Evaluation of tools for analysis and quantification of *Fusarium* mycotoxins

By: Ida Lindell

Master's dissertation in Biology, 30 hp

Department of Forest Mycology and Plant Pathology
Uppsala 2011
Swedish University of Agricultural Sciences

Evaluation of tools for analysis and quantification of *Fusarium mycotoxins*

Ida Lindell

Uppsala, 2011

Supervisors: Richard Davidsson, Group manager Analysis lab, Lantmännen SW seed
Annika Djurle, Dept. Forest Mycology and Plant Pathology, SLU
Ingrid Happstadius, Breeder, spring wheat, Lantmännen SW seed
Tina Henriksson, Breeder, winter wheat, Lantmännen SW seed

Examiner: Dan Funck Jensen, Dept. Forest Mycology and Plant Pathology, SLU

Project initiated by Lantmännen SW Seed in collaboration with Swedish University of Agricultural Sciences

Credits: 30 hp
Level: D
Course title: Degree project in Biology
Course code: EX0564

Front-page picture: Ears of winter wheat infected by *Fusarium* sp., Laberweinting, Germany. Photo: Robert Persson

Preface

This Master's dissertation, worth 30 credits (Swedish hp) is the agronomist degree project for me, Ida Lindell. The project has been a collaboration between the Department of Forest Mycology and Plant Pathology, Swedish University of Agricultural Sciences and Lantmännen SW seed, who also initiated the project. Senior university lecturer Annika Djurle, Department of Forest Mycology and Plant Pathology, Richard Davidsson, Lantmännen SW seed, Ingrid Happstadius, Lantmännen SW seed and Tina Henriksson, Lantmännen SW seed supervised the work.

I would like to express my particular thanks to my supervisors, Annika Djurle, Richard Davidsson, Ingrid Happstadius and Tina Henriksson, who have given me very valuable input during my work with this project. Annika, especially for your help with the statistical analysis, Richard, for your advices with how to present the results in carts and tables, Ingrid with your proof-reading not leaving anything to the chance and Tina, for your help with practical issues.

I would also like to thank everyone who has helped me during my practical work. In the laboratory; specially Rita Svensson who helped me in Svalöv and Rena Gadjieva who supervised me in Uppsala, and in the field trials, the staff in Bjertorp, Svalöv, Harzhof and Laberweinting for all their valuable information and help with scoring and harvest.

Last but not least, I would like to give a large bunch of flowers to friends and family who have been very supportive and helped me with everything from picking ears in the field to serving me with sandwiches during my writing.

Abstract

Fusarium head blight is a large problem world wide which reduces the yields and the quality of small grains. Shrunken kernels with decreased thousand kernel weight as an effect and production of mycotoxins are some of the problems caused by infection of *Fusarium* spp. To avoid toxin contaminated bulks to enter the food and feed chain it is important to have tools for prediction and analysis of the fungi and toxins. Good prediction methods are also important in the breeding industry since early identification in trials of lines with high resistance or susceptibility simplify the work.

To compare different techniques for these objectives this project was initiated. The project aims to investigate the relationships between visible symptoms of Fusarium head blight, DON and T-2 toxin concentrations in the crop at different stages of crop development and ergosterol concentrations. Furthermore some fungi possibly causing the infections were identified. The project was carried out in oats, spring wheat and winter wheat at four locations in Northern Europe; two in Sweden and two in Germany during 2010.

The study shows that DON concentrations increase during crop development but for T-2 toxin no such relationship could be observed. It also shows that the relation between toxin concentrations and ergosterol concentrations is quite complex; high ergosterol concentrations does not necessarily indicate high toxin concentrations but high toxin concentrations are always associated with high ergosterol concentrations. This means that a low threshold value of ergosterol concentrations eliminates all volumes with high toxin concentrations but may as well remove some of the material with low toxin concentrations.

The results of the isolation of fungi are somehow confusing since some samples contaminated with toxin did not contain any of the investigated *Fusarium* species that could explain the toxin production. This is partly explained by other toxin producing fungi not included in this study but might also be explained with trichothecenes (*e.g.* DON or T-2 toxin) produced by *F. avenaceum*. *F. avenaceum* showed positive results for samples containing DON and T-2 toxin but no study has confirmed this fungus to produce DON or T-2 toxin. Some earlier research has, however, suggested the connection between trichothecenes and *F. avenaceum*.

The study concluded that the tools used for analysing Fusarium head blight and mycotoxins produced by *Fusarium* spp. have to be adapted to the objectives of the analysis and to the cultivar and conditions in the area. The most precise method may not be the best if a fast answer is desired while the faster method may be enough to make a prediction or an estimation of the toxin concentrations.

Keywords: DON, Ergosterol, *Fusarium*, Mycotoxin, T-2 toxin, Visible symptoms

Sammanfattning

Axfusarios är en svampsjukdom orsakad av *Fusarium* spp. och är ett stort problem världen över. Följderna av en infektion är dels skördeminskningar, då skrumpna kärnor leder till lägre tusenkornvikt, men infektionen kan även orsaka kvalitetsproblem då det i vissa fall produceras mykotoxiner. För att undvika toxinkontamination av foder och livsmedel är det viktigt att det finns effektiva metoder för att förutspå och analysera svampen och de toxiner som innebär risker vid konsumtion. Effektiva metoder för att förutspå kontamination av *Fusarium*, och toxiner bildade av svampen, är också viktiga inom förädlingsindustrin där tidiga observationer av linjer med hög eller låg tolerans mot *Fusarium*-infektion och toxinproduktion förenklar arbetet.

För att jämföra olika metoder för kvantifiering och analys av *Fusarium*-infektion och mykotoxiner initierades detta projektet med målet att undersöka sambandet mellan synliga symtom av axfusarios, koncentrationen av mykotoxinerna DON och T-2 toxin i grödan vid olika tidpunkter och ergosterolkoncentrationen. Dessutom analyserades vissa arter av *Fusarium* spp. som möjligen skulle kunna orsaka infektionen. Studien utfördes i havre, vårvete och höstvete på fyra platser i norra Europa under 2010.

Studien visar att DON-koncentrationen ökar med grödans utveckling medan man för T-2 toxin inte kan se något liknande samband. Studien visar också att sambandet mellan toxinkoncentration och ergosterolkoncentration är komplext då hög ergosterolkoncentration inte nödvändigtvis tyder på hög toxinkoncentration, men hög toxinkoncentration kan alltid kopplas till en hög ergosterolkoncentration. Detta innebär att ett lågt gränsvärde för ergosterolkoncentration skulle utesluta samtliga kvantiteter med hög toxinkoncentration men dessutom skulle kvantiteter med låga toxinkoncentrationer kunna uteslutas i detta urval.

Resultatet från identifieringen av *Fusarium*-arter är något förbryllande då det i vissa toxinkontaminerade prover inte kunde identifieras någon av de undersökta svamparterna. Detta kan troligen förklaras med toxinproduktion av andra *Fusarium*-arter som inte ingick i studien. Dessutom kunde det i flera fall konstateras att den enda av de undersökta svamparterna som identifierades var *Fusarium avenaceum*, vilken tidigare endast i ett fåtal studier har föreslagits som producent av DON och T-2 toxin.

Slutsatserna av studien är att metoder för att analysera axfusarios och mykotoxiner producerade av *Fusarium* spp. måste anpassas väl till det syfte analysen har och till grödan och de förutsättningar som råder i det aktuella området. Den mest noggranna analysen är inte alltid den bästa om det är snabba analyssvar som krävs medan en sådan metod kanske inte uppfyller de krav som finns på att uppskatta omfattningen av angreppet.

Nyckelord: DON, Ergosterol, *Fusarium*, Mykotoxin, T-2 toxin, Synliga symptom

Content

1 Introduction

Disease caused by *Fusarium* spp. in small grain crops is a problem with increased focus since the consequences of an infection can be very severe, both for the producers and for the consumers. The fungi can cause Fusarium head blight (FHB), which is of large economic importance due to losses in yield and quality (Parry *et al.*, 1995).

Fusarium head blight affects thousand kernel weight since infected kernels become small and shrunken, which also decreases yield (Sneijders and Perkowski, 1990). In addition to yield losses, infected material may cause problems in the refining of primary products, *e.g.* decreased baking quality (Schwarz, 2003). The fungi may also produce mycotoxins which, if consumed, are a risk for human and animal health (Gärtner *et al.*, 2007).

To minimize the risk of contamination of mycotoxins in feed and food the European Union has legislated about thresholds for toxin content both for the primary product and for the product (European Commission, 2010a and European Commission, 2010b). The content of mycotoxins can be analysed with various techniques *e.g.* high performance liquid chromatography-mass spectrometry (HPLC-MS) or enzyme-linked immunosorbent assays (ELISA) (Lattanzio *et al*., 2009). HPLC-MS is a powerful technique for identification and quantification of toxins, however, it is very expensive, and in many routine analyses the more cost-efficient ELISA is commonly used, but the analysis is quite time consuming and not very effective for large-scale analyses (Mirocha *et al.*, 2003). Another fast and cost-efficient technique is near infrared spectroscopy, which for example can be used for analysing ergosterol in grains. Ergosterol is a sterol in the cell membrane of fungi and is used as an indicator for hygiene and contamination of fungi in small grains. High levels of ergosterol indicate high content of fungi, which may be associated with mycotoxin contamination, but not necessarily so (Börjesson and Gruvaeus, 2009).

A fast way of analysing *Fusarium* spp. and mycotoxins is an important issue in breeding of cereals where selection for *Fusarium* resistant material is one important trait. A quick method, *e.g.* scorings, where varieties with high (or low) resistance to diseases caused by *Fusarium* spp. could be identified, would be preferable (Davidsson, Pers. comm., 2010). This kind of tool could also be useful in the work of preventing *Fusarium* and mycotoxin contaminated material to enter the food and feed chain (Klem *et al.*, 2007).

To investigate these aspects of *Fusarium* and mycotoxin contamination Lantmännen SW Seed initiated a project on the subject with Richard Davidsson, Group manager analysis lab, Lantmännen SW Seed, Ingrid Happstadius, Spring wheat breeder, Lantmännen SW Seed and Tina Henriksson, Winter wheat breeder, Lantmännen SW Seed, as the main responsible.

1.1 Objectives and hypothesis

The objectives of this project were to investigate the possibilities of forecasting mycotoxin contamination from visible symptoms of Fusarium head blight in the field and to study how the mycotoxin content varies over the season and the development of the crop.

The specific objectives were to use scorings of visible symptoms, quantification of toxins with ELISA, analysis of ergosterol content with Near Infrared Transmittance (NIT) and identification of *Fusarium* spp. with PCR and species specific primers. A comparison of the results should indicate whether scorings and different analyses were correlated. To investigate how toxin content developed over time samples from different development stages of the crop were to be analysed.

Four hypotheses were formulated;

Firstly, severe visible symptoms increase the risk of high mycotoxin content in the crop.

Secondly, the highest mycotoxin content will be found in the ripe material.

Thirdly, the mycotoxin content will not increase after harvest during drying of the grains.

Finally, high content of ergosterol does not necessarily indicate high content of *Fusarium* spp. or high levels of mycotoxins.

2 Literature review

This literature review contains information from earlier research about the biology of *Fusarium* in small grains in general, different *Fusarium* species, production of mycotoxins and forecasting and analysis of the fungi and its metabolites.

2.1 Biology of *Fusarium* species in small grains

The genus *Fusarium* contains fungi which are soil borne and necrotrophic plant pathogens (Agrios, 2005). *Fusarium* spp. are causing diseases on a lot of genera of cultivated plants, including Gramineae and thereby small grains. Four of the *Fusarium* spp., *F. culmorum, F. avenaceum, F. graminearum* and *F. poae* (Parry *et al*., 1995), are causing one of the most common diseases on small grains in the world, Fusarium head blight (FHB; Hörberg, 2001a; Agrios, 2005). It occurs in most areas where small grains are grown and causes big losses in yield and quality (Parry *et al*., 1995). The yield reduction is mainly a result of shrunken kernels as an effect from occlusion of vascular tissue in the rachis and thereby lack of water in the developing grain (Shaner, 2003). This has a negative impact on the thousand kernel weight (Sneijders and Perkowski, 1990).

Some *Fusarium* spp. may both reproduce sexually and asexually either with ascospores in the sexual stage or with conidia in the asexual stage (Figure 1). It is spread by infested seeds or stubble and crop debris either with spores or fragments of hyphae but the spores can also be spread by wind or rain splash (Parry *et al*., 1995; Bushnell *et al*., 2003; Klem *et al*., 2007) and even by several insects and mites (*Siteroptes graminum*; Parry *et al*., 1995). The fungus is a soil inhabitant, which means that it can survive in the soil as a saprophyte indefinitely. A soil inhabitant often has a wide range of hosts (Agrios, 2005) which is also the fact for *Fusarium* spp. which can infect a lot of different species of grasses and small grains (Parry *et al*., 1995). Previous results from a Canadian study of *Giberella zeae*[1] showed that infection mainly occurs from airborne ascospores rather than from conidia spread with rain splash (Fernando *et al*., 1997). However, in contrast to this result Klem *et al*. (2007) and Parry *et al*. (1995) stated that the reproduction most commonly occurs with the asexual spores (conidia).

FHB caused by *Fusarium* spp. is most often referred to as a flowering disease (Sutton, 1982) where anthers are the primary infection site, but Fernando *et al*. (1997) concluded from studies in Canada that most of the FHB infections are established during primary infection when the perithecia are developed and that the disease thereby should be regarded as a monocyclic disease.

[1] *Giberella zeae* is the teleomorph, the sexual reproductive stage, of *F. graminearum* (*e.g.* Fernando *et al.*, 1997)

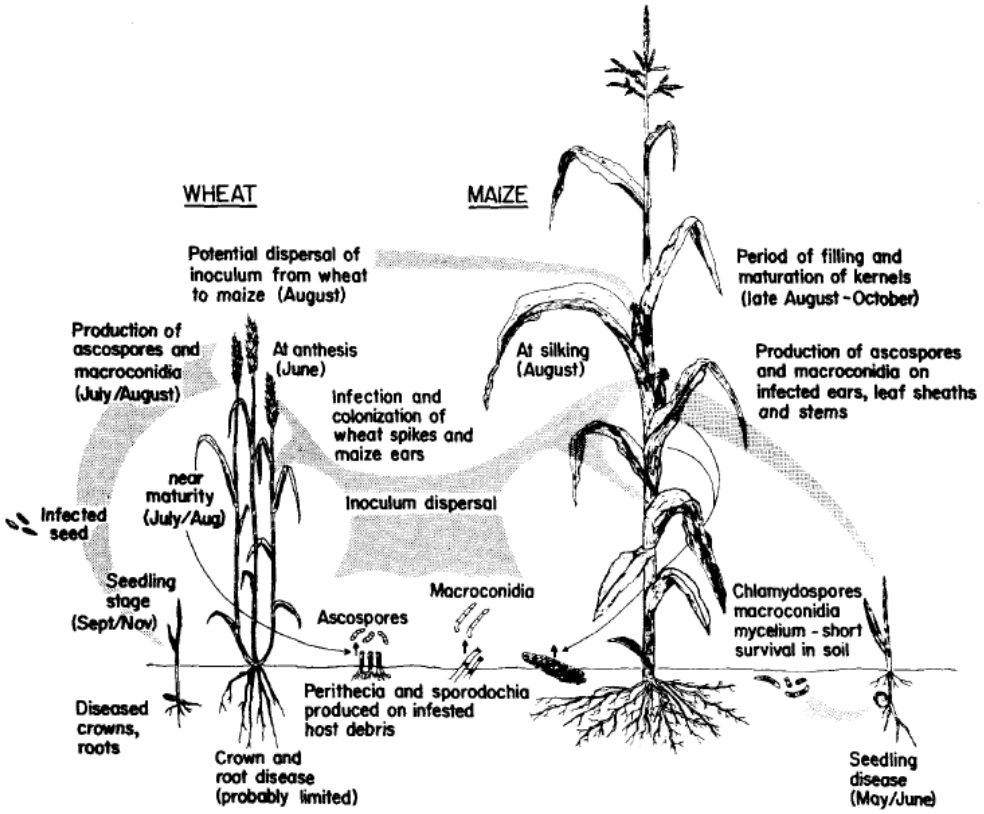


Figure 1. Life cycle of *Fusarium* in wheat and maize. Source: Sutton (1981).

For an infection to occur the weather at the time of development of perithecia and time of flowering is of large importance (e.g. Parry *et al*., 1995; Musa *et al*., 2007). The temperature and humidity will to a large extent decide how severe the infection of FHB will be (Figure 2). Warm weather and high humidity when the perithecia are supposed to develop on the crop debris of the plants and around the time of anthesis when spores are supposed to infect the ear favours the fungus (Klem *et al*., 2007). However, a period of dry weather when the spores are to be dispersed from the perithecia can improve the dispersal (Perry *et al*., 1995).

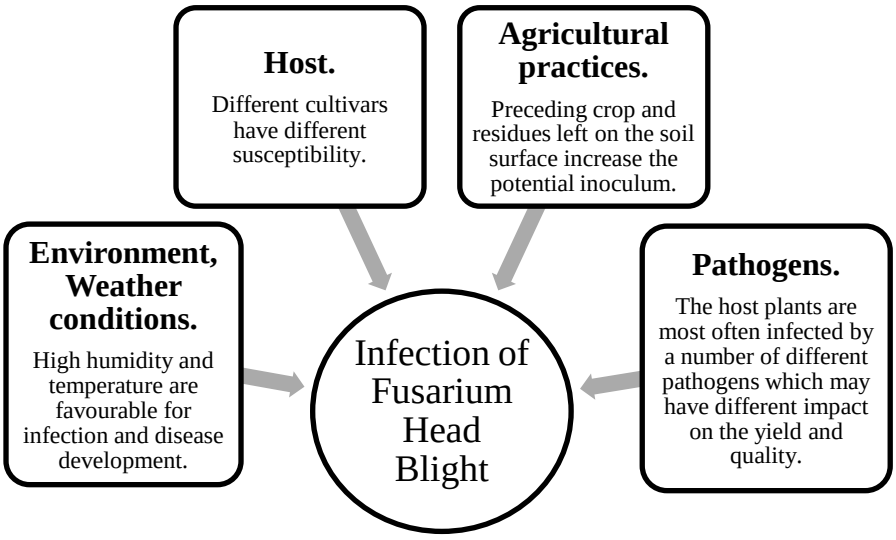


Figure 2. Factors important for the severity of the infection of Fusarium head blight. Based on Chelkowski (1998).

Along with the sum of precipitation five days before anthesis Klem *et al*. (2007) stated that the preceding crop was the most important factor for *Fusarium* infection (Figure 2). Some species of the fungus are, according to Khonga and Sutton (1988), able to form perithecia on crop residues, such as maize stems and wheat spikelets. McMullen *et al*. (2008) states that *F. graminearum* and other pathogenic *Fusarium* spp. causing rots in stalk, root and cobs of maize can survive on plant residues for several years. The consequence of this is that the most effective methods for controlling diseases caused by *Fusarium* spp. is to practice crop rotation and avoid maize as preceding crop to small grains and to use cultivars tolerant to infections by *Fusarium* spp. (Gärnert *et al*., 2007).

To infect the crop the pathogen can penetrate the anthers in the ears of small grains during anthesis. The fungus spreads through the xylem (Kang and Buchenauer, 1999; Agrois, 2005) and phloem and can infect the entire ear (Kang and Buchenauer, 1999), however it is not fully known how the entire ears is infected (Bushnell *et al.*, 2003; Brown *et al*., 2010). Visible symptoms can be observed three days after inoculation if the plant is kept in a moist chamber (Bushnell *et al*., 2003) but according to Bechtel *et al*. (1985) much of the damage from the fungus is done between the second and the third week after anthesis.

Some cultivars have a higher tolerance, or resistance against the pathogen. Resistance can occur as type I or type II resistance. Cultivars with type I resistance are able to limit the penetration and initial infection of the ear, while type II resistant cultivars are able to limit the spread in the ear (Schroeder and Christensen, 1963). Agrios (2005) stated that the severity of an infection also depends on the height of the crop where taller varieties seem to have more severe infections. However, Fauzi and Paulitz (1994) conducted a study where spring wheat was infected with *F. graminearum* in different ways and treated with different amounts of growth regulators. From the results of this study they suggested that plants treated with growth regulators, shorter plants, had closer distance to the ejected ascospores and inoculum on the ground and thereby higher risk of infection. They also suggested that dwarfed plants may have certain impact on the microclimate which favours the production of perithecia and ascospores (Fauzi and Paulitz, 1994).

The first symptom of an infection is small moist brownish spots on the spikelet (Hörberg, 2001a; Bockus *et al*., 2010). Later on symptoms as partly white and partly green ears can be observed as a result of necrotic spikelets. If the infection is severe the entire ear will turn white and wilt. If the weather is humid and favourable for the pathogen and the infection occurs late in the development of the crop the kernels and glumes turn pink since this is the colour of the macroconidia forming sporodochia (Figure 3) (McMullen *et al*., 2008; Bockus *et al*., 2010).

Figure 3. Ear of winter wheat infected with *Fusarium* sp., Laberweinting, Germany. Photo: Robert Persson, 2010.

2.2 Mycotoxins

Fusarium spp. can produce a wide range of toxins which can be dangerous for human and animal health and fertility. The four most toxic groups of mycotoxins are trichothecenes, zearalenone, moniliformin and fumonisins. This report will focus on trichothecenes. The trichothecenes can be divided into four different groups; among them type A and type B trichothecenes. Type A trichothecenes are more toxic than type B and include e.g. T-2 toxin and HT-2 toxin (metabolite to T-2 toxin; Hörberg, 2001a) which can cause disturbance in the reproduction of sows. Deoxynivalenol (DON) appertains to the type B trichothecenes and can trigger feed rejection in pigs (Placinta *et al.,* 1999).

It is not fully known why the fungus produces toxins but Maier *et al*. (2006) and Ilgen *et al*. (2009) describes the effect of the toxins as virulence factors. For the induction of trichothecenes the *TRI5* gene plays an important role since it is coding for the key enzyme (trichodiene synthase) in the pathway for the production of trichothecenes. Ilgen *et al*. (2006) showed that the gene expression is induced in plant tissue penetrated with *Fusarium* spp. and has to be present for a successful colonization. The same study also revealed that the highest induction of *TRI5* was associated with the rachis node, between the rachilla and the rachis. This is confirmed by Savard *et al.* (2000) who showed that the infection mainly is spread from the penetrated spikelet downwards in the ear. First in the floral parts but from day six the toxin concentrations are higher in the rachis and peduncle.

In a study of Swedish winter wheat El Khosht (2010) detected the *TRI5* gene associated with six different species of *Fusarium*; *F. acuminatum, F. avenaceum, F. flocciferum, F. graminearum, F. poae* and *F. tricinctum*. Out of these species Leslie and Summerell (2006) confirm the link between *TRI5* and *F. acuminatum, F. graminearum* and *F. poae, w*hile *F. avenaceum, F. flocciferum* and *F. tricinctum* not have been associated with *TRI5* earlier. Studies by Dejardins *et al.* (1989) have, however, showed a linkage between *TRI5* and *F. sporotrichioides* and Hussein *et al.* (1991) and Abramson *et al.* (1993) could show relationships between trichotecene production and *F. avenaceum*.

Detection of the toxins is possible from 0.1-1 µg g^{-1} but they typically occur in the range of 1 to 10 µg g^{-1} in wheat kernels (Bockus *et al*., 2010). To decrease the risk of *Fusarium* infected small grains in food and feed the European Commission has set limits for allowed amounts of mycotoxins in these products (Table 1).

Table 1. Threshold values for DON and T-2/HT-2 in food and feed in the European Union

Use	Toxin	Product	Threshold value	Source
Feed (12% water content)	DON	Cereals and cereal products	8 mg kg^{-1}	European Commission (2010a)
	T-2 and HT-2	Cereals and cereal products	No value	European Commission (2010a)
Food	DON	Unprocessed cereals (wheat, barley, rye)	1250 µg kg^{-1}	European Commission (2010b)
		Unprocessed oats	1750 µg kg^{-1}	European Commission (2010b)
		Flour and other products for consumption	750 µg kg^{-1}	European Commission (2010b)
	T-2 and HT-2	Unprocessed cereals and cereal products	No value	European Commission (2010b)

The production of mycotoxins has most often been associated with areas where small grains adapted to a temperate climate are grown. This is because *Fusarium* spp. are favoured by lower temperatures than e.g. *Aspergillus* species producing aflatoxins (Placinta *et al*., 1999; D'Mello and Macdonald, 1998). The production of toxins from *Fusarium* spp. is also dependent on a high water activity level which means that the production of toxins cannot occur in very dry places. These characteristics also make it hard for the fungi to synthesize the toxin when the kernels are dry, why the toxins most often are produced before or immediately after harvest (Hocking, 2010).

As well as for the pathogen, the amount of mycotoxins produced is dependent on several different factors (Figure 2). Shaafsma *et al*. (2005) stated that the DON content in wheat depended on environmental effects to 48%, on variety of the crop to 27% and preceding crop to 14 to 27%. The accumulation of DON in kernels seems to depend on how severe the infection of the plant is, if the plant has any resistance to mycotoxin accumulation or infection in the kernels and if the kernels have any mechanism of detoxification.

2.2.1 Different *Fusarium* species and toxin production

Fusarium infection on small grains is mainly caused by *F. graminearum, F. culmorum, F. avenaceum* and *F. poae* (Leslie & Summerell, 2006). Out of these species *F. culmorum, F. avenaceum* and *F. poae* are the most common in Northern Europe (Hörberg, 2001b), but according to Börjesson (2004) *F. graminearum* appears more and more frequent. Other species which have become more common are *F. sporotrichioides* and *F. langsethiae*, but these species are still quite rare (Kosiak *et al*., 2003).

F. poae is not, like the other *Fusarium* spp., favoured by rainy and humid weather which makes the circumstances of severe infections a bit different compared to the other species (Hörberg, 2001b). Infections in the same area, with the same environmental conditions, can cause dissimilar severities of infections depending on which species is causing the infection. Some species such as *F. poae* and *F. langsethiae* infect the crop early, from the time of ear emergence, while other species can start to colonize the crop during the following two weeks after ear emergence. This makes the infections of the different species dissimilar depending on the conditions and weather during certain periods when an infection can occur (Parikka *et al*., 2008).

It is not only the severities of the infections that diverge between different species, also the production of mycotoxins is partly controlled by the fungi. For example, type A trichothecene producers such as *F. poae, F. sporotrichioides* and *F. langsethiae* are more related to production of T-2 toxin while production of DON is more related to type B trichothecene producers *e.g. F. culmorum* and *F. graminearum* (Kristensen *et al.*, 2005; Kosiak *et al.*, 2003; Hörberg, 2001b). Other species related to production of T-2 toxin are *F. equiseti* (Hocking, 2010) and *F. acuminatum* (Bottalico and Perrone, 2002) while *F. avenaceum* mainly is associated with the toxin moniliformin and enniatines (Uhlig *et al.*, 2007).

2.2.2 Visible symptoms of Fusarium head blight and mycotoxin content

The correlation between visible symptoms of Fusarium head blight and content of mycotoxins is not fully known, however, a review of 163 studies could explain 27-53% of the variation of DON content. The largest reason for the variation was explained by disease intensity and the strongest relation to *Fusarium* damaged kernels was the DON concentration (Paul *et al*., 2005). According to Hooker *et al*. (2002) there is a close association between concentration of mycotoxins in mature grains and environmental factors such as the weather at the time of inoculum production and infection. D´Mello and Macdonald (1998) presented a high positive correlation between FHB caused by *F. culmorum* and concentration of DON in wheat grain.

Perkowski (1998) studied the relationship between mature kernels with symptoms of FHB, shrunken kernels and the content of DON. He found that the fraction with the most shrunken kernels had the highest concentration of DON (Table 2).

Table 2. Concentration of DON in barley kernels separated depending on size of the kernels (Perkowski, 1998)

Size of kernel (mm)	DON (mg kg^{-1})
<2.2	43.3
>2.5 – 2.2	35.5
≤2.8 – 2.5	9.1
>2.8	3.5

The concentration of DON has also been correlated with visible symptoms of FHB before harvest (Figure 4) (Gärnert *et al.*, 2007). A similar correlation was found by Chelkowski (1998). His study showed that the concentration of DON depended on the percentage of *Fusarium* damaged kernels (r=0.9).

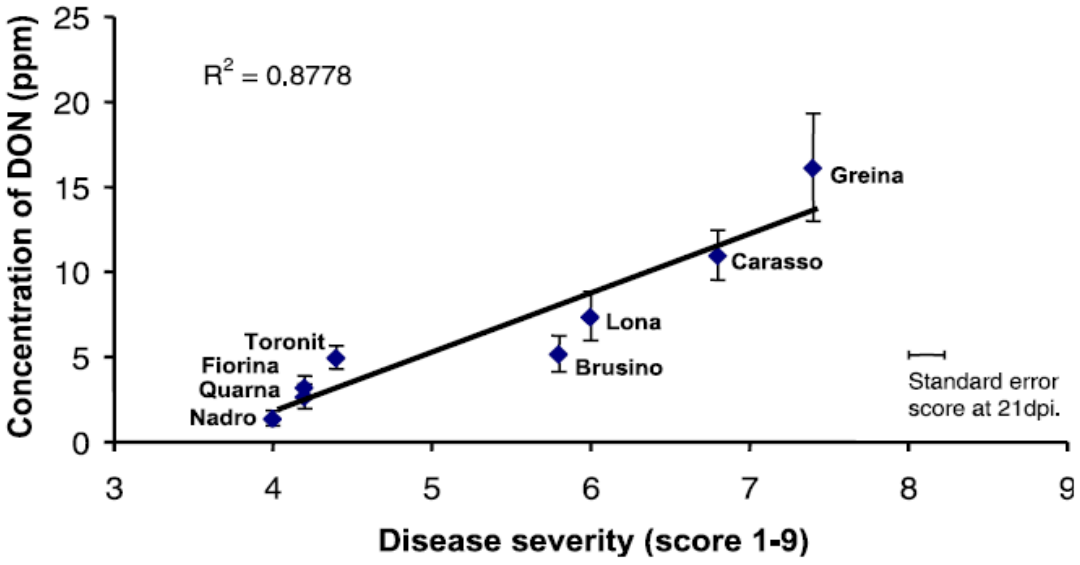


Figure 4. Correlation between scoring of Fusarium head blight and concentration of deoxynivalenol in an inoculated field trial in Swiss spring wheat cultivars. Source: Gärnert *et al.* (2007).

Confirming the correlations found by Gärnert *et al.* (2007) and Chelkowski (1998) other studies indicate a correlation where the concentration of DON in kernels is a function of how severe the visible symptoms are (Jones and Mirocha, 1999; Snijders and Perkowski, 1990). Snijders and Perkowski (1990) also showed that FHB was related to kernel toxin content and yield reduction. However, a healthy looking kernel could also contain mycotoxins and a kernel with visible symptoms may not have any accumulation of mycotoxins (Chelkowski, 1998). Fauzi and Paulitz (1994) showed in a study with wheat inoculated with *F. graminea-*

rum that 75% of the kernels contained the fungus but during scorings only 9 to 13% of the ears in the population showed symptoms.

2.2.3 Forecasting and analysis of Fusarium head blight and mycotoxins

There have been several studies of how to predict the severity of FHB infections and mycotoxin production (*e.g.* Hooker *et al.*, 2002; De Wolf *et al.*, 2003; Musa *et al.*, 2007). These are often based on a combination of environmental factors, such as temperature, relative humidity and precipitation (Klem *et al.*, 2007), cultivars and management (Musa *et al.*, 2007). In a previous study three periods with high risk of infection was defined and a model was based on weather data for those periods to forecast the concentrations of DON (Table 3). In more than 89 % of the cases studied the predictions of DON content <1.0 µg g^{-1} with this model were made correctly (Hooker *et al.*, 2002).

Table 3. Periods defined as high risk in terms of *Fusarium* infection and factors that increase or decrease the DON concentration in winter wheat in Ontario, Canada (Hooker *et al.*, 2002)

	4-7 days before heading		**3-6 days after heading**		**7-10 days after heading**	
	Increased	Decreased	Increased	Decreased	Increased	Decreased
Temperature		< 10°C		> 32 °C		
Precipitation	> 5 mm		> 3 mm		> 3 mm	

To predict the risk of mycotoxin contamination in small grains, analysis of the ergosterol content in grain can be used (Börjesson, 2004). Ergosterol is a component in the cell membrane of fungi and an analysis gives, thereby, only information about the total amount of fungi in the material, not about the actual levels of *Fusarium* spp. or mycotoxins. However, ergosterol analysis can hint about the risk of mycotoxin contamination (Börjesson and Gruvaeus, 2009). Lamper *et al*. (2000) showed a strong and positive correlation between DON concentration and ergosterol concentration (0.87) and for these two factors and kernel infection (r=0.90 for DON and r=0.89 for ergosterol). Also Bechtel *et al*. (1985) had earlier showed that the content of ergosterol in wheat also reflects the DON contamination of the material (Table 4), but a study by Miller and Young (1985) showed that non-inoculated winter wheat contained ergosterol but had no detectable levels of DON. In a report by Börjesson (2010) the risk of high DON concentrations increases if the ergosterol content is higher than 20 mg kg^{-1}. Also Dowell *et al*. (1999) show a correlation between visible symptoms, ergosterol and DON content where all kernels with visible symptoms contained DON and could be identified by analysing the ergosterol content. However there were more kernels that contained DON than what was forecasted from the observation of visible symptoms.

Table 4. Content of deoxynivalenol (DON) and ergosterol in wheat kernels separated depending on how severe the infection of *Fusarium* seems to be. Based on Bechtel *et al.* (1985)

	DON (ppm)	**Ergosterol (ppm)**
Normal kernels	0.43	2.8
Lightly to moderately infected kernels	22.7	29.0
Shriveled kernels	68.7	103.0

To measure the ergosterol content in a fast and effective way, near infrared spectroscopy could be used either by using reflectance (NIR) or transmittance (NIT) (Börjesson *et al.*,

2007). Börjesson *et al.* (2007) showed that these two methods gave quite equal results even though the NIR-method measure in a wider range of wavelengths (400-2500 nm) compared to NIT (850-1050 nm; Börjesson *et al.*, 2007).

To analyse the quantity of mycotoxins enzyme-linked immunosorbent assays (ELISA), high-pressure liquid chromatography or gas chromatography normally is used (Placinta *et al.*, 1999). To determine where the kernel accumulates the highest amounts of mycotoxins Gärtner *et al.* (2007) analysed different milling fractions and found that DON mainly was accumulated in the outer parts of the kernel. This is confirmed by Savard *et al.* (2000) who stated that the infection is spread more rapidly in the rachis compared to the spikelet and that the concentration of DON is higher in the rachis compared to the spikelet.

2.3 *Fusarium* and mycotoxin content depending on growth stage of the crop

Sneijders and Perkowski (1990) stated that the time of infection of the crop is not correlated to the content of DON but in a study by Del Ponte *et al.* (2007) the opposite was shown. Del Ponte *et al.* (2007) saw that wheat in all stages was susceptible to infection but the highest yield reduction and DON contamination occurred if the ears were infected past flowering to late milk development stage (DC 69-77; Zadoks *et al.*, 1974). The same study also stated that infection as late as hard dough stage (DC 87) resulted in DON accumulation but no reduction of yield. However, the findings shows that that infection in early development stages of the crop leads to higher accumulation of DON, higher yield reduction and more visible symptoms on the kernels (Del Ponte *et al.*, 2007).

Miller and Young (1985) showed that the contamination of mycotoxins (DON) increased for six weeks after infection and then dropped. The same pattern was showed for ergosterol; the content of ergosterol increased during the six weeks following inoculation, and then decreased slightly (Figure 5). The DON production was correlated with the ergosterol content or fungal biomass ($r=0.64$, $P<0.05$; Miller and Young, 1985).

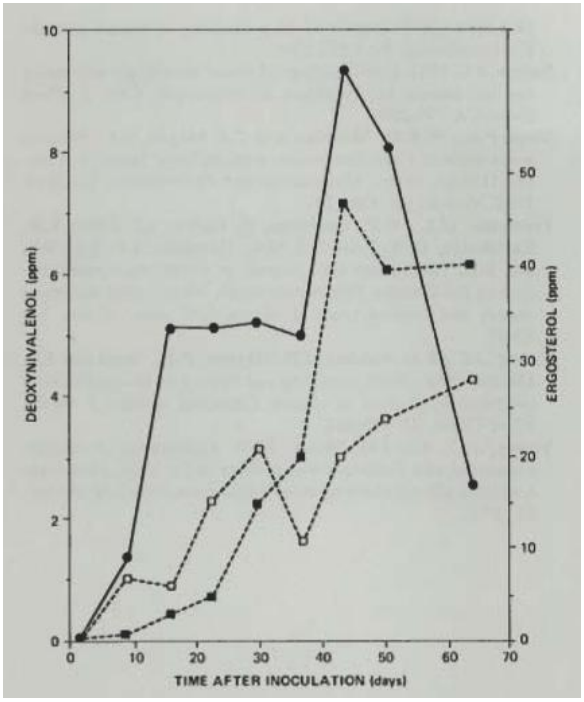


Figure 5. Levels of DON (circles) and ergosterol (squares) in ears of winter wheat as a function of time after inoculation with *Fusarium graminearum* in a field trial in Ottawa, Canada. Open symbols represent uninoculated material. Source: Miller and Young (1984).

3 Materials and methods

Data was collected from four different locations in Northern Europe, Bjertorp and Svalöv in Sweden, and Harzhof and Laberweinting in Germany (Figure 6). At each location samples were taken on two occasions in 2010, one approximately in milk development stage (DC 70-80) and one at the time of harvest (DC 90-95; Table 6).

Figure 6. Map of Europe. Locations (showed in red) for data collection to study correlation between visible symptoms of fusarium head blight and mycotoxin content and development of mycotoxins content in small grains.

In the study ten varieties of winter wheat, five varieties of oats and four varieties of spring wheat were included (Table 5). One of the winter wheat varieties, Olivin was not included in the trial referred to as Trial B (Table 6) in Svalöv and all the varieties of oats were excluded in Harzhof.

Table 5. Varieties included in the study of correlation between visible symptoms of fusarium head blight and mycotoxin content and development of mycotoxins content in small grains.

Oats[1)]	**Spring wheat**	**Winter wheat**
Belinda	Vinjett	Olivin[2)]
SW Kerstin	Diskett	Oakley
SW Ingeborg	Triso	Aurora
Circle	SW 45544	Lans
Galant		Loyal
		Kranish
		Boomer
		Hereford
		Ellvis
		Nimbus

[1)] Not sampled in Harzhof, [2)] Not sampled in Svalöv, Trial B

3.1 Field work

For information about the trials included in the study see Appendix I.

To evaluate the visible symptoms scores between 1 and 9 were given to each plot included in the study. The numbers represent how large part of the spikelet that was infected and is a result from counting infected spikelets on 25 ears in each plot. A score of 1 represents healthy ears and a score of 9 represents severe visible symptoms (Figure 7).

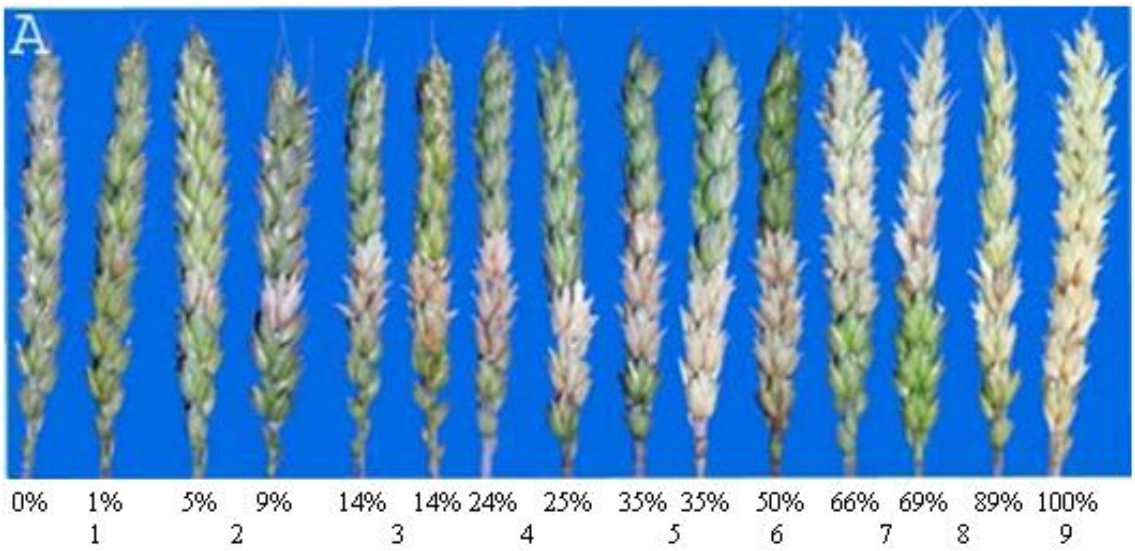


Figure 7. Scale of Fusarium head blight severity used as guide during scorings. The upper number represent the percentage of ear affected and the number below represent the score. Source: Engel *et al*. (2003).

The first sampling (Table 6) took place approximately when the crop was in milk development stage (Appendix I). Twenty ears from each plot were collected randomly, put in plastic bags and frozen. The ears collected in Laberweinting, Harzhof and Bjertorp were kept cold during transport until a freezer was accessible.

Table 6. Schedule of sampling for data collection to study correlation between visible symptoms of Fusarium head blight and mycotoxin content and development of mycotoxins in small grains

	1st sampling	**2nd sampling**	
	Freeze	Freeze	Dried
Laberweinting	20 ears	Plot harvested	Plot harvested
Harzhof	20 ears	100 ears cut and threshed	100 ears cut and threshed
Svalöv (Trial A)	20 ears	Plot harvested	Plot harvested
Svalöv (Trial B)	20 ears	100 ears cut and threshed	100 ears cut and threshed
Bjertorp	20 ears	Plot harvested	Plot harvested

The second sampling (Table 6) took place at the time of harvest. In some trials the whole plots were threshed and in these cases the plot harvest was divided into two bags, one bag was put in the freezer to stop the development and one was dried in 30°C for one week. The entire plot harvest in Laberweinting was frozen immediately after harvest and sent frozen to Svalöv.

The delivery was however delayed and the samples were unfrozen with condensation on the inside of the plastic bag for five days. When arriving to Svalöv half of the harvests were re-frozen and the rest were dried in 30°C for one week. Half of the plot harvest from Bjertorp was dried and then sent to Svalöv while the other half was sent to Svalöv immediately after harvest and frozen approximately one day after threshing.

In the trials where the whole plots were not harvested (see Table 6) 200 ears were collected, evenly spread over the plot. Out of these ears 100 were frozen and 100 were dried in 30°C for one week. The ears were threshed manually and after threshing kernels from the frozen ears were refrozen.

3.2 Laboratory work

All samples were milled in a mixer. The frozen samples (milk development stage and harvest, not dried) were dried in room temperature before milling. For the ears collected at milk development stage the entire ear was milled since it was impossible to collect enough amounts of kernels. The samples were analysed according to Table 7.

Table 7. Schedule of analyses of the material. Bjertorp means that only samples from Bjertorp were included for the particular analysis and development stage of the crop, and (---) indicates that no such analysis was run

	Milk development	**Threshed, not dried**	**Threshed and dried**
Ergosterol concentrations, NIT	---	Bjertorp	All samples
Toxin concentrations (DON och T-2 toxin), ELISA	All samples	Bjertorp	All samples
Identification of *Fusarium* spp., PCR	All samples	---	All samples

3.2.1 Toxin concentrations

To determine the concentrations of DON and T-2 toxin in the material Enzyme-linked immunosorbent assay (ELISA) was used. ELISA is a method where antibodies and antigens are utilized for detection of certain substances.

Wells are covered with capture antibodies for anti antibodies for a certain substrate. Enzyme conjugate, anti antibodies and samples are applied to the coated wells and free particles of the substance and enzyme conjugate of the same are captured at the antibody binding site on the coated wells and the overflow is washed away. To be able to detect the antibodies with enzyme conjugate they are stained and the absorbance, which is proportional to the concentrations of the assayed substance, is measured (Figure 8; Ridascreen, 2007; Beuch, 2009).

Ridascreen test kits for DON and T-2 toxins from R-Biopharm AG, Germany, were used to detect the mycotoxins and the analyses were made at *Cereal laboratory*, Lantmännen SW Seed in Svalöv. All samples collected at milk development stage and samples threshed and dried were analysed for toxin concentrations. In addition, the samples collected in Bjertorp at harvest but not dried were analysed to get a full picture of the development of the toxin concentration (Table 7). The computer program RIDASOFT Win (R-Biopharm AG) was used for calculation of the final results from raw data.

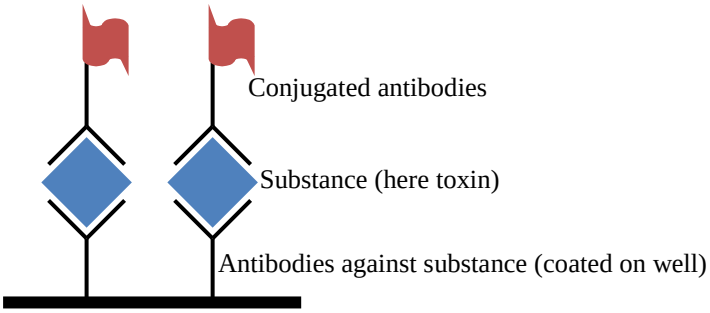


Figure 8. Schematic sketch of the mechanism in an ELISA test. A well coated with antibodies for a certain substance, here a toxin, binds free particles of the substance. Conjugated antibodies are applied and bind to the free part of the substance. The conjugated antibody can be detected.

DON

The samples were ground in a mixer and diluted with water (1:5)[2] . Five g of each sample were mixed with 25 ml of distilled water and mixed on a shaker for three minutes. The sample solutions were centrifuged and 50 µl of sample solutions and DON standards (0 ppb, 3.7 ppb, 11.1 ppb, 33.3 ppb and 100 ppb) were added to separate wells coated with capture antibodies directed against anti DON antibodies. In addition 50 µl of enzyme conjugate (peroxidase conjugated DON) and 50 µl of anti-DON antibody (monoclonal) was added and shaken manually before incubation for 30 minutes at room temperature (Ridascreen, 2007; Svensson, Pres. comm.).

After incubation the wells were emptied and washed three times with 250 ml of washing buffer (10 mM phosphate buffer, pH 7.4). Between each washing the wells were tapped onto a towel to remove remaining liquid. When washed, 100 µl of red stained Substrate/Chromogen was applied and shaken manually before incubation in room temperature and darkness for 15 minutes (Ridascreen, 2007; Svensson, Pers. comm.).

After incubation 100 ml of stop solution was added and gently mixed into the wells before the absorbance was measured at 450 nm (Ridascreen, 2007; Svensson, Pers. comm.).

T-2 toxin

The samples were prepared by grinding in a mixer and 5 g of each sample were dissolved in 70% methanol for extraction. The extracts were stirred for 10 minutes and centrifuged. The solution (50 µl) was diluted with 300 µl dilution buffer[3] and 50 µl per well were used for the assay. In addition to the samples six standard solutions with T-2 toxin in aqueous solution (0 ppb, 0.1 ppb, 0.2 ppb, 0.4 ppb, 0.8 ppb, and 1.6 ppb) were used. To each well 50 µl of enzyme conjugate and 50 µl anti T-2 toxin antibody were applied, the plate was gently mixed and incubated in room temperature for 1 hour. The liquid was poured out of the wells and washed with distilled water three times. Between each wash the wells were tapped onto a towel to remove remaining liquid (Ridascreen, 2009; Svensson, Pers. comm., 2010).

After application of 50 µl of substrate containing urea peroxide and 50 µl chromogen with tetramethylbenzidine the wells were gently shaken manually and incubated at room tempera-

[2] For some of the samples with high toxin content the dilution was increased to 1:12 to fit into the standard curve.

[3] PBS dilution buffer, pH 7.2: 0.55 g NaH_2PO_4 x H_2O + 2085 g Na_2HPO_4 x 2 H_2O + 9 g NaCl; filled up to 1000 ml with methanol/distilled water (10/90).

ture for 30 minutes. To stop the reaction 100 µl of stop solution with 1 N sulphuric acid was applied, gently mixed and the absorbance was measured at 450 nm (Ridascreen, 2009; Svensson, 2010).

3.2.2 Ergosterol

To determine the concentration of ergosterol the samples were run in a NIT, Infratec 1241 Grain analyzer (FOSS, Höganäs, Sverige). Ten subsamples were analysed with near infrared transmittance and a mean value of the relation between incoming and outgoing light was calculated. The equipment is calibrated continuously through the national Ergnet network.

All threshed and dried samples and the threshed but not dried samples from Bjertorp were analysed for ergosterol (Table 7).

3.2.3 Identification of fungi

In order to identify the species causing the *Fusarium* infection polymerase chain reactions, PCR, were used. These analyses were made at the Department of Forest Mycology and Plant Pathology, Swedish University of Agricultural Sciences, Uppsala, by the author tutored by Research engineer Rena Gadjieva. All samples collected at milk development stage and all threshed and dried samples were analysed (Table 7).

Extraction of DNA

The DNA was extracted using the CTAB-method (Garden and Burns, 1993) with some modifications.

The milled samples were put into 2 ml screw cap tubes with three glass beads in each tube and homogenized (Precellys 24, Bertine technologies). To each tube 1000 µl of CTAB[4] was added and the tubes were shaken (Vortexgenie 2, Scientific industries) and homogenized. The samples were incubated in 65^{o}C and shaken once during incubation. After one hour the samples were centrifuged (Heraeus Pico 17 centrifuge, Thermo scientific) at 13 000 rpm for 10 minutes and the supernatants were moved to new eppendorf tubes. The same volume of chloroform as for the supernatants was added and the tubes were shaken. The samples were centrifuged at 10 000 rpm for 7 minutes and the upper phase was transferred to new eppendorf tubes. The DNA was precipitated by adding 1.5 volumes of cold isopropanol (2-propanol) and the tubes were shaken and left at room temperature for 30 minutes. After incubation the samples were centrifuged at 13 000 rpm for 15 minutes and the supernatant was carefully poured out and thrown away. The pellets were washed with 200 µl 70% cold ethanol, centrifuged at 13 000 rpm for 5 minutes and the supernatant was again poured out and thrown away. The pellets were air dried for about 30 minutes and then re-suspended in 100 µl milliQ water.

The samples were stored at -20^{o}C.

PCR

The concentrations of nucleic acids were determined by measuring the absorbance (260/280 nm and 260/230 nm) and were used to calculate the dilutions to reach 1 ng DNA/ml.

The PCR were run with specific primers for *F. avenaceum, F. culmorum, F. graminearum, F. poae, F. langsethiae* and *F. sporotrichioides*. Mixtures of primers were prepared according to Appendix II and 7 µl of the primers were mixed with 7 µl of the diluted DNA solutions. In addition to the samples one positive control and one negative control were used in each set of

[4] CTAB (3%), 30 ml: 9 g CTAB solved in 9.78 ml milliQ water. Mixed with 4.5 ml 1 M TRIS-HCl, 15.6 ml 5 M NaCl and 0.12 ml 0.5 M EDTA.

samples. The positive controls for each set of primers were isolates of the respective *Fusarium* species kept at the department and they are presented in Appendix II. The negative controls contained milliQ water.

The PCR were run according to programmes in Appendix II.

Electrophoresis
The PCR-products were put on agarose gel made from agarose and TBE-buffer and stained by Gel green (Biotium). The concentration of the gel used is shown in Appendix II The gels were loaded with 4 µl Gene ruler (100 bp) in the edges and 3 µl PCR product in each well. The gels were run in 220V for 55 minutes and finally the results were detected with UV-light (GelDoc, Bio-Rad Laboratories).

3.3 Data analysis

The relationship between toxin concentration in material from different stages of crop development were analysed with the procedures CORR and GLM in SAS (SAS, 2010). The relationship between ergosterol and toxin concentrations in the threshed and dried material were analysed with logistic regression using the procedure GENMOD in SAS and a Bayesian method (Yuen and Mila, 2006). The Bayesian method calculates the possibility of making a correct prediction, here to predict a DON concentration above 1250 µg kg^{-1} using different concentration thresholds of ergosterol. The threshold of ergosterol concentration was varied between 11 and 18 since no sample had a higher content than 18 mg kg^{-1}. Specificity specifies the true negative values; values below 1250 µg DON kg^{-1} with lower ergosterol concentration than the tested threshold. Sensitivity specifies true positive values; toxin concentrations above 1250 µg kg^{-1} and ergosterol concentrations above the tested threshold. The method could yield a good prediction if both the Specificity and the Sensitivity is high (close to 1) since that indicates mainly correct predictions (Yuen and Mila, 2006)

4 Results

For weather data for the different locations see Appendix III.

4.1 Visible symptoms and toxin concentrations

The symptoms were vague in Harzhof and Svalöv and the symptoms varied between none and 2 on the scale. The visible weak symptoms in Svalöv and Harzhof were noticeable very late in the season why the scorings took place at the time of the second sampling. In Bjertorp no symptoms were observed. In Laberweinting the visible symptoms were enough to make a scoring between 1 and 9, for winter wheat twice and for spring wheat once (Table 8). The early scoring in winter wheat ranged between 1 and 3 while the later ones ranged between 1 and 6. For spring wheat the scorings ranged between 1 and 4.

As Laberweinting had the largest variation in scoring the correlation between scoring of visual symptoms and DON or T-2 concentration was analysed in both winter and spring wheat (Table 9). The data is also plotted in Figure 9 and Figure 10. Oats were not evaluated with this scoring system because of the difficulties to detect the symptoms.

Table 8. Results from scorings of visible symptoms of Fusarium head blight in winter wheat and spring wheat. A scale between 1 and 9 is used where 1 represents a healthy plant and 9 represents severe visible symptoms of Fusarium head blight. Plots marked with (-) could not be scored since no symptoms were observed

		Laberweinting			**Harzhof**	**Svalöv**
		2010-07-07	2010-07-15	2010-07-19	2010-08-13	2010-08-10
Spring wheat	Vinjett			4	-	-
	Diskett			1	2	1
	Triso			2	-	-
	SW45544			3	-	1
Winter wheat	Olivin	1	1		-	-
	Oakley	3	5		2	1
	Aurora	1	3		-	-
	Lans	1	5		1	-
	Loyal	3	6		-	1
	Kranich	1	3		1	-
	Boomer	2	5		1	-
	Hereford	2	5		-	1
	Ellvis	1	4		-	1
	Nimbus	2	4		-	1

Table 9. Correlation coefficients (r) between toxin concentration (DON or T2) and visual scoring in winter wheat and spring wheat from Laberwainting

		1st sampling (milk development)		**2nd sampling (threshed and dried)**	
	Scoring	DON	T2	DON	T2
Winter wheat	2010-07-07	0.50	0.51	0.20	-0.17
	2010-07-15	0.48	0.52	0.08	0.34
Spring wheat	2010-07-19	0.71	-0.72	0.62	-0.26

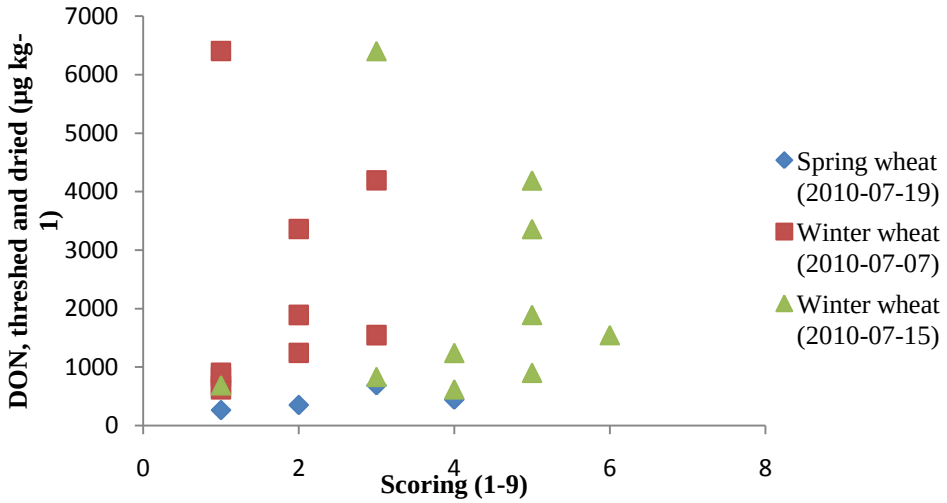


Figure 9. Scorings of winter wheat and spring wheat in Laberweinting and the concentrations of DON for the threshed and dried material.

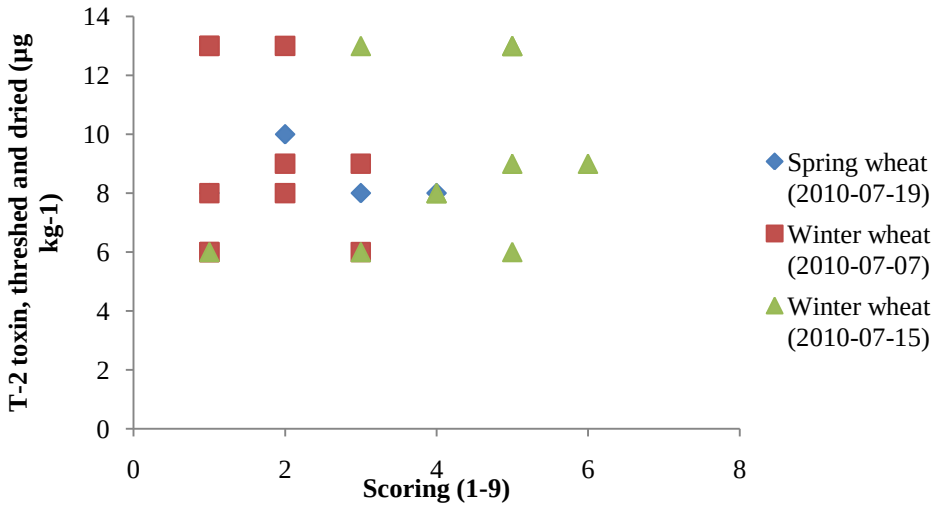


Figure 10. Scorings of winter wheat and spring wheat in Laberweinting and the concentrations of T-2 toxin for the threshed and dried material.

4.2 Toxin concentration and development of the crop

The ELISA showed presence of both DON and T-2 toxin in all the samples. DON varied between 15 µg kg^{-1} and 15 208 µg kg^{-1} at milk development stage, and between 14 µg kg^{-1} and 6407 µg kg^{-1} in the threshed and dried material. For T-2 toxin the range was 7 µg kg^{-1} to 865 µg kg^{-1} at milk development stage and 2 µg kg^{-1} to 57 µg kg^{-1} in the threshed and dried material.

Out of the five samples of oats from Bjertorp three had higher DON concentrations in the threshed material compared to milk development. When drying, the concentrations in four of the samples increased even more. For spring wheat the DON concentration increased for all the four samples from milk development stage to ripe material and for three of them during drying. Winter wheat was represented with 9 samples and out of these seven samples had increasing DON concentrations between milk development stage and threshing. After drying eight of the samples had increased DON concentrations (Figure 11).

The progress of toxin concentration for the entire material was studied and the concentrations at milk development stage were compared to the levels in the threshed and dried material. Out of these samples eight out of 15 samples (53%) of oats had increased levels of DON and five (33%) had increased levels of T-2 toxin. For spring wheat 10 out of 20 samples (50%) had increased DON concentrations but none had increased T-2 toxin concentrations. Out of the 48 samples of winter wheat 8 (16%) had increased levels of DON and three (6%) had increased levels of T-2 toxin.

T-2 toxin showed reverse results for the progress of concentrations between milk development stage and threshing, where one of the oat samples and none of the spring wheat and winter wheat samples had increased levels of T-2 toxin when threshing was compared to milk development stage. During drying the change of T-2 toxin concentrations were more similar to the DON concentrations where three of the oat samples, all four of the spring wheat and nine of the winter wheat samples showed increased T-2 toxin concentrations (Figure 12).

These results of toxin concentrations over time of crop development from Bjertorp shows significant increases of DON concentrations if all samples are analyzed independent of crop

(p<0.01). When analysing each crop separately the increase in DON concentration in spring wheat is significant (p<0.01) (Figure 11). For T-2 toxin a significant decrease is observed when studying all crops together (p<0.01) and also for winter wheat (p<0.01) (Figure 12).

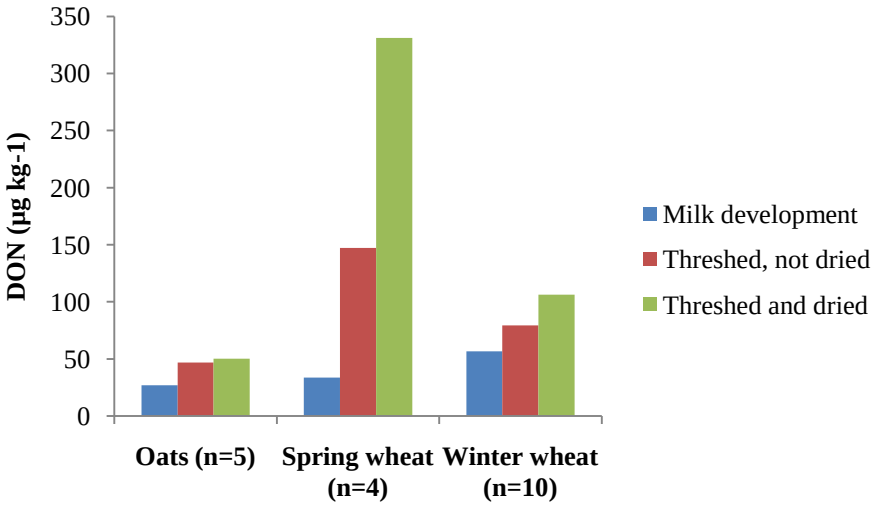


Figure 11. Development of DON concentration over time (mean values), from milk development stage, via threshed but not dried kernels and finally for the dried small grains for oats (StdDev: 11.3, 33.5, 39.7), spring wheat (StdDev: 13.9, 93.7, 195.6) and winter wheat (StdDev: 70.4, 102.2, 76.6), sampled in Bjertorp.

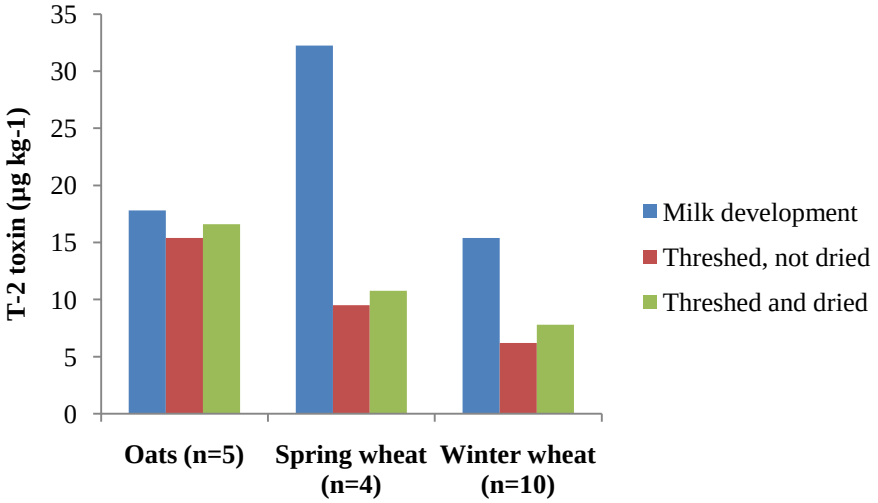


Figure 12. Development of T-2 toxin concentration over time (mean values), from milk development stage, via threshed but not dried kernels and finally for the dried small grains for oats (StdDev: 1.92, 2.19, 1.67), spring wheat (StdDev: 29.2, 1.00, 1.50) and winter wheat (StdDev: 7.90, 1.81, 2.97), sampled in Bjertorp.

The ELISA showed that a high content of DON in winter wheat at milk development stage also generates a high content of DON after harvest and drying (r=0.75***). Oats and spring wheat showed no such correlations (r=0.02 and r=-0.29 respectively) (Figure 13), neither did

the results for T-2 toxin for oats (r=-0.04), spring wheat (r=0.15) or winter wheat (r=0.15) (Figure 14). In Laberweinting all samples of winter wheat contained more than 1000 µg DON kg^{-1} at milk development stage and more than 500 µg DON kg^{-1} at harvest. For spring wheat all samples in Laberweinting and in Bjertorp had DON concentrations above 100 µg kg^{-1} at harvest while all samples in Hartzhof were below 150 µg kg^{-1}.

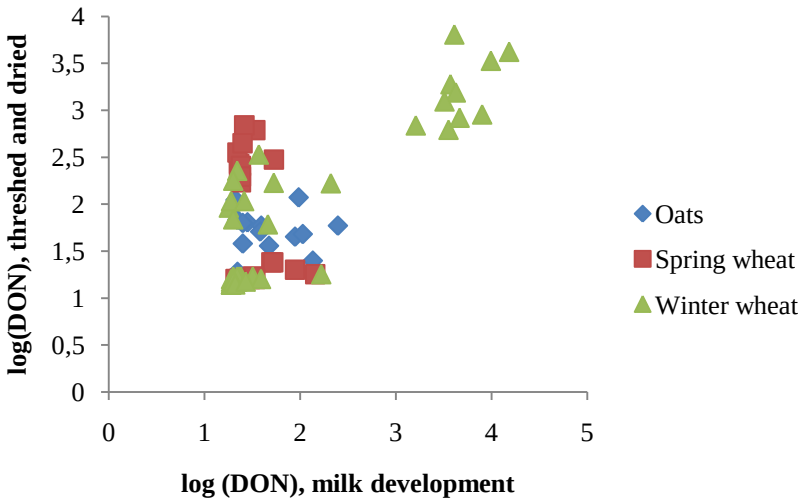


Figure 13. The relationship between DON concentration in different development stages of oats (r=0.02), spring wheat (r=-0.29) and winter wheat (r=0.75***). The DON concentration in the threshed and dried material as a function of the DON concentration at milk development stage. All values are shown as logarithms to fit into the chart, original data presented in µg kg^{-1}.

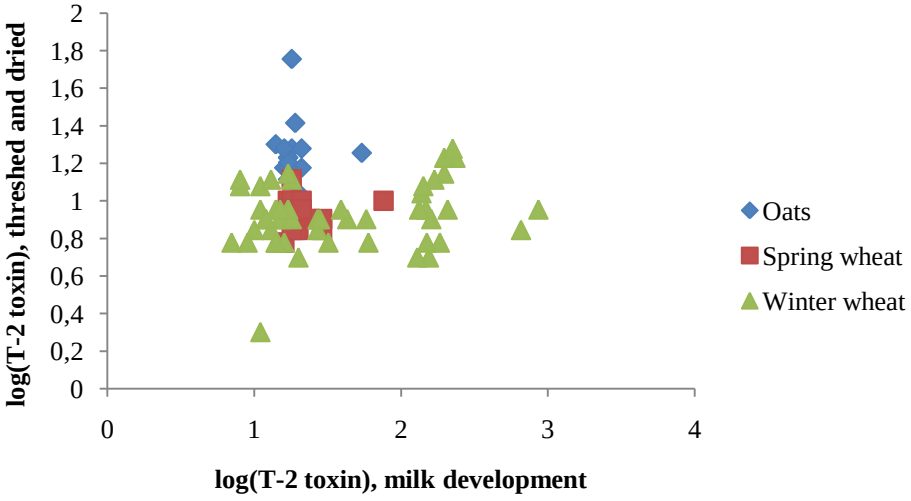


Figure 14. The relationship between T-2 toxin concentration in different development stages of oats (r=-0.04), spring wheat (r=0.15) and winter wheat (r=0.15). The T-2 toxin concentration in the threshed and dried material as a function of the T-2 toxin concentration at milk development stage. All values are shown as logarithms to fit into the chart, original data presented in µg kg^{-1}.

4.3 Toxin concentrations and ergosterol

Ergosterol was detected in all the samples and the concentrations varied between 8.3 mg kg^{-1} and 16.8 mg kg^{-1} (Figure 15).

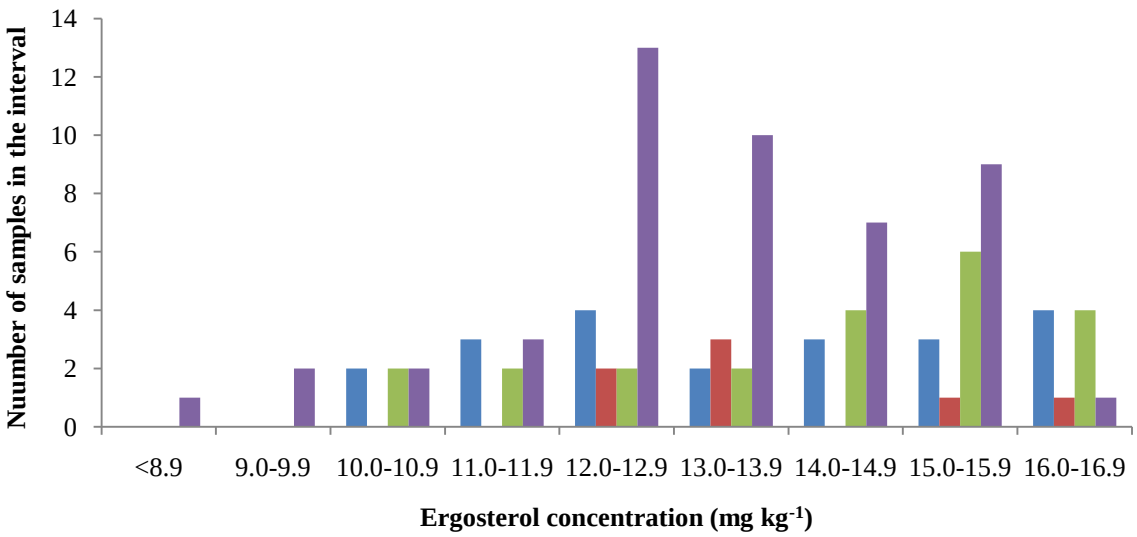


Figure 15. Frequency of ergosterol concentrations in the threshed and dried material. Each species of *Fusarium* is represented with a colour; *F. avenaceum* – blue, *F. culmorum* – red, *F. graminearum* – green and purple represent those samples where none of the *Fusarium* spp. were detected.

The relationships between ergosterol and DON concentrations are weak but there might be a tendency of increased concentrations of ergosterol in samples with higher toxin concentrations. For T-2 toxin no such tendency is observed. In winter wheat the relation between DON and ergosterol are weak and significant both at milk development stage (r=0.50**; Figure 16) and for the threshed and dried material (r=0.42**; Figure 17) but spring wheat and oats shows no significant results. Neither were ergosterol and T-2 toxin concentrations (Figure 18 and Figure 19) significantly correlated.

The samples from different countries; Sweden and Germany, showed now large differences in correlation coefficients for the relationship between ergosterol concentration and toxin concentration (Table 10).

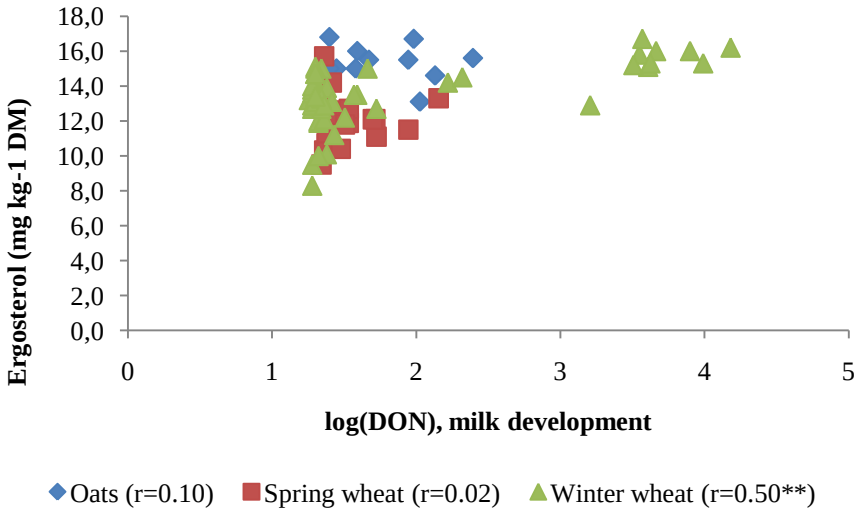


Figure 16. Relationship between ergosterol concentration and DON concentration at milk development stage. Correlations coefficients are shown in the legend. Toxin concentrations are, originally, presented in µg kg^{-1}.

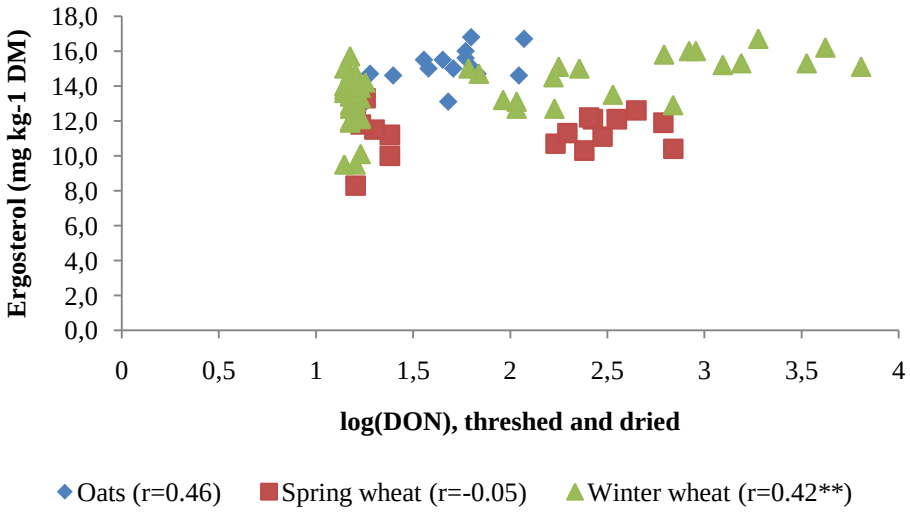


Figure 17. Relationship between ergosterol concentration and DON concentration in the threshed and dried material. Correlations coefficients are shown in the legend. Toxin concentrations are, originally, presented in µg kg^{-1}.

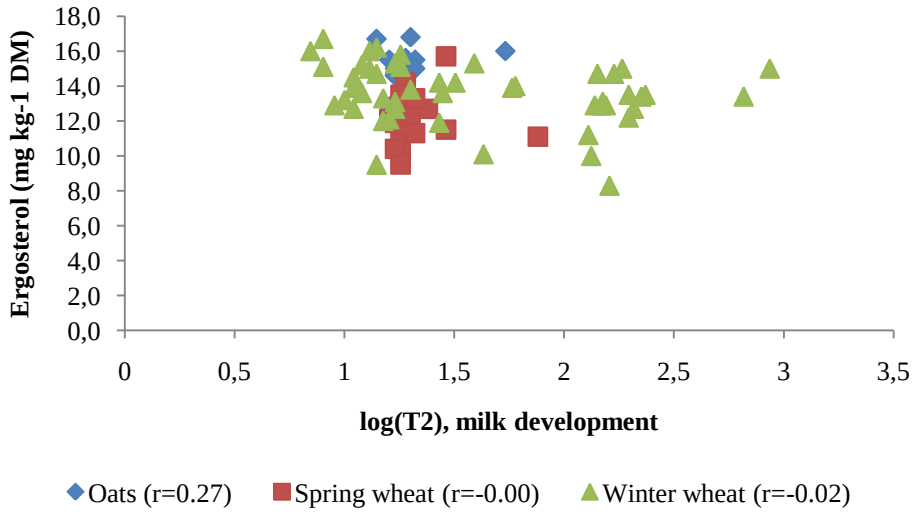


Figure 18. Relationship between ergosterol concentration and T-2 toxin concentration at milk development stage. Correlations coefficients are shown in the legend. Toxin concentrations are, originally, presented in µg kg^{-1}.

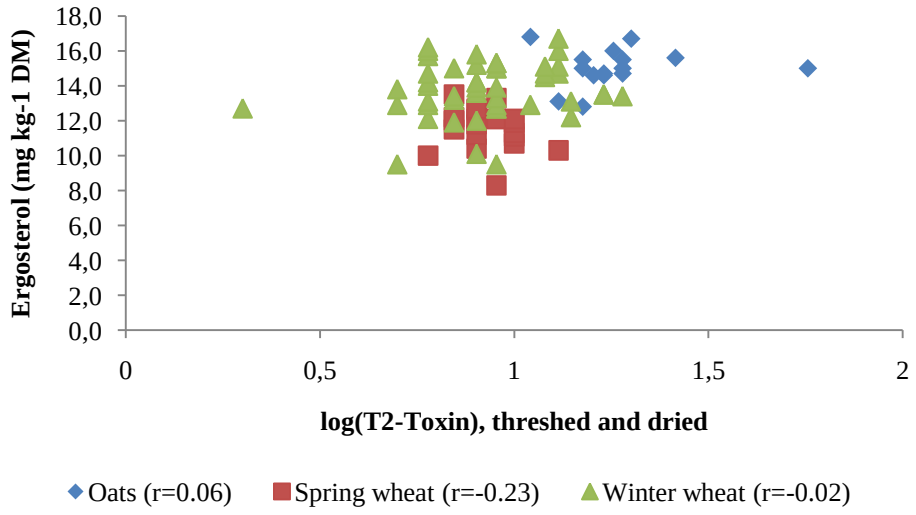


Figure 19. Relationship between ergosterol concentration and T-2 toxin concentration in the threshed and dried material. Correlations coefficients are shown in the legend. Toxin concentrations are, originally, presented in µg kg^{-1}.

Table 10. Correlation coefficients (r) for the relationship between ergosterol concentration and DON and T-2 toxin concentration at different development stage of the crops; milk development stage and after threshing and drying

			Correlation coefficients			
		n	DON (milk development)	DON (threshed and dried)	T-2 toxin (milk development)	T-2 toxin (threshed and dried)
Oats	Sweden	10	-0.06	0.17	0.44	0.56
	Germany	5	-0.17	0.49	-0.05	-0.87
Spring wheat	Sweden	12	0.40	-0.42	-0.22	-0.73**
	Germany	8	-0.04	0.35	0.42	-0.08
Winter wheat	Sweden	29	0.17	0.14	0.12	-0.01
	Germany	20	0.61**	0.49*	-0.10	0.36

Out of the samples of threshed and dried material six had toxin concentrations higher than, or close to, the, by the European commission, set limit for DON at 1250 µg kg^{-1}. None of these samples had concentrations of ergosterol above 20.0 mg kg^{-1}, which is a threshold for ergosterol concentration to differentiate different qualities from each other at delivery of small grains to Lantmännen in Sweden (Table 11) (Börjesson and Gruveaus, 2009).

In Table 12 results from the logistic regression of toxin concentration in the threshed and dried material and ergosterol content are presented. Specificity indicates the portion of correct predictions, in the specific interval of ergosterol, when the toxin concentrations transcend the set limits for DON (1250 µg kg^{-1}). Sensitivity indicates the portion of correct predictions in absence of DON concentrations above the set limits. It is desirable to have values close to one both for sensitivity and for specificity since this increases the chance for a correct prediction.

The screening of T-2 toxin did not generate toxin levels high enough to make a similar analysis.

Table 11. Values of DON concentration higher than, or close to, the, by the European commission, set limit at 1250 µg kg^{-1} and their corresponding ergosterol concentrations

DON concentration (µg kg^{-1})	**Ergosterol concentration (mg kg^{-1})**	**Variety**	**Crop**	**Location**
1243	15.2	Nimbus	Winter wheat	Laberweinting
1548	15.3	Loyal	Winter wheat	Laberweinting
1893	16.7	Boomer	Winter wheat	Laberweinting
3362	15.3	Hereford	Winter wheat	Laberweinting
4193	16.2	Oakley	Winter wheat	Laberweinting
6407	15.1	Aurora	Winter wheat	Laberweinting

Table 12. Possibility to make a correct prediction of DON concentrations ≥1250 µg kg^{-1} at different ergosterol thresholds. Specificity specifies correct predictions when the toxin concentration is above the set limits and sensitivity specifies the portion of correct predictions when the toxin concentration is below the set limit (Yuen and Mila, 2006)

		Ergosterol, mg/kg						
		≤11	≤12	≤13	≤14	≤15	≤16	≤16.5
DON	**Specificity**	0.101	0.19	0.418	0.62	0.772	0.937*	0.975
	Sensitivity	1	1	1	1	1	0.4	0.2

In Figure 20 the receiver-operating characteristics (ROC; Yuen and Mila, 2006) curve for the sensitivity and specificity of DON concentration (threshold: 1250 µg kg^{-1}) and varying ergosterol concentration is presented. The table and the curve both show that when all true positive samples (correct predictions) are picked up also 23% (0.23) of the false positive samples (wrongly identified as having a DON concentration above the limit) will be included.

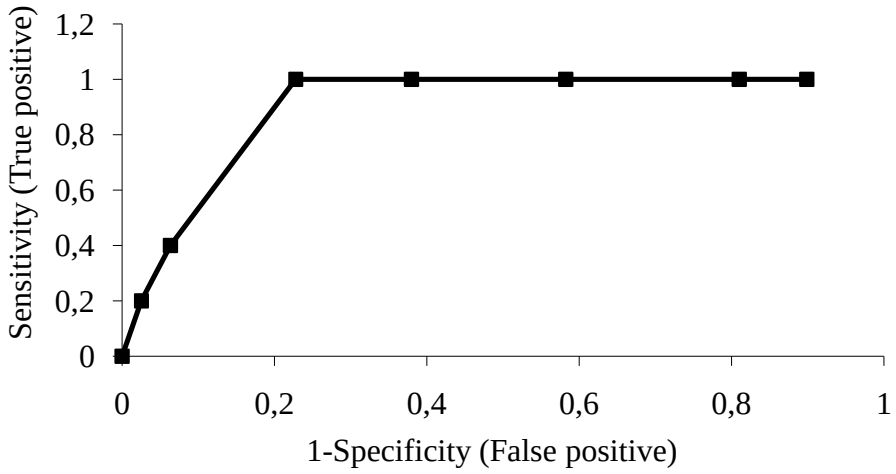


Figure 20. ROC curve showing the possibility for correct prediction of DON contamination using ergosterol as an indicator.

4.4 Fungal identification and toxin concentration

In the samples collected at milk development stage, and tested with Fusarium specific PCR primers, at least one Fusarium species was detected in 31 out of 84 samples. For the samples taken from the threshed and dried material 36 of 84 samples contained at least one *Fusarium* species. The results of identified fungi are presented for the respective location in Table 13 and for the respective crop in Table 14. Several species might have been detected from the same sample, in 18 out of 67 samples containing any of the analysed species more than one species were detected.

Laberweinting is the location with the most frequent detection of fungi with 37 positive PCR results from 19 samples (37/19) followed by Bjertorp (23/19), Svalöv (25/32) and Harzhof (5/14). The different crops were about equally frequently infected (oats: 15/15, spring wheat: 21/20 and winter wheat: 50/49).

F. avenaceum was detected in all locations and in all crops both at milk development stage and in the threshed and dried material. At milk development stage it was the most common species in Svalöv but in the threshed and dried material it occurred with similar frequency in both Bjertorp and Laberweinting. *F. culmorum* and *F. graminearum* were detected in all locations at both development stages except from Harzhof where no detection was made in the threshed and dried material and in Bjertorp where no *F. graminearum* was detected at milk development stage. *F. culmorum* and *F. graminearum* were also detected in all crops with one exception for oats in milk development stage. *F. poae* was not detected in any sample and *F. langsethiae* and *F. sporotrichioides* were only detected in a few winter wheat samples. Those samples containing *F. langsethiae* and *F. sporotrichioides* were equally divided between different locations; *F. langsethiae* in Svalöv, Harzhof and Laberweinting and *F. sporotrichioides* in Svalöv and Laberweinting (Table 13 and Table 14).

Table 13. Number of samples with the different *Fusarium* spp. detected and the mean DON and T-2 toxin concentrations and ergosterol concentrations for each location.

		Mean																	
		DON (µg kg^{-1})		**T-2 toxin (µg kg^{-1})**		**Ergosterol (mg kg^{-1})**	***F. avenaceum***		***F. culmorum***		***F. graminearum***		***F. poae***		***F. langsethiae***		***F. sporotrichioides***	
	n	Milk dev.	Threshed, dried	Milk dev.	Threshed, dried	Threshed, dried	Milk dev.	Threshed, dried	Milk dev.	Threshed, dried	Milk dev.	Threshed, dried	Milk dev.	Threshed, dried	Milk dev.	Threshed, dried	Milk dev.	Threshed, dried
Bjertorp	19	43.9	138.8	19.6	10.7	13.5	3	9	2	2	-	7	-	-	-	-	-	-
Svalöv	32	37.2	47.2	109.8	10.8	13.5	10	3	2	2	3	3	-	-	1	-	1	-
Harzhof	14	30.6	17.1	87.9	7.3	11.6	1	1	1	-	1	-	-	-	1	-	-	-
Laberweinting	19	3078	496.5	19.2	11.8	2.0	2	9	1	2	9	12	-	-	1	-	1	-

Table 14. Number of samples with the different *Fusarium* spp. detected and the mean DON and T-2 toxin concentrations and ergosterol concentrations for each crop.

		Mean																	
		DON (µg kg^{-1})		**T-2 toxin (µg kg^{-1})**		**Ergosterol (mg kg^{-1})**	***F. avenaceum***		***F. culmorum***		***F. graminearum***		***F. poae***		***F. langsethiae***		***F. sporotrichioides***	
	n	Milk dev.	Threshed, dried	Milk dev.	Threshed, dried	Threshed, dried	Milk dev.	Threshed, dried	Milk dev.	Threshed, dried	Milk dev.	Threshed, dried	Milk dev.	Threshed, dried	Milk dev.	Threshed, dried	Milk dev.	Threshed, dried
Oats	15	64.1	54.7	20.1	19.8	15.0	2	5	-	1	2	5	-	-	-	-	-	-
Spring wheat	20	38.7	185.9	22.6	8.6	11.6	2	7	2	2	2	6	-	-	-	-	-	-
Winter wheat	49	1208	482.9	96.5	9.0	13.8	11	9	1	4	9	11	-	-	3	-	2	-

The results show that samples picked at milk development stage (n=84) were contaminated by five of the six investigated species of *Fusarium*; *F. avenaceum* (16 samples), *F. culmorum* (3 samples), *F. graminearum* (15 samples), *F. langsethiae* (3 samples) and *F. sporotrichioides* (2 samples) (Table 13 and Table 14). In Figure 21 and Figure 22 the frequencies of samples in different intervals of toxin concentrations are presented.

In the threshed and dried material (n=84) three different species of *Fusarium* were detected; *F. avenaceum* (21 samples), *F. culmorum* (7 samples) and *F. graminearum* (22 samples). Figure 23 and Figure 24 present the frequency of *Fusarium* species detected in intervals of toxin concentrations for DON and T-2 toxin respectively. *F. poae*, *F. langsethiae* and *F. sporotrichioides* were not detected in any of the samples of threshed and dried material.

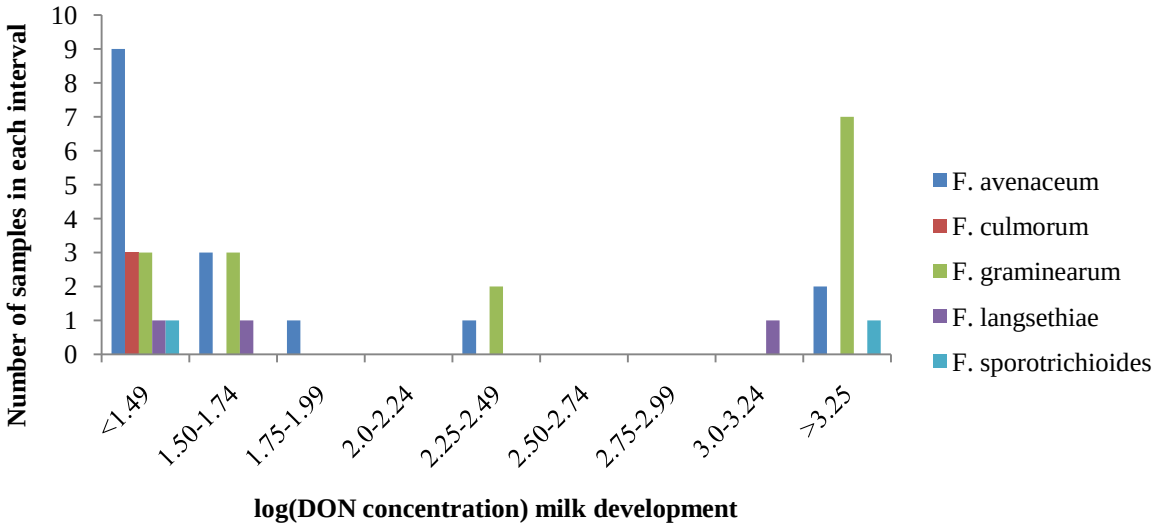


Figure 21. Number of samples in each interval of concentration of DON (all values as logarithms) at milk development stage. Each *Fusarium* spp. Is represented with one colour; *F. avenaceum* – blue, *F. culmorum* – red, *F. graminearum* – green, *F. langsethiae* – purple, *F. sporotrichioides* – turquoise. Original data presented in µg kg^{-1}.

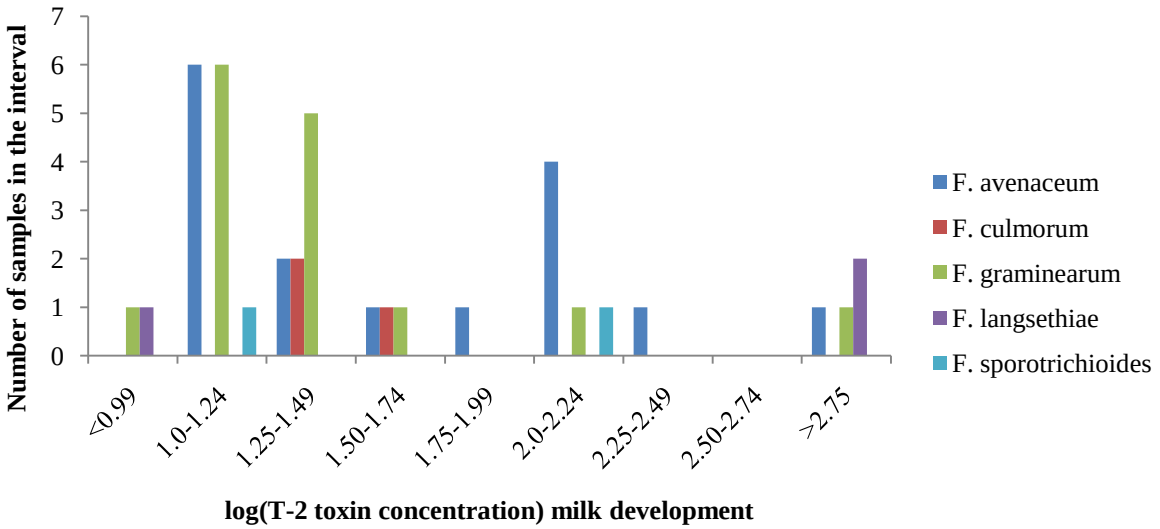


Figure 22. Number of samples in each interval of concentration of T-2 toxin (all values as logarithms) at milk development stage. Each *Fusarium* spp. is represented with one colour; *F. avenaceum* – blue, *F. culmorum* – red, *F. graminearum* – green, *F. langsethiae* – purple, *F. sporotrichioides* – turquoise. Original data presented in µg kg^{-1}.

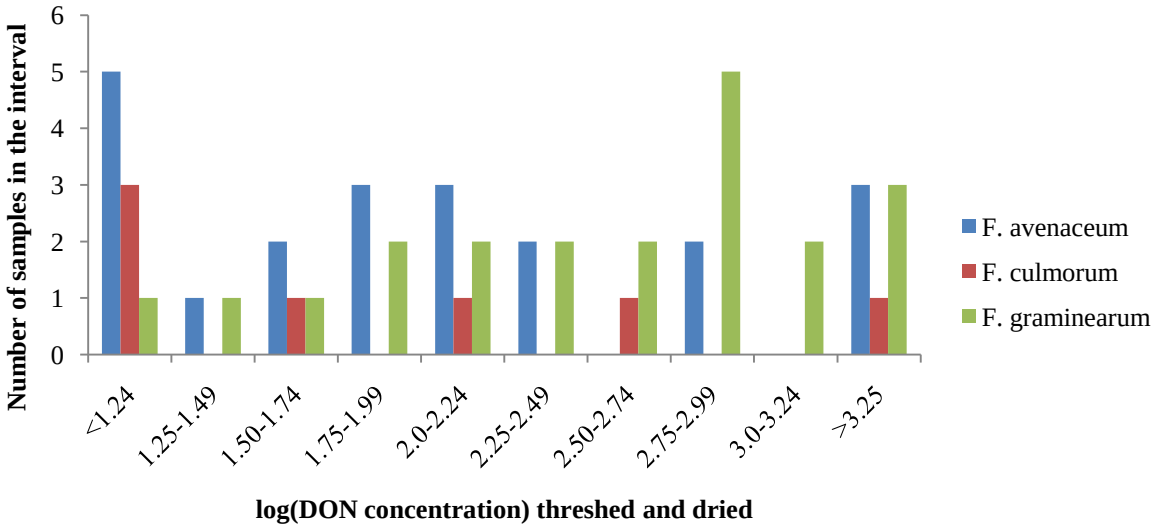


Figure 23. Number of samples in each interval of concentration of DON (all values as logarithms) in the threshed and dried material. Each *Fusarium* spp. is represented with one colour; *F. avenaceum* – blue, *F. culmorum* – red and *F. graminearum* – green. Original data presented in µg kg^{-1}.

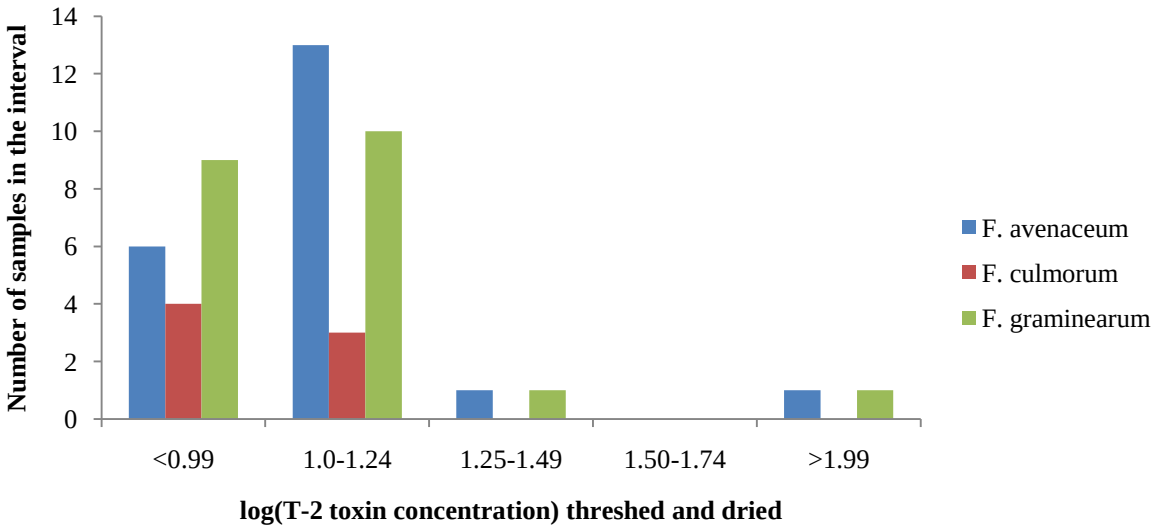


Figure 24. Number of samples in each interval of concentration of T-2 toxin (all values as logarithms) in the threshed and dried material. Each *Fusarium* spp. is represented with one colour; *F. avenaceum* – blue, *F. culmorum* – red and *F. graminearum* – green. Original data presented in µg kg^{-1}.

5 Discussion

This project aimed to investigate different methods of forecasting mycotoxin contamination using scorings of visible symptoms of Fusarium head blight, analysis of ergosterol and analysis of toxin concentrations at different stages of the crop development.

The results show that it is only DON contamination of winter wheat that shows a strong positive correlation between the toxin concentrations at milk development stage (DC69-77) and for the threshed and dried material. There is however a trend of increased DON concentrations over the crop development for all crops included in the study. For T-2 toxin no such trend is observed which, however, might be associated with generally low concentrations.

No correlations between toxin contamination and ergosterol content could be shown from the results. It is however observed that samples with high toxin content have high ergosterol concentrations but a high concentration of ergosterol does not necessarily indicate high concentrations of toxins.

To have a better infection frequency in the trials and thereby more data to process it would be good to replicate some parts of this project with trials in locations more favourable for *Fusarium* infection. This might generate more obvious visible symptoms and thereby a larger sample size to analyse. It is however important to have a wide range of data, from samples with light infections to samples with severe infections.

5.1 Visible symptoms

According to the first hypothesis severe visible symptoms should increase the risk of mycotoxin contamination in the crop. The result shows that positive correlations between scorings of visible symptoms of FHB and toxin concentrations only were found for spring wheat and DON. It is hard to draw any general conclusions about the linkage between visible symptoms and toxin contamination since only one of the locations included in this study had severe visible symptoms. Thereby the hypothesis can neither be confirmed nor discarded.

Nevertheless, spring wheat in Laberweinting shows, not significant but, high positive correlation between symptom scorings and DON concentration both at milk development stage (r=0.71) and in the threshed and dried material (r=0.62). This relationship is confirmed by earlier research *e.g.* Gärnert *et al.* (2007), Jones and Mirocha (1999), Chelkowski (1998) and Snijders and Perkowski (1990) and is illustrated in Figure 4. However, the figure shows results from inoculated plants and the circumstances are thereby different. Inoculated trials often give extremely high infection rates and can be hard to compare with trials with natural infection. For the trials included in this study it also has to be considered that the sample size for spring wheat was small (n=4), which may have influence on the result both in a positive and in a negative way. The scores of the samples are evenly spread which gives one deviating value a large influence on the conclusion.

In winter wheat no clear relationship was found between scoring of visible symptoms and DON or T-2 toxin contamination either at milk development stage or in the threshed and dried materials. This result is in line with the result of Fauzi and Paulitz (1994) who showed that scorings and mycotoxin contamination not always are correlated.

For T-2 toxin there are no correlations with visible symptoms neither for winter wheat nor for spring wheat. This might be explained by the species causing the infection since T-2 toxin here seems to be produced by *e.g. F. langsethiae* (see further discussion in chapter 5.4) which is a slow growing species with scarce mycelium (Kristensen *et al.*, 2005). This might affect the occurrence of visible symptoms.

When studying results of the scorings it should, however, be considered that the scale is ordinal and the relationship between toxin concentration and scoring should therefore not be expected to be linear. This makes the charts hard to interpret since it is not known what the optimal case would look like.

To have higher possibilities of infection it would be preferable that the location and the setup of the trials would be more favourable for *Fusarium*. The trial in Laberweinting, which had the most severe infection was probably favoured both by the climate (Klem *et al.*, 2007) with a higher precipitation in the beginning of the season compared to the weather in Harzhof which had similar relative humidity and temperature (Appendix III). The fungi might also be favoured by the cropping system and in particular the preceding crop, maize (Khonga and Sutton, 1988).

5.2 Toxin concentration and development stage of the crop

This study shows that it is only DON contamination of winter wheat that shows a strong positive correlation (r=0.75***) between the toxin concentrations at milk development stage (DC69-77) and for the threshed and dried material. It is also shown that spring wheat in Bjertorp has a significant increase of DON concentration during the crop development. A similar trend of increased DON concentrations during the crop development is shown for both winter wheat and oats but could not be confirmed statistically. It is more difficult to find similar relationships between crop development and T-2 toxin which might depend on the general low concentrations and since few samples reached concentrations above 20 µg kg^{-1} it is hard to detect differences.

When studying the development of toxin concentrations in the crops it was noticed that the DON concentration increased over time and crop development. This is in line with the second hypothesis and also with the results of Miller and Young (1984) who stated that the levels of DON increase during six weeks after inoculation. It is, however, hard to draw any conclusions about the time of inoculation for the trials included in this project since all infections were natural infections.

A reason for increased concentrations of DON in the samples from Bjertorp might be the weather. The samples from milk development stage were picked at the end of a dry period with low relative humidity. Immediately after this a period with rainy weather and high relative humidity occurred (Appendix III). This weather has probably favoured the pathogen and thereby perhaps also the production of toxins (Klem *et al.*, 2007). Especially fungi producing DON, *e.g. F. culmorum* and *F. graminearum* are favoured by humid weather (Hörberg, 2001b) which might explain the increased DON concentrations.

The study shows a significant increase ($p<0.01$) of DON concentration during crop development if the material is analysed independent of crop. However, it should be considered that these results originate from few samples and a lot of samples showed no increase of concentrations between the different development stages.

The concentrations of T-2 toxin did not seem to change over time which is explained by generally low concentrations and low variation in concentrations. Samples with T-2 toxin in the range of 7 - 865 µg kg^{-1} at milk development stage and 2 - 57 µg kg^{-1} in the threshed and dried material were represented which should be compared to the variation of DON concentrations which covered a larger range (15 - 15208 µg kg^{-1} at milk development stage and 14 - 6407 µg kg^{-1} in the threshed and dried material). This homogeneity in the samples makes it impossible to draw any general conclusions.

When comparing the different varieties no obvious trends can be observed and the toxin concentrations were often similar. At milk development stage SW Kerstin had slightly higher concentrations of both DON and T-2 toxin, while Belinda seemed to contain more toxins in the threshed and dried material compared to the other varieties. For spring wheat SW 45544 and Triso had the highest concentrations of T-2 toxin but for DON no trends could be observed. For winter wheat the results are equivocal and no variety with extreme toxin concentrations could be distinguished.

According to Börjesson (2004) most of the toxin content is found in the outer parts of the kernel and in the rachis. Based on this conclusion the toxin concentration would be higher in the samples from milk development stage where the entire ear was used for analysis compared to only the kernels for the samples from the threshed material. It is however hard to determine how these facts might have affected the results of this study since the concentrations of DON at milk development stage are the lowest in the comparison and for T-2 toxin the concentrations did not change.

The third hypothesis, which states that the toxin concentrations would not increase during drying of the grains, is in line with the findings of Hocking (2010) who showed that the toxin concentrations after harvest were constant. Based on results from this present study and in contrast to the result from Hocking (2010) this hypothesis is discarded since most of the samples had higher levels of toxins after drying compared to not dried material. However the sample size is small and the hypothesis cannot for sure be discarded.

Another difficulty might be when comparing the material from the different stages with each other. Since the procedure of drying is a bit different between the different times of sampling and the water content before drying is very different between the ripe material and the material sampled at milk development stage a comparison might be unfair. In the samples picked at milk development stage a larger percentage of the weight is removed during drying which influences the results since the values are measured per weight (μg kg^{-1}).

The likelihood of detecting DON and T-2 toxin in all samples could be discussed, but according to Davidsson (Pers. comm., 2010) it is probably difficult to find entirely clean material and thereby it should be possible to have this high frequency of toxin contaminated samples. It is however possible that some of the samples with low toxin concentrations not actually are detection of toxins but rather the result of a cross reaction, however the methodology is very well suited for the screening purpose in this project. Often the ELISA also generated higher values than *e.g.* MS or HPLC depending on the matrix used. The certainty in the method used in this study is not investigated but similar methods have shown uncertainty as high as 20% why it is reasonable to suspect some unsecure values.

5.3 Toxin concentrations and ergosterol

Confirming the last hypothesis, there is no strong positive correlation between the concentration of DON or T-2 toxin and ergosterol. Even if the ergosterol concentration is high the toxin concentration might be low. These results are confirmed by Miller and Young (1985) who showed that samples with ergosterol did not have any detectable levels of DON. However, the results in this project show that a high concentration of toxins always is related to high concentrations of ergosterol. This makes it hard to use ergosterol concentration for prediction of concentration of DON and T-2 toxin.

One could suspect that the samples from Sweden would show stronger correlations to the ergosterol concentrations since the calibration of the method only is made for samples from Sweden. However, Table 10 shows no large differences in these relationships between the Swedish samples and the German samples which eliminates this factor as an explanation of low correlation coefficients.

If ergosterol thresholds similar to the ones used by Lantmännen are used there is a risk that quantities with high toxin concentrations are accepted since the ergosterol concentrations are lower than the limits (Table 11). If the threshold of ergosterol concentration is set to <16 mg kg^{-1} there is a 6.3%* risk to include quantities with DON levels above 1250 μg kg^{-1}. As illustrated in Table 12 and Figure 20 this risk is reduced to 2.5% if the threshold of ergosterol concentration are set to <16.5 mg kg^{-1}. The results shows that the threshold for ergosterol concentration has to be set to <15 mg kg^{-1} if all the samples (five samples) with higher toxin concentrations than the by the European commission set limit should be detected. A threshold of ergosterol at <15 mg kg^{-1} would, on the other hand, mean that 18 samples (20%) with ergosterol concentrations higher than 15 mg kg^{-1} but DON concentrations lower than the limit at 1250 μg kg^{-1} would be discarded.

If the threshold of ergosterol is higher the industries, using the primary products, are exposed to a risk of quantities with high toxin concentrations contaminating the rest of the batch and if the lower threshold is used the provider may be afflicted since there is a risk of wrong classification of the quality of the batch. The lower threshold would increase the demands for screening and the selection of toxins included in the screening, which may vary depending on year and district (see chapter 5.4).

Since no similar limit for T-2 toxin concentration is set there could be no comparable assessment. However, T-2 toxin is considered to be more toxic that DON (Placinta *et al*., 1999) and the National Food Administration in Sweden have set a limit of the tolerable daily intake to 0.2 µg kg bodyweight^{-1}. The corresponding value for DON is five times higher, 1 µg kg bodyweight^{-1} (Fredlund *et al.,* 2009).

Considering the recommendations of daily intake the set limit for T-2 toxin in small grains as primary products hypothetically could be set to 250 µg kg^{-1}, five times lower than the same corresponding limit for DON. None of the threshed and dried samples reached 250 µg kg^{-1} T-2 toxin but two samples from milk development stage transcended the value. Unfortunately no ergosterol analysis could be made on these samples but for further projects it would be very interesting to investigate how the ergosterol concentration behaves with high concentrations of T-2 toxin.

5.4 Fungal identification and toxin concentration

The study showed contamination at milk development stage of five out of six tested species of *Fusarium*; *F. avenaceum, F. graminearum, F. culmorum, F. langsethiae* and *F. sporotrichioides*. *F. poae* was not detected in any of the samples. When the results from the identification are studied it is revealed that all of the identified fungi are present in samples contaminated both with DON and T-2 toxin. This is somehow confusing since the only producers of DON of the investigated species are *F. graminearum* and *F. culmorum* and for T-2 toxin *F. langsethiae* and *F. sporotrichioides* are possible producers (Hörberg, 2001b; Kosiak *et al.*, 2003; Kristensen *et al.*, 2005). *F. avenaceum* should, according to the literature, not produce DON neither T-2 toxin (Uhlig *et al.*, 2007).

Some of these cases where the identified *Fusarium* species should not be able to produce the specific toxin can be explained by other species detected in the same sample. The toxin production could in these cases be derived to the latter. However in most cases there is no such explanation and the reason for toxin production has to be found in other species than those investigated or the expected ones. For instance *F. equiseti* and *F. acuminatum* both produces T-2 toxin, and none of these fungi were included in the PCR analysis. Both of these have been found in environments with similar conditions as the locations included in this study (El Khosht, 2010). The same author also found the *TRI5* gene in *F. avenaceum* which, if confirmed by other studies, suggest *F. avenaceum* as a possible producer of trichothecenes and among these DON and T-2 toxin. If this statement is true more of the toxin concentrations not associated to the expected fungal species could be explained.

The same lane of thought as above could be used for the threshed and dried material where only *F. avenaceum, F. graminearum* and *F. culmorum* were detected. Here the DON production can be explained by the presence of *F. graminearum* and *F. culmorum*, but the T2- toxin production has no confirmed explanation.

The results from milk development stage show no difference in toxin production between *F. avenaceum, F. culmorum, F. graminearum* and *F. sporotrichioides*. For *F. langsethiae* a difference can be seen when comparing DON concentrations at milk development stage with T-2 toxin concentrations, where the T-2 toxin concentrations are higher than the DON concentrations. This is in line with the reports of Placinta *et al*. (1999), D'Mello and Macdonald (1998) and Hörberg (2001b) who stated that production of T-2 toxin is associated with *F. langsethiae, F. sporotrichioides* and *F. poae.* Since no *F. poae* was found in the material used in this study it is reasonable to suspect that the T-2 toxin detected might be produced by *F. langsethiae* and *F. sporotrichioides.* It is however possible that some of the T-2 toxin has

been decomposed to HT-2 toxin (Hörberg, 2001a) and thereby not detected with the screening method for toxins used in this project.

The results show that the toxin production to a large extent depends on the location and the crop (Table 13, Table 14). In Bjertorp no infections of *F. langsethiae* or *F. sporotrichioides* were detected, neither were any infections of these fungi detected in oats or spring wheat in any of the locations. When comparing the toxin concentrations for these crops at milk development stage it is observed that the samples of winter wheat (containing *F. langsethiae* or *F. sporotrichioides*) has higher mean T-2 toxin concentrations compared to oats and spring wheat. This result is confirmed by *e.g.* Kristensen *et al.*, (2005) who stated that T-2 toxin is produced by *e.g. F. langsethiae* or *F. sporotrichioides*. In the threshed and dried material it is possible that the T-2 toxin has been decomposed to HT-2 toxin why the mean concentrations are more similar between the crops. If studying the same result divided per location a similar pattern might be observed. For Svalöv and Harzhof where *F. langsethiae* and/or *F. sporotrichioides* were detected the mean T-2 toxin concentrations were higher compared to Bjertorp and Laberweinting. In Bjertorp no *F. langsethiae* or *F. sporotrichioides* were detected but in Laberweinting both species were present which makes the result less univocal. No similar pattern is noticed for DON and DON producing fungi (*F. graminearum* and *F. culmorum*).

When studying the results from the PCR analysis some samples containing a particular species at milk development stage did not show any positive results for the sample of threshed and dried material. These circumstances can be described by the collection of samples where ears picked and analysed at milk development stage impossibly could be sampled at the time of threshing. This means that, even if the material is picked from the same plot, different ears are analysed at each time of sampling. In the cases where the severity of the infection was very light there is a large possibility that there are ears not infected, or at least not containing detectable amounts of the fungi. Because of this there is also a chance that ears with infection was picked at one occasion of sampling and ears without infection were picked at the other time of sampling.

The possible margin of error for the toxin analysis (see chapter 5.2) may also have influence on the results. If the values with the lowest toxin concentrations were ignored (considered as zero values), then some of the samples with no possible fungus for toxin production present would be eliminated.

6 Conclusions

As shown in this study it is important to choose the method for prediction or analysis of *Fusarium* and mycotoxins depending on the objective of the analysis. The choice of tool should be different depending on if the analysis should give a fast or exact result, if the main objective is to predict later toxin concentrations or if the results are used to study biological systems.

One suggested way to predict the risk of mycotoxin contamination is to make scorings of visible symptoms in field. Based on this field study no conclusion about this method can be made but earlier research reviewed in this report indicates that a scoring might be possible to use to predict the toxin contamination.

This study reveals a possibility to use values of DON concentrations from different times during the crop development to have an idea of the toxin concentration in the threshed and dried material, this because the concentrations continuously increase. Since the variation of T-2 toxin concentrations of the samples in this study were much smaller, no such relation could be seen for T-2 toxin.

Ergosterol analysis might hint about bulks with high risk of mycotoxin contamination but a high ergosterol concentration does not necessarily indicate high toxin concentrations. If ergosterol content is used as a criterion to get rid of toxin contaminated material the threshold of ergosterol concentration has to be legitimate. A high threshold allows samples with high toxin concentrations to pass the selection while a too low threshold increases the risk of elimination of material with low toxin concentrations. This is a matter of cost-benefit reasoning where different participants in the production chain probably will have different opinions.

As shown in this study toxin production caused by *Fusarium* infection is associated both with crop and with location. This makes it important to adapt the identification and quantification methods to different conditions. For example, it does not seem to be necessary to screen for *F. sporotrichioides* and *F. langsethiae* in oats and spring wheat neither in samples that origin form the area around Bjertorp. This result may however differ depending on different conditions between different years (Djurle *et al.*, 2010).

As a subject for further research it would be interesting to complete this project with a similar study where the toxin concentrations are correlated to the results of quantitative PCR for the fungi. These results would give a hint about if the amount of fungi in small grains affect the production of mycotoxins. Quantitative PCR analysis has earlier been made by *e.g.* Fredlund *et al*. (2008) and by Doohan, Parry and Nicholson (1999) as a greenhouse experiment but would be interesting to make as a field study with natural infection including the parameters used in this study: ergosterol analysis, toxin analysis and scorings of visible symptoms. It would also be interesting with further analysis of the material included in this study to investigate if *F. avenaceum* might play a role in the production of trichothecenes and what is causing the toxin production in the samples without any fungi detected.

The question of *Fusarium* and mycotoxin produced by *Fusarium* species is an important issue. An effective tool to prevent toxin contamination in food and feed would help both the farmer responsible for the small grains delivered and the industry responsible for the product bought by the consumer.

7 References

Abramson, D., Clear, R.M., Smith, D.M., 1993. Trichotecene production by *Fusarium* spp. Isolated from Manitoba grain, *Canadian plant pathology 15*: 147-152.

Agrarmeterologi, 2010. Bayerische Landesanstalt für Landwirtschaft, http://www.lfl.bayern.de/agm/, 2010-12-02.

Agrios, G.N., 2005. *Plant pathology*, Fifth edition, Elsevier Academic press.

Bechtel, D.B., Kaleikau, L.A., Gaines, R.L., Seitz, L.M., 1985. The effects of *Fusarium graminearum* infection on wheat kernels, *Cereal Chemistry 62*: 191-197.

Beuch, U., 2009. Lab practical, Viral diseases, Course BI0990, Plant pathology and entomology, Swedish University of Agricultural Sciences.

Bockus, W.W., Bowden, R.L., Hunger, R.M., Morrill, W.L., Murray, T.D., Smiley, R.W., 2010. *Compendium of wheat diseases and pests*, Third edition, American Phytopathological Society, Minnesota, USA.

Bottalico, A., Perrone, G., 2002. Toxigenic *Fusarium* species and mycotoxins associated with head blight in small-grain cereals in Europe, *European journal of Plant Pathology 108*: 611-624.

Brown, N.A., Urban, M., van de Meene, A., Hammond-Kosack, M.L., Kin E., 2010. The infection biology of *Fusarium graminearum*: Defining the pathways of spikelet to spikelet colonisation in wheat ears, *Fungal Biology 114*: 555-571.

Bushnell, W.R., Hazen, B.E., Pritsch, C., 2003. Histology and physiology of Fusarium head blight, In: Leonard, K.J., Bushnell, W.R. (Eds). *Fusarium head blight of wheat and barley*, The American phytopathological society, St. Paul, Minnesota.

Börjesson, T., 2004. Mykotoxiner i stråsädeskärna – Förekomst i Sverige, Meddelande från Södra jordbruksförsöksdistriktet 57.

Börjesson, T., Stenberg, B., Schnürer, J., 2007. Near-infrared spectroscopy for estimation of ergosterol content in barley: A comparison between reflectance and transmittance techniques, *Cereal chemistry 83*: 231-236.

Börjesson, T., Gruvaeus, I., 2009. Ergosterolhalter – indirekt bestämning av svampförekomst. Kan odlingsåtgärder påverka?, Växteko, Meddelande från Södra jordbruksförsöksdistriktet, nr 62, SLU, Södra jordbruksförsöksdistriktet.

Börjesson, T., 2010. Utredning av skillnader i svampflora i vete och kornprover med olika ergosterolinnehåll predikterat med NIT, Final report H0760013.

Chelkowski, 1998. Distribution of *Fusarium* species and their mycotoxins in cereal grains, *Mycotoxins in agriculture and food safety.* Sinha, Kaushal K. and Bhatnagar (Eds.), Deepak, 1998.

D'Mello, J.P.F., Macdonald, A.M.C., 1998. Fungal toxins as disease elicitors, In: Rose, J. (Ed.), Environmental Toxicology: Current Developments. Gordon and Breach, Amsterdam, pp. 253-289.

Dejardins, A.E., Spemcer, G.F., Plattner, R.D., Beremand, M.N., 1989. Furanocumarin phytoalexis, trichotecene toxins, and infection of *Pastinaca sativa* by *Fusarium sporotrichioides*, *Phytopathology 79*: 170-175.

Djurle, A., Grudzinska-Sterno, M., Rasmussen, P.H., Yuen, J., 2010. Incidence of *Fusarium* species and mycotoxins in Swedish winter wheat fields, *Bioforsk Focus 5*: 7.

Del Ponte, E.M., Fernandes, J.M.C., Bergstrom, G.C., 2007. Influence of growth stage on Fusarium head blight and deoxynivalenol production in wheat, *Journal of Phytopathology 155*: 577-581.

De Wolf, D.E., Madden, L.V., Lipps, P.E., 2003. Risk assessment models for wheat Fusarium head blight epidemics based on within-season weather data, *Phytopathology 93*: 428-435.

Doohan, F.M., Parry, D.W., Jenkinson, P., Nicholson, P., 1998. The use of specific PCR-based assays to analyse Fusarium ear blight of wheat, *Plant pathology 47*: 197-205.

Doohan, F.M., Parry, D.W., Nicholson, P., 1999. Fusarium ear blight of wheat: the use of quantitative PCR and visual disease assessment in studies of disease control, *Plant pathology 48*: 209-217.

Dowell, F.E., Ram, M.S., Seitz, L.M., 1999. Predicting scab, vomitoxin, and ergosterol in single wheat kernels using near-infrared spectroscopy, *Cereal Chemistry 76*: 573-576.

El Khosht, F., 2010. Species diversity and geographical distribution of *Fusarium* species on winter wheat in two regions in Sweden, Undergraduate thesis, Dept. of Forest Mycology and Pathology, Swedish University of Agricultural Sciences.

Engel, J.S., Lipps, P.E., Mills, D., 2003. Fusarium head blight severity scale for winter wheat, The Ohio State University Extension, Extension fact sheet 49-03.

European Commission, 2010a. Kommisionens rekommendation (2006/576/EG) om förekomst av deoxynivalenol, zearalenon, ochratoxin A, T-2 och HT-2 och fumonisiner i produkter avsedda för foder, http://eur-lex.europa.eu/Notice.do?mode=dbl&lang=lv&ihmlang=lv&lng1=lv,sv&lng2=cs,da,de,el,en,es,et,fi,fr,hu,it,lt,lv,nl,pl,pt,sk,sl,sv,&val=431952:cs&page=, available: 2010-09-23.

European Commission, 2010b. Kommisionens förordning (EG) nr 1881/2006 om fastställande av gränsvärden för vissa främmande ämnen i livsmedel, http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=CONSLEG:2006R1881:20100701:SV:PDF, available: 2010-09-23.

Fauzi, M.T., Paulitz, T.C., 1994. The effect of plant growth regulators and nitrogen fertilization on Fusarium head blight of the spring wheat cultivar Max, *Plant Disease 78*: 289-292

Fernando, W.G.D., Paulitz, T.C., Seaman, W.L., Dutilleul, P., Miller, J.D., 1997. Head blight gradients caused by *Giberella zeae* from area sources of inoculums in wheat field plots, *Phytopathology 87*: 414-421.

Fredlund, E., Abrahamsson Zetterberg, L., Thim, A.M., Olsen, M., 2009. Riskprofil Mögel och mykotoxiner i livsmedel, Rapport 4.

Fredlund, E., Gidlund, A., Olsen, M., Börjesson, T., Hytte Spliid, N.H., Simonsson, M., 2008. Method evaluation of *Fusarium* DNA extraction from mycelia and wheat for downstream real-time PCR quantification and correlation to mycotoxin levels, *Journal of Microbiological methods 73*: 33-40.

Garden, M., Burns, T.D., 1993. ITS primers with enhance specificity for basidiomycetes – application to the identification of mycorrhizae and rusts, *Molecular Ecology 2*: 113-118.

Gärnert, B.H., Munich, M., Kleijer, G., Mascher, F., 2007. Characterization of kernel resistance against *Fusarium* infection in spring wheat by baking quality and mycotoxin assessments, *European Journal of Plant Pathology 120*: 61-68.

Halstensen, A.S., Norby, K-Ch., Sletner Klemsdal, S., Elen, O., Clasen, P-E., Eduard W., 2006. Toxigenic *Fusarium* spp. as determinants of thrichothecene mycotoxins in settled grain dust, *Journal of occupational and environment hygiene 3*: 651-659.

Hocking, A.D., 2010. Common mycotoxigenic species of *Fusarium*, FAO Corporate document repository, http://www.fao.org/docrep/x5036e/x5036E07.HTM, available 2010-07-05.

Hooker, D.C., Schafsma, A.W., Tamburic-Ilincic, L., 2002. Using weather variables pre- and post-heading to predict deoxynivalenol content in winter wheat, *Plant disease 86*: 611-619.

Hussain, H.M., Baxter, M., Andrew, I.G., Franich, R.A., 1991. Mycotoxin production by *Fusarium* species isolated from New Zealand maize fields, *Mycopathologia 113*: 506-511.

Hörberg, H., 2001a. *Fusarium*-svampar i stråsäd, SLU, Inst. för ekologi och växtproduktionslära, Faktablad om växtskydd, 103J.

Hörberg, H., 2001b. Dispersal of mycotoxin producing *Fusarium* spp., Licenciate thesis, Dept. of Ecology and Crop production Science, Swedish University of Agricultural Sciences, Uppsala.

Ilgen, P., Hadeler,B., Maier, F.J., Schäfer, W., 2009. Developing kernel and rachis node induce the trichothecenes pathway of *Fusarium graminearum* during wheat head infection, *Molecular plant-microbe interactions 22*: 8: 899-908.

Jones, R.K., Mirocha, C.J., 1999. Quality parameters in small grains from Minnesota affected by Fusarium head blight, *Plant disease 83*: 506-511.

Kang, Z., Buchenauerm, H., 1999. Immunocytochemical localization of *Fusarium* toxins in infected wheat spikes by Fusarium culmorum, *Physiological and molecular Plant Pathology 55*: 275-288.

Khonga, E.B., Sutton, J.C., 1988. Inoculum production and survival of *Gibberella zeae* in maize and wheat residues, *Canadian Journal of Plant Pathology 10*: 232–239.

Klem, K., Váňová, M., Hajšlová, J., Lancová, K., Sehnalová, M., 2007. A neural network model for prediction of deoxynivalenol content in wheat grain based on weather data and preceding crop, *Plant soil environment 53*: 421-429.

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, *Plant and Soil science 53*: 168-176.

Kristiensen, 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.

Lamper, C., Téren, J., Bartók, T., Komoróczy, R., Mesterházy, Á., Sági, F., 2000. Predicting DON contamination in *Fusarium*-infected wheat grains via determination of the ergosterol content, *Cereal research communications 28*: 3: 337-344.

LANTMÄT, 2010. FältForsk, SLU, http://www.ffe.slu.se/lm/stationsdata.cfm, available 2010-11-23.

Lattanzio, V.M.T., Pascale, M., Visconti, A., 2009. Curent analytical methods for trichothecene mycotoxins in cereals, Trends in Analytical Chemistry 28: 6: 258-768.

Leslie, F.J., Summerell, B.A., 2006. *The fusarium laboratory manual*, First edition, Blackwell publishing, Oxford.

Maier, F.J., Miedaner, T., Hadeler, B., Felk, A., Salomon, S., Lemmens, M., Kassner, H., Schäfer, W., 2006. Involvement of trichothecenes in fusarioses of wheat, barley and maize evaluated by gene disruption of the trichodiene synthase (Tri5) gene in three field isolates of different chemotype and virulence, *Molecular plant pathology 7*: 449-461.

McMullen, M., Zhong, S., Neate, S., 2008. Fusarium Head Blight (Scab) of small grains, Plant disease management, NSDU Extension Service, North Dakota State University, Fargo, North Dakota.

Miller, J.D., Young, J.C., 1985. Deoxynivalenol in an experimental *Fusarium graminearum* infection of wheat, *Canadian Journal of Plant Pathology 7*: 132-134.

Mirocha, C.J., Xie, W., Filho, E.R., 2003. Chemistry and detection of *Fusarium* mycotoxins, In: Leonard, K.J., Bushnell, W.R. (Eds). *Fusarium head blight of wheat and barley*, The American phytopathological society, St. Paul, Minnesota.

Musa, T., Hecker, A., Vogelgsang, S., Forrer, H.R., 2007. Forecasting of Fusarium head blight and deoxynivalenol content in winter wheat with FusaProg, *Bulletin 37*: 283-289, Agroscope Reckenholz-Tänikon.

Nicholson, D.R., Simpson, G., Weston, H.N., Rezanoor, A.K., Lees, D.W., Parry, D., 1998. Detection and quantification of *Fusarium culmorum* and *Fusarium graminearum* in cereals using PCR assays, *Physiological and molecular plant pathology 53*: 17-37.

Parikka, P., Rämö, S., Hiettaniemi, V., 2008. *Fusarium* species and mycotoxins in Finnish cereals and development of infection under different cultivation practices, Presentation 2008-03-12, Agrifood research, Finland.

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

Parry, D.W. and Nicholson P., 1996. Development of a PCR assay to detect *Fusarium poae* in wheat, *Plant pathology 45*: 383-391.

Paul, P.A., Lipps, P.E., Madden, L.V., 2005. Relationship between visual estimates of Fusarium head blight intensity and deoxynivalenol accumulation in harvested wheat grain: A meta-analysis, *Phytopatology 95*: 1225-1236.

Perkowski, J., 1997. Distribution of deoxynivalenol in barley kernels infected by *Fusarium*, *Nahrung 42*: 81-83.

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

Ridascreen, 2007. *Ridascreen® DON*, Instructions for enzyme immunoassay for the quantitive analysis of deoxynivalenol, R-Biopharm, Darmstadt, Germany.

Ridascreen, 2009. *Ridascreen® T-2 toxin*, Instructions for enzyme immunoassay for the quantitive analysis of T-2 toxin, R-Biopharm, Darmstadt, Germany.

SAS, 2010. Version 9.1, SAS Institute Inc., Cary, NC, USA.

Savard, M.E., Sinha, R.C., Seaman, W.L., Fedak, G., 2000. Sequential distribution of the mycotoxin Deoxynivalenol in wheat spikes after inoculation with *Fusarium graminearum*, *Canadian Journal of Plant Pathology 22*: 280-285.

Schroeder, H. W., Christensen, J. J., 1963. Factors affecting resistance of wheat to scab caused by *Gibberella zeae*, *Phytopathology, 53*: 831–838.

Schwarz, P.B., 2003. Impact of Fusarium head blight on malting and brewing quality of barley, In: Leonard, K.J., Bushnell, W.R. (Eds). *Fusarium head blight of wheat and barley*, The American phytopathological society, St. Paul, Minnesota.

Shaafsma, A., Hooker, D.C., Miller, J.D., 2005. Progress and limitations with respect to preharvest forecasting *Fusarium* toxins in grains, *Phytopathology 95*: S123.

Shaner, G., 2003. Epidemiology of Fusarium head blight of small grain cereals in North America, In: Leonard, K.J., Bushnell, W.R. (Eds). *Fusarium head blight of wheat and barley*, The American phytopathological society, St. Paul, Minnesota.

Snijders, C.H.A., Perkowski, J., 1990. Effects of head blight caused by *Fusarium culmorum* on toxin content and weight of wheat kernels, *Phytopathology 80*: 566-570.

Sutton, J.C., 1982. Epidemiology of wheat head blight and maize ear rot caused by *Fusarium graminearum*, *Canadian Journal of Plant Pathology 4*: 195–209.

Uhlig, S., Jestoi, M., Parikka, P., 2007. *Fusarium avenaceum* – the North European situation, *International Journal of Food Microbiology 119*: 17-24.

Yuen, J., Mila, A., 2006. Are Bayesian approaches useful in plant pathology?, Wageningen UR frontis series, Volume 3, Bayesian Statistics and quality modelling in the agro-food production chain.

Zadoks, J.C., Chang, T.T., Konzak, C.F., 1974. A decimal code for the growth stages of cereals, *Weed research 14*: 415-421.

7.1 Personal communication

Svensson, 2010. Rita Svensson, Laboratory technician, Lantmännen SW seed, 2010-10-15.

Davidsson, 2010. Richard Davidsson, Group Manager Analysis Lab, Lantmännen SW seed, 2010-12-08.

Appendix I

	Winter wheat					Spring wheat					Oats		
	Svalöv		**Laberweinting**	**Harzhof**	**Bjertorp**	**Svalöv**		**Laberweinting**	**Harzhof**	**Bjertorp**	**Svalöv**	**Laberweinting**	**Bjertorp**
	Trial A	Trial B				Trial A	Trial B						
Soil type	15-25% clay	15-25% clay	50-70% sand, 15-20% clay	70-85% sand, 10-15% clay	15-25% clay	15-25% clay	15-25% clay	50-70% sand, 15-20% clay	70-85% sand, 10-15% clay	25-40% clay	15-25% clay	50-70% sand, 15-20% clay	25-40% clay
Soil preparation	Ploughing and harrowing	Ploughing and harrowing	Cultivation and harrowing	1 deep cultivation, 1 shallow cultivation, 1 shallow cultivation in front of sowing machine	1 cultivation and 2-3 times harrowing	Ploughing and harrowing three times	Ploughing and harrowing three times	1 cultivation, 1 harrowing	1 deep cultivation, 1 shallow cultivation, 1 shallow cultivation before sowing machine	Ploughing in autumn and 2-3 times harrowing	Ploughing and harrowing three times	1 cultivation, 1 harrowing	Ploughing in autumn and 2-3 times harrowing
Preceding crop	Oats	Oats	Maize	Winter oilseed rape	Winter oilseed rape	Oil radish	Oil radish	Maize	Winter oilseed rape	Spring oilseed rape	Broad bean	Maize	Spring oilseed rape
Sowing date	2009-09-26	2009-07-28	2009-10-31	2009-10-14	2009-09-27	2010-04-25	2010-04-25	2010-03-23	2010-04-09	2010-04-16	2010-05-22	2010-03-27	2010-04-16
Time of flowering	2010-07-01	2010-07-01	2010-06-20	2010-06-26	2010-07-03	2010-07-14	2010-07-14	2010-06-25	2010-07-07	2010-07-12	2010-07-14	2010-06-25	2010-07-12
Fertilisation	90+60 kg N	90 kg N	54+46 kg N	36+25+70+45 kg N	100+60 kg N	50+90 kg N	60 kg N	54+46 kg N	36+25+46 kg N	66+40+22 kg N	60+45 kg N	54+46 kg N	66+40 kg N
Pesticide application	Herbicides, Insecticides, Fungicides	Herbicides, Insecticides	Herbicides, Insecticide	Herbicides, Growth regulator, Insecticides	Herbicides, Fungicides	Herbicides, Insecticides, Fungicides	Herbicides, Insecticides	Herbicides, Insecticide	Herbicides, Growth regulator, Insecticides	Herbicides, Fungicides	Herbicides, Insecticides, Fungicides	Herbicides, Insecticide	Herbicides
Time of 1st sampling (~DC 70-80)	2010-07-15	2010-07-15	2010-07-12	2010-07-13	2010-07-20	2010-07-21	2010-07-21	2010-07-12	2010-07-13	2010-07-26	2010-07-21	2010-07-12	2010-07-20
Time of 2nd sampling (DC90-95)	2010-08-10	2010-08-10	2010-08-14	2010-08-13	2010-08-18	2010-08-27	2010-08-27	2010-08-14	2010-08-13	2010-08-24	2010-08-27	2010-08-14	2010-08-19

Appendix II

– Primer mixtures and PCR programmes

	Specific primer	Positive control	PCR programme	Agarose gel concentration	Source
F. avenaceum	Fa F Fa R	evp 65 (0.1 ng/ml)	Programme 1	1 %	Doohan *et al.*, 1998
F. culmorum	Fc01 F Fc01 R	evp 5 (0.1 ng/ml)	Programme 1	1 %	Nicholson *et al.*, 1998
F. graminearum	Fg16 F Fg16 R	m1b (0.1 ng/ml)	Programme 1	1 %	Nicholson *et al.*, 1998
F. poae	Fp82 F Fp82 R	evp 20 (0.1 ng/ml)	Programme 2	1.5 %	Parry and Nicholson, 1996
F. langsethiae	Pfus F Pfus R	F.L. 1 ng/ml	Programme 2	1.5 %	Halstensen *et al.*, 2006
F. sporotrichioides	Pfus F Fspor R	evp 61 (0.1 ng/ml)	Programme 1	1.5 %	Halstensen *et al.*, 2006

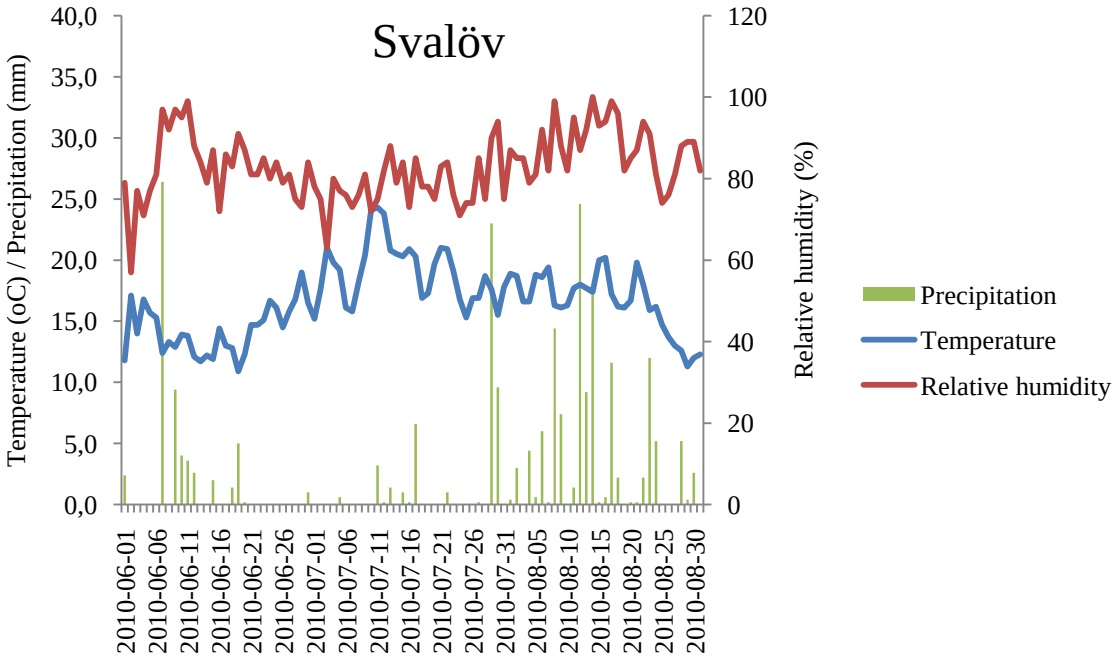
Mastermix, Primers	
MilliQ water	2 v.u.
Buffer (RB)	1 v.u.
dNTP	1 v.u.
Primer, 1F	0.2 v.u.
Primer, 4	0.2 v.u.
$MgCl_2$	0.3 v.u.
Taq-polymeras	0.06 v.u.

PCR programme 1		
1 cycle	94 °C	5 minutes
5 cycles	94 °C	30 seconds
	66 °C	20 seconds
	72 °C	45 seconds
5 cycles	94 °C	30 seconds
	64 °C	20 seconds
	72 °C	45 seconds
25 cycles	94 °C	30 seconds
	62 °C	20 seconds
	72 °C	45 seconds
1 cycle	72 °C	5 minutes

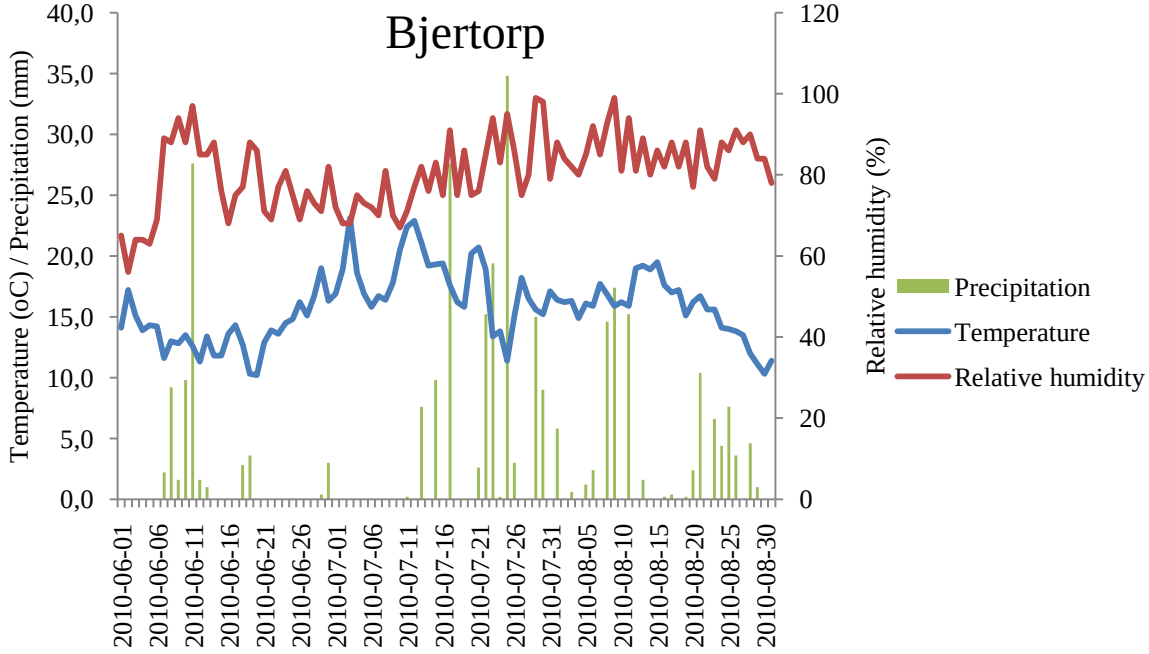
PCR programme 2		
1 cycle	94 °C	2 minutes
30 cycles	94 °C	30 seconds
	66 °C	30 seconds
	72 °C	30 seconds
1 cycle	72 °C	5 minutes

Appendix III

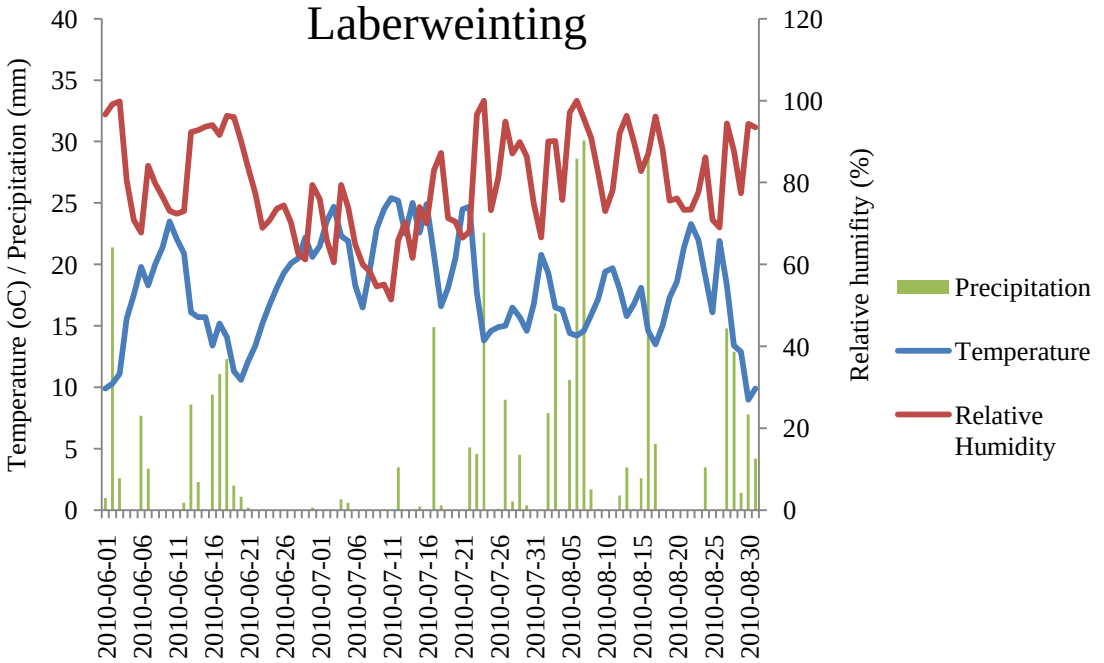
Weather data for the period of 2010-06-01 – 2010-08-31. Data for precipitation, temperature (200 cm) and relative humidity in Svalöv, Bjertorp, Laberweinting and Harzhof.



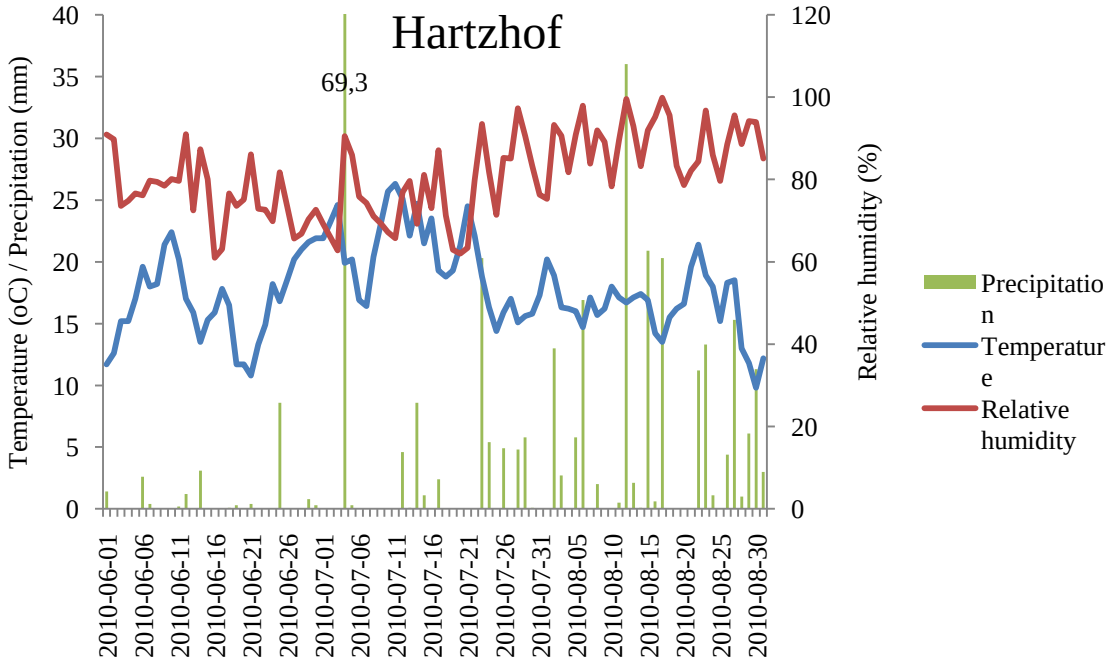
Source: LANTMÄT, 2010, Weather station: Svalöv, no.23483.



Source: LANTMÄT, 2010. Weather station: Bjertorp, no.25640.



Source: Agrarmeterologi, 2010. Weather station: Feistenaich, no.47.



Source: Agrarmeterologi, 2010. Weather station: Birkenmoor, no.92.