains unclear and more research is required to clarify this issue (Parry et al. 1995).

The protein content of the grain is a determining factor in the cultivation of bread wheat. The price that farmers receive for wheat, for example in Sweden, is dependent on the grain protein content (Jordbruksverket 2016). At the same time, there is a high request from the bakeries for wheat flour with high protein content. Thus, farmers tend to apply higher nitrogen fertilizer rates for wheat to increase N availability during the kernel development and consequently increase the protein content (van der Burgt et al. 2011).

However, as mentioned above, there is some scientific proof that *Fusarium* infection is favored by increasing N application rates. Therefore, nitrogen fertilization strategy could be counterproductive and have a negative effect on grain quality by increasing mycotoxin contamination. For this reason, the investigation of the relationship between N-input and *Fusarium* infection is very important.

In the present study, winter wheat spikes were collected shortly before harvest from a field experiment fertilized with different rates of nitrogen. The spikes were inoculated with *F. graminearum* (*Fg*) and the aim was to investigate the effect of N status of the spikes on (1) *Fg* infection at late stage of spikes maturity and on (2) the resulting DON contamination in the grain and chaff, by using accurate quantification techniques (real-time PCR for fungal DNA estimation and ELISA for toxin quantification).

I hypothesized that higher N contents in winter wheat spikes, realized by higher N application rates, would lead to higher *Fg* infection and DON production. I tested this hypothesis in relation to late infection, when the environmental conditions allow for infection late in the wheat cycle, close to the harvest time.

2 Materials and Methods

2.1 Plant material

Winter wheat spikes of cultivar Olivin were sampled from a field experiment close to Uppsala on 15th August 2016. Sampling was undertaken one week before harvesting of the entire field. Detached spikes were collected, put in clean plastic bags and stored at -20 °C until used.

The field experiment consisted of blocks distributed in three areas of a field with different expected yield potentials according to previous annual harvest maps (Myrbeck 2016). Within each block, there were three treatments that comprised application of different rates of applied nitrogen. These nitrogen treatments represented: scarce 0 kg N /ha, normal 160 kg N/ ha (representing normal N application for the actual use field according to recommendations from the Swedish Board of Agriculture (Jordbruksverket 2016)) and an excessive nitrogen rate of 324 kg N /ha. For each treatment, there were three replications. The nitrogen treatments are described in table 1. Further details about the field experiment are reported elsewhere (Myrbeck 2016).

Plants of block 1 which represented an area in the field with a low yield potential and the plants of block 3 which represented an area in the field with a high yield potential, were collected for this study (Appendix I).

Table 1. Nitrogen fertilizers supply to winter wheat crop.

Date	N0	N160	N324	Developing stage
2015-10-29	-	-	30 kg	DC* 11-12
2016-03-31	-	-	81 kg	DC 21-23
2016-04-12	-	100 kg	100 kg	DC 21-23
2016-05-17	-	60 kg	60 kg	DC 33
2016-06-01	-	-	53 kg	

0N, 160N and 324N denote 0, 160 and 324 kg N/ha application rate, respectively.

* Zadoks decimal code (Zadoks et al. 1974).

2.2 Inoculum preparation

Three isolates of *Fusarium graminearum* (VPE 104, VPE 105 and VPE 90) to be used for inoculation of detached wheat spikes were obtained from the fungal culture collection of the department of Crop Production Ecology. The isolates were previously identified and proved to be pathogenic on wheat.

A spore suspension was obtained by growing the isolates individually on potato dextrose agar (PDA, 39 g /L) in Petri dishes at room temperature for one week (Fig.1). Pieces of PDA with mycelia for the three isolates were transferred to a synthetic nutrient agar (SNA) medium for macroconidia production. SNA medium was prepared by 1 g KH₂PO₄, 1 g KNO₃, 0.5 g MgSO₄·7H₂O, 0.5 g KCl, 0.2 g Glucose, 0.2 g Sucrose added to 1 L distilled water and autoclaved in 121°C for 20 minutes. The SNA medium is commonly used for obtaining macroconidia which are produced more abundant on this medium than on richer media (Summerell et al. 2003). Fungal isolates were left to grow at room temperature under white

artificial light for two weeks. Subsequently, macroconidia were harvested by washing the culture surface with tap water using a plastic wash bottle.

A suspension of 2×10^5 macroconidia /ml tap water was used to inoculate wheat spikes. The suspension was obtained by mixing equal volumes of spore suspensions of three *F.graminearum* isolates. Inoculum concentration was measured with a hemocytometer (Bürker, Germany). Conidial suspensions were stored at 4°C until used for inoculation, which took place within two days.



Fig. 1. *F. graminearum* isolate VPE 90 (left) and VPE 104 (right) after one week of growth on PDA.

2.3 Spike inoculation

For inoculation, spikes were kept at 4 °C for 24 hours. Inoculation was performed by immersing each spike separately in a 50 ml conical tube filled with spore suspension for approximately 20 seconds (Fig. 2). This brought about approximately 1.20 ml of suspension for each spike. For each replication, 5 spikes were randomly selected for inoculation with *F.graminearum* or with tap water for the control groups of spikes.

The inoculated spikes were incubated for two weeks in a greenhouse chamber equipped with a sprinkler system that intermittently produced a fine mist. Spikes were left in plastic dishes (≈ 20 cm diameter) and put in random order on a greenhouse bench (Fig. 3). Every two days, spikes were exposed to the direct mist by placing the dishes under the sprinklers for 5-7 minutes. This kept the spikes moist and helped to create wet conditions similar to field conditions. Control, non-inoculated spikes were incubated in a separate greenhouse chamber in order to avoid any contamination from inoculated spikes.



Fig. 2. Inoculation method, spikes were inoculated by immersing them in *F. graminearum* spore suspension.

Greenhouse temperatures were set to 22 °C during the day (12h) and 12 °C during the night (12h), while relative humidity was set 80 % during the day and night. However, during the experiment period, greenhouse temperatures and relative humidity varied from the set values. The chosen weather data represented the average values of temperature and relative humidity for August in Uppsala area, in an attempt of mimicking the climate of harvesting period of winter wheat in this region. The data were collected from the SLU nearby weather station (Ultuna/lantmetweatherstation, <http://www.ffe.slu.se/lm/LMHome.cfm?LMSUB=0&ADM=>) during the last 5 years.

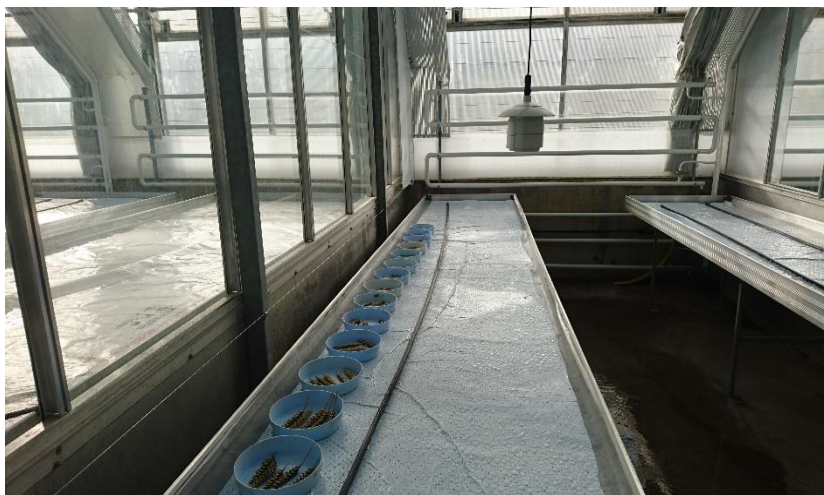


Fig. 3. Greenhouse chamber with inoculated wheat spikes.

2.4 *F. graminearum* biomass determination

Inoculated spikes were manually threshed to separate grain from the chaff. Grain and chaff (glumes and rachis) were ground in liquid nitrogen with a mixer mill (Retsch Mixer Mill MM 400) to fine powder and stored at – 20 °C until the time for analysis.

DNA was extracted from 100 mg grounded material using DNeasy Plant Mini kit (Qiagen, Hilden, Germany) according to manufacturer's protocol. However, 530 µl of the lysis buffer AP1 was used for the chaff instead of 400 µl, as recommended by the manufacturer. The DNeasy kit was used with the QiaCube (QIAGEN AB, Sweden) with the standard plant cells and tissues protocol.

DNA concentration and purity of each DNA template was measured using Spectrophotometer ND- 1000 (NanoDrop technologies, USA). Deionised water was used as a blank. DNA samples with concentrations above 10 ng/ µl were diluted to 10 ng/ µl before real-time PCR analyses.

Real-time PCR was performed in 96-well plates using the SSO fast Eva Green Master Mix (Bio-rad). Each 12.5 µl qPCR consisted of 3.65 µl of deionized water, 0.05 µl each of the two Fgram primers (Table 2; Nicolaisen et al 2009), 2.5 µl of the template DNA and 6.25ul of Eva Green master mix. PCR was performed in a CFX Connect TM Real-Time PCR Detection System (Bio-Rad, CA, USA). Wells with no template DNA served as controls (NTC). The amplification conditions used were 98 °C for 2 min, followed by 40 cycles of 98 °C denaturation for 5 sec and 62 °C annealing for 10 sec, followed by a melt curve from 95 to 65 °C.

Five *F.graminearum* DNA standards, previously extracted from mycelia of PDA plates of *F. graminearum* using DNeasy Plant Mini kit (Qiagen, Hilden, Germany), were serially diluted 1:10 with deionized water and used to develop a standard curve. Bio-Rad CFX manager 3.1 software was used to determine the Cq of each reaction and the efficiency of the standard curve (>90%). The number of cycles of standard dilutions ranged from 21 to 36, and wheat DNA samples yielded above 36 cycles were considered as negative. Each DNA sample including standard dilution was tested in duplicates to ensure repeatability.

The estimation of *F.graminearum* DNA was considered as an estimation of the *F.graminearum* biomass in the samples.

Table 2. List of primers used for DNA amplification in qPCR.

Primer	Sequence (5' → 3')	Length(pb)
FgramB379 fwd	CCATTCCTGGGCGCT	16
FgramB411 rev	CCTATTGACAGGTGGTTAGTGACTGG	26

2.5 DON analysis

To determine the concentrations of DON in the different samples, Enzyme-linked immunosorbent assay (ELISA) was used. For this assay, 5g of grounded plant material was mixed with 25 ml of distilled water (1:5) and thoroughly mixed by vigorous shaking manually for 3 minutes. The mix was centrifuged at 3300 rpm for 3 minutes and the supernatant was then used to determine DON concentration. For the chaff samples, the supernatant was further filtered using Whatman No. 1 filter paper.

Ridascreen test kit from R-Biopharm AG, Germany, was used to measure the mycotoxin DON. The computer program RIDASOFT Win (Art. No. Z9999) was used for calculation of the final results from raw data. The detection limit of this assay for cereals (wheat) was 18.5 µg/kg. Absorbance was measured at 450 nm with a microtiter plate spectrophotometer (Thermoscan, Australia), and amount of DON was calculated as a mean of two technical replicates according to standards provided in the kit.



Fig. 4. Laboratory Mixer Mill MM 400 with grinding jar.



Fig. 5. Microplate Spectrophotometers.

2.6 Determination of nitrogen content in grain

The grain was analyzed for N by near infrared transmission spectroscopy (InfratecTM1241 Grain Analyzer). The analysis was performed by Soil and plant laboratory at the Department of Soil and Environment at SLU, Uppsala.

2.7 Data analysis

Statistical analyses were carried out with JMP statistical software (version 12.2.0). Variables were analyzed with a two-way Analyses of Variance (ANOVA) with the factors block and N application rate. If a normal distribution of the residuals was not confirmed, the data were log transformed before statistical analyses. Pairwise comparison of treatment means was done using HSD Tukey's test at $P = 0.05$.

3 Results

3.1 Effect of Yield potential and nitrogen on yield and grain and straw parameters

Winter wheat plants harvested from high yield potential block 3 yielded significantly higher grain yield kg/ha compared with winter wheat plants harvested from low yield potential block 1. Grain from block 1 showed significantly higher values in moisture content, nitrogen content and protein content compared with those from block 3. No significant difference between the two blocks with respect to the percentage of nitrogen of straw was found (Table 3).

In both blocks, the two N application rates 160 and 324 kg N/ha significantly increased nitrogen content in grain and the percentage of protein, compared with N application rate 0 kg/ha. The N application rate 324 kg N/ha showed the highest nitrogen content (2.5%) and protein content (14.1%) in grain but without significant differences with N application rate 160 kg N/ha, for any of these two parameters (Table 3). No significant differences could be shown between the three N application rates with respect to the percentage of nitrogen of straw (Table 3).

Table 3. Yield and grain and straw characteristics of differently fertilized winter wheat plants from two different harvest potential blocks.

Factor	Yield kg/h	Moisture% Grain	N % Grain	Protein % Grain	N% Straw
Yield potential (A)					
Block 1^a	8134 b	16.6 a	2.4 a	13.7 a	0.45
Block 3	9490 a	16.1 b	2.2 b	12.6 b	0.45
F test	***	***	**	**	
Nitrogen rate kg/ha (B)					
0	7937 b	16.42 a	2 b	11.5 b	0.40
160	8987 a	16.25 b	2.4 a	13.8 a	0.45
324	9513 a	16.37 ab	2.5 a	14.1 a	0.50
F test	***	*	***	***	
Interactions					
A and B	ns	*	ns	ns	ns

Means followed by the same letter within a column are not significantly different by HSD Tukey's Test at P = 0.05. ns, *, ** and *** = not significant, significant at $p \leq 0.05$, $p \leq 0.01$, $p \leq 0.001$.

a) Block 1 and block 3 represent an area in the field with a low and high yield potential, respectively.

3.2 *F. graminearum* biomass

Real-time PCR results showed that the concentration of *F. graminearum* DNA in the grain was very low or undetectable in both control and fungal treated spikes for the three N application rates in the two yield potential blocks (Table 4). Low levels of *F. graminearum* DNA in non-inoculated samples indicated low contamination from the field environment.

However, real-time PCR analysis displayed enhanced *F. graminearum* DNA content in the chaff of *F. graminearum* inoculated spikes compared to control spikes, which confirmed the success of artificial inoculation in the experiment (Table 4). The concentrations of *F. graminearum* DNA in the chaff of control spikes was very low or undetectable, whereas in fungal treated spikes, considerable amount of *F. graminearum* DNA was found (Table 4) but without significant differences between the N application rates in any of the yield potential blocks (Table 5).

Table 4. The amount of DNA (pg) of *Fusarium graminearum* in grain and chaff of differently fertilized winter wheat plants from two different yield potential blocks. The values for DNA are means over three replicates. Treatments: C- control (*F. graminearum* non- inoculated spikes), T- treatment (*F. graminearum* inoculated spikes). Block 1 and block 3 represent an area in the field with a low and high yield potential, respectively

Block	N rate kg/ha	Chaff C pg DNA	Grain C pg DNA	Chaff T pg DNA	Grain T pg DNA
Block 1	0	0	0	55	0
Block 1	160	0	0	48	0
Block 1	324	0	0	118	1
Block 3	0	0	0	61	0
Block 3	160	1	0	147	0
Block 3	324	0	0	81	5

Table 5. The amount of DNA (pg) of *Fusarium graminearum* in chaff of *F. graminearum* inoculated spikes (T).

Factor	Chaff T
Block (A)	
Block 1 ^a	74
Block 3	96
F test	ns
N rate kg/ha (B)	
0	58
160	98
324	99
F test	ns
Interactions	ns
A and B	

^a) Block 1 and block 3 represent an area in the field with a low and high yield potential, respectively. ns: not significant.

3.3 DON accumulation

DON was only detectable in the chaff. In all grain samples, DON concentrations were below the detection limit of 18.5 µg DON/kg wheat grain. The maximum DON concentration was 73 µg/kg in control spikes and 178 µg/kg in *F. graminearum* treated spikes (Table 6).

The differences in DON concentrations in the chaff among the N application rates were not statistically different in *F. graminearum* treated spikes (Table 7). The total mean DON levels of chaff were higher in fungal treated spikes than control spikes (124 versus 66 µg/kg) regardless of the effect of N application rate (Table 8).

Table 6. The amount of DON (µg/kg) in grain and chaff of differently fertilized winter wheat plants from two different harvest potential blocks. The values for DON are means over three replicates. Treatments: C- control (*F. graminearum* non- inoculated spikes), T- treatment (*F. graminearum* inoculated spikes).

Block	N rate kg/ha	Chaff C	Grain C	Chaff T	Grain T
Block 1 ^a	0	73	nd	119	nd
Block 1	160	60	nd	68	nd
Block 1	324	61	nd	119	nd
Block 3 ^a	0	69	nd	178	nd
Block 3	160	73	nd	79	nd
Block 3	324	63	nd	177	nd

^a) Block 1 and block 3 represent an area in the field with a low and high yield potential, respectively. nd: below detection limit.

Table 7. The amount of DON ($\mu\text{g/kg}$) in chaff of *F. graminearum* inoculated spikes (T).

Factor	Chaff T
Block (A)	
Block 1 ^a	90
Block 3	108
F test	ns
Nitrogen rate (B)	
0	120
160	71
324	123
F test	ns
Interactions	ns
A and B	

^a) Block 1 and block 3 represent an area in the field with a low and high yield potential, respectively. ns: not significant.

Table 8. The amount of DON ($\mu\text{g/kg}$) in chaff with respect to the effect of nitrogen application rate and artificial inoculation (*F. graminearum*).

Factors	Control spikes	Fungal treated spikes
0N	71	149
160N	67	74
324N	62	148
Means	66b	124a

Means followed by the same letter within a row are not significantly different by HSD Tukey's Test at $P = 0.05$. 0N, 160N and 324N denote 0, 160 and 324 kg N/ha application rate, respectively.

4 Discussion

Nitrogen is an important nutrient in winter wheat production, and nitrogen fertilizers are almost always necessary to achieve desired yield and protein content. N fertilization above levels required for maximum yield in bread wheat are commonly applied to increase grain protein content (Marschner 2012). This is done because only after all the N required for maximum yield is supplied, additional N fertilizers will increase the protein content of developing grain. However, there seems to be in some years a cost connected to this strategy. As the N application rate increases, the severity of many fungal diseases may also increase. For wheat diseases, leaf rust (*Puccinia triticina*), stripe rust (*Puccinia striiformis*), septoria leaf blotch (*Mycosphaerella graminicola* (*Zymoseptoria tritici*)) and powdery mildew (*Blumeria graminis*) are all diseases that have been observed to increase as the N application rates increase (Robert et al. 2004; Neumann et al. 2004; Olesen et al. 2003). Rate of nitrogen application can also influence FHB severity in winter wheat, but the results have been inconsistent (Fauzi and Paulitz 1994; Lemmens et al. 2004b; Heier et al. 2005). Thus, it is considered important to study the relationship between N-input and *F. graminearum* infection, the main causal agent of FHB in Sweden and worldwide.

The objective of this study was to evaluate the impact of N level and protein content of spikes of winter wheat on *F. graminearum* late infection, under climatic conditions conducive for infection close to harvest. Late infection of winter wheat by *Fusarium spp.* has received less attention by farmers or agronomists. Unfortunately, at this late stage of the growing season where the harvest is right around the corner, there is no easy way to manage *Fusarium* infections. Forecasting models usually ignore late infections and instead tend to focus only on the risk of infection around flowering. Although anthesis is the most susceptible period for infection, where the anthers are the main sites of infection, under favorable conditions of temperature and humidity, late infections (post-anthesis) are highly probable to occur. Several studies have showed that late infections can result in significant levels of disease and toxin accumulation (Del Ponte et al. 2007; Siou et al. 2014).

In this experiment, a wet season was simulated with a misting water system, keeping the inoculated spikes wet during the whole experimental period. The risk of late infection is manifested in wet years, after prolonged post-anthesis rainfall (through grain maturation). High moisture conditions favour ascospore production on crop residues, and hence increase incolumn pressure and infection severity which may lead to higher DON accumulation. Moreover, during wet periods in summer at harvesting time, water content of ripening grain may increase, as they absorb moisture from the atmosphere, to the levels (20-30%) that may also favour fungal infection and toxin production. The wheat grain is harvested when its moisture content is between 14 and 20%. Farmers sometimes have to wait for longer periods until they can combine. They wait for the moisture content of mature grain drops to levels that are safe for storage. During this waiting period, the risk of infection is high particularly if this combined with warm temperatures. Furthermore, as ascospores have a capacity to move over long distance by wind, they can land on mature spikes. If this coincides with high humidity,

they may germinate and cause infections. In that context, it is worth mentioning that local crop residue management may be less effective in disease management, especially in regions with high wind conditions.

4.1 *Fusarium graminearum* biomass

In this study, fungal DNA of *F.graminarum* (Fg) was less abundant in the grain than in the chaff. Such a difference has already been observed for late inoculations. Xu et al. (2008) found that fungal DNA was significantly greater in chaff than in the corresponding grain sample at harvest. At this late stage of spike maturation, the water content of grain decrease and pericarp cell walls thicken, which makes infection more difficult for the fungus (Leand and Bushnell 2003). When grain are mature, the testa (seed coat) is more resistant to hyphal penetration (Pugh et al. 1933). Hope et al. (2005) showed that the growth of *F.graminarum* in wheat grain only occurred above 19% water content and no mycelial growth observed under 19% water content. It has also been shown in another study that the minimum water content for *F.graminarum* germination was 18% and for growth was 19-20%. These moisture content levels are marginal for germination of conidia of most fusaria, under which growth would not normally occur (Magan and Lacey 1984). In this study, the moisture content level in the grain were at max 16.4% (Table 2) when the experiment started and may have increased some during the experiment. However, it is still probably not high enough for fungal growth as found by previous authors. This may explain the very low or undetectable DNA concentrations in the grain. Another possible explanation is that, as stated by Siou et al. (2014), the fungus has easier access to nutrients in the chaff than in the grain at this stage of spike maturity. Several reports have shown that susceptibility of the wheat spikes to *F. graminearum* infection declines sharply after the start of dough stage (Strange and Smith 1971; Lacey et al. 1999; Pugh et al. 1933). Del Ponte et al. (2007) showed that wheat plants were susceptible to infection from flowering to hard dough stages; but significant infections resulted from inoculations post flowering to late milk stages.

The different N application rates (0, 160 and 324 kg/ha) did not affect the fungal biomass levels in the chaff significantly, although there was a trend towards a lower DNA level at lowest N application rate (0 kg/ha) compared with other two N application rates (160 and 324 kg /ha) (Table 5). The N application rates 160 and 324 kg/ha significantly increased the grain N content and consequently the protein content compared with these of 0 kg/ha (Table 3). Straw N contents between the three N application rates were not significantly different (Table 3). N content of glumes (chaff) was not measured, but it is expected to be in the same range of N content of straw (Table 3). Glumes are a major source of N for grain development. They act as a temporary sink of N that will be soon mobilized to grain at early grain-filling stages (Lopes et al. 2006). In a study by Lopes et al. (2006) it was found that total N content of glumes consisted of 11% of that present in the grain at early dough stage. In a study by Wang et al. (2003) on different spring wheat cultivars, nitrogen concentration at maturity in non-grain tissue was found in range (0.51-0.62%) in glumes, (0.44-0.53%) in rachis and (0.49-0.51%) in

stems. It is not expected that the low levels of N content in glumes and rachis at harvest time will have a significant impact on fungal infection. In this study, DNA of *F. graminearum* was only detectable in chaff, except a very little amount in grain of the highest N application rate. As a result, a clear relationship could not be established between N grain levels and fungal biomass levels; thereby examine how the N status of winter wheat spike might influence the interaction with *F. graminearum*.

In a study of effect of N application at anthesis on FHB incidence by Yoshida et al. (2008), N application led to a significant increase in grain protein, but did not significantly influence the levels of FHB or *Fusarium*-damaged kernels. In another related study, molasses N fertilizer did not significantly increase grain protein content, but the symptoms of FHB doubled at higher N rates (Lemmens et al. 2004b). The authors of this study concluded that higher N or protein in grain and/or spike tissues *per se* are not responsible for higher FHB infection level. In agreement with this, Wang et al. (2005) found a weak correlation between the degree of infection with *F.culmorum* and the crude protein content of winter wheat. In an investigation done by Yang et al. (2010), authors suggested that the observed effect of different N levels on *F. graminearum* infection is not caused by a change in a composition or amount of protein in the grain. Fauzi and Paulitz (1994) suggested that N would not change the inherent susceptibility of wheat spikes to *F.graminearum*.

Nitrogen is essential to improve growth of the crop and general plant vigor. It increases plant biomass and enable higher yields. It also affects plant response to pathogens infections. Plants suffering from nitrogen deficiency are weaker, grow slowly, age faster and become more vulnerable to pathogen attacks (Snoeijers et al. 2000). Generally, high concentrations of nitrogen increase susceptibility of plants to biotrophic pathogens, whereas it increases resistance of plants to necrotrophic plant pathogens. The form of nitrogen (ammonium or nitrate) that available to plant and pathogen may also affect the severity of disease or resistance (Huber and Watson 1974). The difference in response to N supply between these two types of pathogens is a result from differences in the mode of nutrition. Biotrophic pathogens rely on nutrients supplied by living cells. Necrotrophic pathogens, on the other hand, need senescing tissue or produce toxins in order to damage or kill the host cells (Marschner 2012) . Necrotrophic pathogens which kill host tissues probably have access to a wider range of N sources than biotrophic pathogens which have only access to N sources available in the apoplast and/or the haustorial matrix (Walters and Bingham 2007). *F.graminarum* can be classified as a hemibiotroph (Goswami and Kistler 2004). Initial infection resembles that of a pathogen that relies on a living host (biotrophs), but eventually switching to killing and consuming host cells (necrotrophs). *F. graminearum* act as a biotroph during the first 48 to 72 hour after infection, then switches to a necrotrophic phase at approximately 72 hour after infection (Kang and Buchenauer 1999).

4.2 DON accumulation

In this study, there was no indication that fungal development in the chaff influenced DON levels in the grain. The concentrations of DON in the grain were very low and below the detection limit of analyzing method used (Table 6). Because DON is a water-soluble compound, it can be translocated to spike tissues that are not invaded by the fungus (Snijders 2004; Kang and Buchenauer 1999). The transportation of DON from chaff to kernel in wheat was demonstrated by Snijders and Krechting (1993). In addition, because the grain of the *Fusarium* inoculated spikes, in the present study, were not infected by the fungus, it was expected that they contained very low amounts of DON. According to Cowger et al. (2009) DON may only be produced after grain infection, but not when the mycelia grow over the surface of the grain before infection. As stated by Xu et al. (2008) once the toxigenic isolates infected the grain, the toxin contamination is inevitable. Furthermore, it is known that DON levels in chaff are higher than DON levels in kernels of small grains (Cowger and Arellano 2013). Miller et al. (1985) found that chaff of wheat inoculated plants had approximately 10 times higher DON concentrations than grain in both resistant and susceptible cultivars.

Chaff of control spikes in the present study was contaminated with DON, although the results of the real-time PCR analysis showed no fungal biomass. This might be due to that even with the very low concentrations of *F. graminearum* DNA in chaff which were under the limit of detection; the fungus was present and able to produce DON. Or might be that other species of *Fusarium* with the ability to produce the DON occurred in the field but not detected by real-time PCR. This could be the case because for this real-time PCR assay, species-specific primers only for *F. graminearum* were used. The potential producer of DON beside *F. graminearum* is *F. culmorum* (Bottalico and Perrone 2002). In a study by Lindblad et al. (2013) the data from 2010 showed that *F. culmorum* in some years significantly contributed to DON production in Swedish grown spring wheat. The same results shown by Yli-Mattila et al. (2008) who found that *F. culmorum* contributed to DON production in barely for the period 2005–2006 in Finland, but was not involved in DON production in spring wheat and oats. There is a third *Fusarium* species where some strains are known to produce DON: “*F. crookwellense*”. However, the strains of *F. crookwellense* which produce DON are considered rare, and the species is not known to be a widespread pathogen in Scandinavia (Miller et al. 1991; Bottalico and Perrone 2002).

F. graminearum prevails in warmer humid European areas such as central and south-eastern Europe, whereas *F. culmorum* is more often found in colder areas such as western and northern Europe (Parry et al. 1995; Osborne and Stein 2007). However, *F. graminearum* increases in concern for Sweden. Because of future climate change, *F. graminearum* will find more optimal growth and spreading conditions in Swedish regions which are expected to experience a more humid climate and warmer mean annual temperatures (SJV 2007; Fogelfors 2009). In fact, as a possible result of changes in climate conditions, the increase of *F. graminearum* over *F. culmorum* as the predominant DON – producing *Fusarium* sp in wheat have been identified in Northern Europe (Waalwijk et al. 2004; Bottalico and Perrone 2002; Jennings et al. 2004). In Sweden, *F. graminearum*, and not *F. culmorum*, has been shown to be the main producer of

DON in cereal grain in recent years (Fredlund et al. 2013; Lindblad et al. 2013; Persson 2016). In addition to climate changes, increasing cultivation of maize (an important host for *F. graminearum*) and more adapting zero-tillage or reduced-tillage systems are among other proposed factors for a shift in dominant species from *F. culmorum* to *F. graminearum* in recent years (Friberg et al. 2015).

In the present study, *F. graminearum* inoculation significantly increased DON concentrations in the chaff of the *F. graminearum* treated spikes compared with the control spikes (Table 7). However, due to background contamination from the field in control spikes, DON concentrations in *F. graminearum* treated spikes cannot be explained only by the *F. graminearum* inoculation. It should also be noted that the spikes that were used for DON analysis in control and *F. graminearum* treated spikes respectively are not the same spikes. Thus, the subtraction of the amount of DON in *F. graminearum* treated spikes from this in control spikes to predict the amount of DON produced due to artificial inoculation is not accurate. For these reasons, no direct correlation between fungal biomass and DON in the chaff can be made. Nevertheless, DON concentrations in the chaff of *F. graminearum* treated spikes were for the most part relatively low compared to the relatively high DNA concentrations (Table 4 and Table 6).

Several factors affect the occurrence and the amount of mycotoxins. One of the most important factors is the genetic capacity of pathogenic strains to produce toxins (Bakan et al. 2002). Some strains of *F. graminearum* produce large amount of DON, whereas others produce small or undetectable amounts of DON (Walker et al. 2001). In this study, three *F. graminearum* isolates previously isolated from wheat kernels were used, but information about isolates aggressiveness and DON production are missing. Other important factors that affect DON production in wheat are susceptibility of wheat cultivars to FHB, fusarium infection pressure and climatic conditions, and according to Oldenburg et al. (2007) these are the main factors.

Most cultivars of common wheat are susceptible to FHB and only a few cultivars with moderate resistance to FHB development or mycotoxin accumulation have been released worldwide (Mesterhazy 1995; Wegulo et al. 2015). Many studies have shown that FHB-resistant wheat cultivars accumulated generally less DON than susceptible cultivars (Mesterhazy et al. 1999; Cowger et al. 2009; Koch et al. 2006). Five types of resistance in wheat have been described: I) resistance to initial infection; II) resistance to spread in infected tissue; III) resistance to kernel infection; IV) tolerance; and V) resistance to toxins accumulation in ears (Schroeder and Christensen 1963; Mesterhazy 1995). In the last type of resistance, DON biosynthesis is prevented or/and DON subjected to degradation by plant enzymes (catabolism) (Miller et al. 1985). In the present study, winter wheat cv. Olivin was used, but information about the level and type of its resistance are missing. According to a Swedish survey from 2009-2014, cultivars Olivin and Ellvis are less susceptible to FHB than the other marketable cultivars (Jordbruksverket 2017). Nonetheless, DON concentrations in chaff may not express the cultivar resistance level. In other words, DON concentrations in chaff cannot be predicted by the resistance level of the cultivar. Rottinghaus (2009) found that DON concentrations in chaff

were poorly correlated with cultivar resistance level and were approximately three times higher than DON in grain and straw.

In the current experiment, the concentration of inoculum used was very high (2×10^5 macroconidia /mL) and higher than in many other studies (Lemmens et al. 2004b; Hope et al. 2005; Cowger and Arellano 2013). Thus, it is unlikely that the low DON concentration in chaff is due to the low infection pressure. During the experimental period, the temperature of greenhouse chamber ranged between 12-22 °C (data not shown) with average temperature 17 °C. As the optimal temperature for DON production by *F. graminearum* may be around 25 °C (Hope et al. 2005) the low temperature in this experiment may be was the factor to decrease DON concentrations. However, low temperature is often connected to humid weather, and high moisture is far more critical in affecting toxin production than temperature (Hope et al. 2005). The percentage relative humidity ranged between 50-80% (data not shown) with average 72 %. Although the optimum percentage relative humidity for DON production is above 93% (Hope et al. 2005; Ramirez et al. 2006) the sporadic wetness of the spikes every 2 days (fine mist produced by the misting system) that kept the spikes moist (free moisture) might compensate the low percentage relative humidity.

A likely reason for low DON concentrations in chaff in spite of relatively high fungal DNA concentrations is presumably because of the saprophytic colonization of chaff at this stage of spikes maturity. Many of FHB pathogens can survive as saprophytes (Grudzinska-Sterno et al. 2016; Leplat et al. 2013) and some of them may colonize wheat tissue without parasitizing them (Ali 2001). In late infections, *Fusarium spp.* may occur as saprophyte, and hence they do not have time to reach the full stage of toxin productivity (El Khosht 2010). This might explain the low DON concentrations that observed in late infections that occurred close to harvest time. In a study by Tunali et al. (2012) authors found that DON levels produced by *F. pseudograminearum*, *F. culmorum* and *F. graminearum* were higher when infecting living host tissue compared with saprophytic colonization of grain or straw. They stated that the levels of DON production in vitro or from saprophytic colonization of substrates are several orders of magnitude lower than those in infected living tissue. This suggests that specific host signals are involved in toxin production in *F. graminearum*.

Moreover, leaching out of DON from the spikes could be another reason for further decrease in DON concentrations in chaff. As mentioned in material and methods, spikes were put in plastic dishes and exposed to a direct mist from sprinklers each two days. However, this action caused aggregation of the water in the bottom of dishes in some replications at the day of exposure to the direct mist, but not the next day. Great availability of free moisture around the spikes (in contact with spikes surface) might facilitate the leaching out of DON from the tissue. DON and its derivatives are water-soluble compounds and the leaching action of water have been reported in many studies (Gautam and Dill-Macky 2012; Bucheli et al. 2008; Lemmens et al. 2004a).

It is clear from this study that chaff (glumes and rachises) can contain significant amounts of DON resulting from late infection of spikes. Attention needs to be paid to the DON content from these resources that may be consumed by animals. Pigs are among farm animals the most susceptible to mycotoxins. EU established guidelines stated 900 µg/kg as a maximum level of DON in pig diets (Anonymous 2006a). However, negative effects of DON levels as low as 350 µg/kg have been reported (Drochner et al. 2004).

5 Conclusions and Recommendations

In this study, the artificial inoculation of the detached winter wheat spikes at late ripening stage by *F. graminearum* resulted in infection of the chaff only. Minor infection occurred in some grain samples. Low moisture content of grain and thickness of pericarp (fruit coat) at this stage of grain maturity might be amongst the reasons for non-infection and/ or reduced infection below the detection limit of real-time PCR. For further work, and to insure successful infection of grain by the *Fusarium* fungus, an earlier time-point of spike development for artificial inoculation than this used in the present experiment would be advisable. For example, the spikes are sampled 3 to 4 weeks before harvesting (instead of 1 week) when the grain moisture contents are still around 20-30%. Since fungal DNA was only detected in the chaff, a direct correlation between grain N content and fungal biomass was not possible to calculate. Accordingly, it was difficult to draw any definite conclusion. The hypothesis that a higher N content in grain will lead to a higher fungal infection can neither be confirmed nor rejected.

In addition, DON was only detectable in the chaff. This suggests that late infections might result in fungal development and toxin production with levels more significant in chaff than in grain at this evaluated growth stage. Non-inoculated control spikes were contaminated by DON although they were not infected by *F. graminearum*, as revealed by real-time PCR results (below the detection limit). Most probably, *F. culmorum*, another DON producer commonly found in Swedish grain samples (Djurle et al. 2010; Lindblad et al. 2013), occurred in the field environment and caused DON contamination. Further real-time PCR analysis with species-specific primers only for *F. culmorum* can be done to prove this issue. Indeed, in the context of field contamination, it was preferred to sterilize and wash (or autoclave) the spikes before the artificial inoculation with *F. graminearum*. Natural microflora that might occur on the wheat spikes (e.g. some other competitive *Fusarium* spp. or/and some saprotrophic fungi such as *Alternaria* and *Cladosporium*) can affect growth and DON production in these spikes (Cooney et al. 2001; Magan et al. 2003). Inoculation with *F. graminearum* significantly increased DON concentrations in *F. graminearum* treated spikes compared with control spikes. However, the DON concentrations obtained were relatively low compared to relatively high fungal biomass. Factors behind reducing DON concentrations in fungal treated spikes can be following up as a subject of further studies.

The experiment may need to be repeated with some adjustments in experimental conditions and methodology. Temperature and humidity of the greenhouse compartment should be controlled more precisely. Preferably, weather conditions of the experiment are chosen according to the optimal conditions for infection rather than mimicking weather conditions of August in Uppsala area, which might not ensure the highest infection level. Moreover, it is preferable to change the incubation technique in a way that does not lead to accumulation of water around the spikes. Hence, the assumption of low DON concentrations due to leaching from spikes tissue would be excluded. In addition to incubation technique it would also be good to adjust the incubation time of the detached spikes. As the effect of temperature on DON production by *F. graminearum* and *F. culmorum* is dependent on the time of incubation

(Vesonder et al. 1982) and since the average temperature in this study was relatively low (17°C), longer periods of incubation than 2 weeks can be tested in later studies. Furthermore, it would be better to grow winter wheat plants in the greenhouse from the beginning with additional N fertilizer to be applied around flowering in order to insure higher protein content in the grain as well as to get higher differences between the different N treatments. Because the N applications during vegetative through boot growth stages usually increase yield, the N applications after heading typically have less impact on yield but increase protein content (Orloff et al. 2012) . However, the limited period of time for this study prevented achievement of this.

Acknowledgements

Although many people helped me along the way, I would especially like to thank **Åsa Myrbeck** from the Department of Soil and Environment for her permission of sampling the wheat ears from the field experiment and providing detailed information about it. Also, for allowing the use of the experiment results in my thesis. Her prompt responses, friendly cooperation and valuable discussions are greatly appreciated.

I would also like to thank **Helena Bötter** for the technical assistance in the lab and for making the work environment more enjoyable.

A lot of thanks to the statistics man **Johannes Forkman** for his excellent assistance in using JMP software for data analysis. His adequate answers to my various questions are gratefully acknowledged. He is a very helpful person with a great sense of humour.

I express my thanks in particular to my supervisor Prof. **Paula Persson** for all her help and support to produce this thesis. Her comments and notes are much appreciated.

Popular scientific summary

Wheat occupies a prominent position in the list of food crops around the world. It is grown on a larger area than any other crop, including maize and rice. It covers around 25 % of the global need of food and contributes about 20 % of protein in the human diet. In addition, it is an important source of animal feed. In Sweden, winter wheat is grown on about 400 000 hectares with three million tons being harvested each year.

Unfortunately, many phytopathogenic fungi can infect wheat, one of the most important being species from genus *Fusarium*. This can cause a nasty disease called head scab. Head scab, or as called by scientists, Fusarium Head Blight (FHB) causes significant financial losses to the farmers not only because of reduction in yield but also because of reduction in quality of grain. Additional losses for the farmers can happen due to contamination of grain with toxic compounds known as mycotoxins. Serious threats to human and animal health are imposed by these mycotoxins when consumed at high enough levels; the reason why contaminated grain is rejected or downgraded at marketing. The toxin deoxynivalenol DON also called “vomitoxin” is the most important *Fusarium* mycotoxin in Swedish wheat which is mainly produced by *Fusarium graminearum*. The toxicity symptoms of DON include vomiting, nausea and diarrhea and feed refusal in some animals.

F. graminearum overwinters in dead crop residue that are left over in the field after harvest. During wet weather, it produces many spores that can spread through the air or splash onto wheat spikes in rain storms. Wheat plants are susceptible to infection from flowering until late stage of grain maturity. Normally, wet weather and high temperatures cause the high level of infection and outbreak of disease. Farmers apply many agricultural practices to control *F. graminearum* infection and mycotoxin production. Among those, cultivar selection, crop rotation with non-host crops and appropriate tillage system. In addition, timely application of fungicide in severe infection cases. Optimizing nitrogen (N) fertilization might also contribute to the control of *F. graminearum* infection. However, the practice with different N application rates has given inconsistent results.

The study of the effect of nitrogen fertilization on *F. graminearum* infection in winter wheat has been the core idea of this project, but in relation to late infection (post flowering). Therefore, winter wheat spikes at late stage of maturity were collected from a field experiment treated with different N fertilization levels. The spikes were artificially inoculated with *F. graminearum* spores and incubated in a greenhouse chamber. To assess the magnitude of infection, *F. graminearum* DNA was estimated and the mycotoxin DON was quantified in both grain and chaff by real-time PCR and ELISA, respectively. It was found that the fungus *F. graminearum* and its mycotoxin DON were only detectable in the chaff. No grain infections were detectable in both control (*F. graminearum* non- inoculated) and treatment (*F. graminearum* inoculated) spikes, except in a few samples. Hence, it was difficult to see how the N content of grain can influence the level of infection by *F. graminearum*. One can conclude that at this late stage of winter wheat growth (close to harvest time), late infections

by *F. graminearum* can lead to a certain level of fungal development and toxin production with levels more significant in chaff than in grain.

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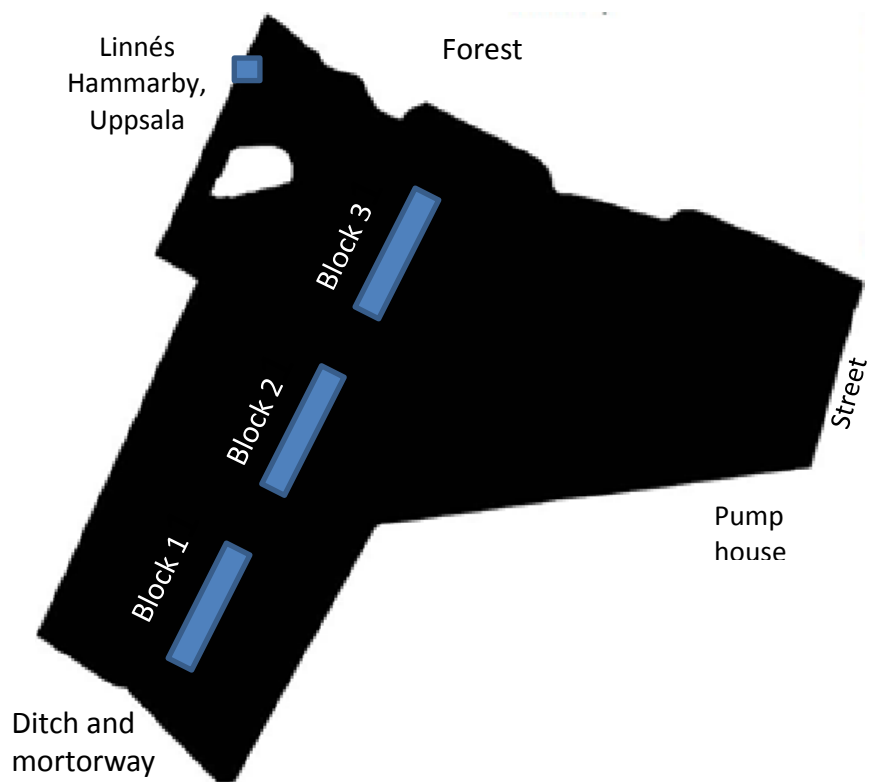
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Appendix I



Sketch of the field from which winter wheat spikes were sampled (by Åsa Myrbeck)