UPTEC X 11 012
Examensarbete 30 hp
Mars 2011

Quantification of Fusarium species in Swedish Spring wheat by real-time PCR and their correlation with mycotoxin content and region

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UPTEC X 11 012	**Date of issue 2011-03**

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Title (English)

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Title (Swedish)

Abstract

Fusarium is a type of mould capable of producing several diseases in cereals. Infection is a worldwide problem associated with yield losses and the accumulation of toxic secondary metabolites, mycotoxins, which are harmful to both humans and animals. *F. graminearum*, *F. culmorum*, *F. avenaceum*, *F. poae*, and *F. tricinctum*, including corresponding mycotoxins were quantified in wheat samples from 6 distinct areas in Sweden. High levels of both *Fusarium* and toxins were detected; however no samples exceeded current limit values. The most dominant species of *Fusarium* were *F. avenaceum* and *F. graminearum* and the most common mycotoxins were deoxynivalenol and enniatin B.

Keywords

Fusarium, mycotoxins, wheat, real-time PCR, region, climate

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Project name	Sponsors
Language **English**	Security
ISSN 1401-2138	Classification
Supplementary bibliographical information	Pages **39**

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Populärvetenskaplig sammanfattning

Fusarium är en mögelsvamp som är vanligt förekommande i spannmål världen över. I släktet *Fusarium* ingår flera arter som kan orsaka allvarliga infektioner i grödor, med i vissa fall stora skördeförluster som följd. I Norden är *F. graminearum*, *F. culmorum*, *F. avenaceum*, *F. tricinctum* och *F. poae* vanligt förekommande arter. Tillväxten av *Fusarium* är starkt kopplad till klimatfaktorer som temperatur och nederbörd. Som en följd av detta kan klimatförändringar komma att medföra en ökad spridning av mögelsjukdomar.

Flera arter av *Fusarium* bildar mögelgifter, så kallade mykotoxiner, som kan påverka hälsan hos både människor och djur. Mykotoxiner är värmetåliga ämnen som inte bryts ner vid livsmedelsproduktion, och kan därför nå konsumenter via kosten. I Sverige är vete det mest odlade spannmålet och en bidragande anledning till att mykotoxiner når livsmedelskedjan. Kunskap om och kontroll av förekomsten av mögelsvampar är därför viktig både för livsmedelssäkerheten och för att kunna förebygga spridningen.

Realtids-PCR är ett effektivt verktyg för detektion och kvantifiering av *Fusarium* i spannmålsprover. Följande arbete kartlägger förekomsten av *Fusarium* arter i vete från regioner i södra och mellersta Sverige med hjälp av denna teknik. Samband mellan kontaminering av mykotoxiner och mögelinfektion samt statistiska skillnader mellan olika geografiska regioner fastställs.

Examensarbete 30 hp

Civilingenjörsprogrammet Molekylär bioteknik

Uppsala universitet februari 2011

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Abbreviations

BEA	Beauvericin
C_t	Threshold cycle
CZID	Czapek-dox iprodione dichloran agar
DG18	Dichloran 18% glycerol
DON	Deoxynivalenol
EFSA	European Food Safety Authority
ENN	Enniatins
EC	European Commission
ESI-MS/MS	Electrospray ionization tandem mass spectrometry
EU	European Union
FAO	Food and Agriculture Organization of the United Nations
FHB	*Fusarium* head blight
GLM	General linear model
HPLC	High performance liquid chromatography
JECFA	Joint Expert Committee on Food Additives
LC-MS/MS	Liquid chromatography tandem mass spectrometry
LOD	Limit of detection
MON	Moniliformin
NIV	Nivalenol
PCR	Polymerase chain reaction
PDA	Potato dextrose agar
TDI	Tolerable daily intake
WHO	World Health organisation
ZEN	Zearalenone

1 Introduction

Fusarium is a type of mould associated with food spoilage and infestation of grains on a global scale. The genus include a wide range of species capable of producing several diseases in cereals and maize, including *Fusarium* head blight (FHB), that cause significant yield and quality losses to infected crops (Parry *et al*., 1995). *Fusarium* outbreaks with great economic consequences have been reported from the U.S., China, Canada, Argentina and Japan, with losses exceeding 50% in some instances (McMullen *et al*., 1997). FHB is caused by a complex of different *Fusarium* species and the composition and interaction pattern differ considerably between countries (Xu *et al*., 2005). In northern European areas, *F. avenaceum*, *F. graminearum*, *F. culmorum*, *F. poae* and *F. tricinctum* are considered to be the dominating species (Uhlig *et al*. 2007). The distribution and growth of *Fusarium* fungi is closely related to climatic factors, such as temperature and moisture (Doohan *et al*., 2003) and the prevalence is consequently expected to increase as a result of emerging climate change.

In addition to yield losses, most species of *Fusarium* produce an array of toxic metabolites, known as mycotoxins, which can cause a wide range of disorders in both humans and animals (D'Mello *et al*., 2009; Peraica *et al*., 1999). *F. culmorum* and *F. graminearum* are by themselves potent producers of approximately 40 and 50 different toxic compounds respectively (Pitt and Hocking, 1997), some of which are closely related to products intended for human and animal consumption. Humans are exposed to mycotoxins through dietary intake of plant based foods and animal products such as milk, meat and eggs from cattle and farm stock given contaminated feed (Bryden, 2007). As a result of economic and health consequences associated with mycotoxins and *Fusarium* the matter has attracted a fair amount of attention over recent years (e.g. Bryden, 2007; Creppy, 2002; Pitt, 2000; Placinta *et al.*, 1999; Peraica *et al*., 1999).

The dominating dietary intake of *Fusarium* mycotoxins is through cereal products and in particular products made from wheat and maize (Food Standards Agency, 2007). Wheat, the most commonly cultivated grain in Sweden (Statistiska centralbyrån, 2010), has been found to be heavily contaminated by *Fusarium* species and corresponding mycotoxins in Norway and Finland (Uhlig *et al*., 2007; Jestoi *et al*., 2004; Langseth and Rundberget, 1999) and results from Swedish surveys of wheat and oats present similar results (Fredlund *et al*., 2010; Fredlund *et al*., 2008). In the temperate regions of America, Europe and Asia, *Fusarium* is considered to be the fungi responsible for most of the mycotoxin production in grains such as wheat (Creppy, 2002).

This master thesis is a part of an ongoing study on the prevalence of *Fusarium* and corresponding mycotoxins in Swedish grains carried out by the Swedish National Food Administration in cooperation with the Swedish Board of Agriculture, the Swedish National Veterinary Institute and Lantmännen, in order

to ascertain the frequency of *Fusarium* contaminated cereals and evaluate the possibility of developing predictive models. During the initial phase of the project, in 2009, common strains of *Fusarium* in Swedish Fall wheat from three geographical areas were identified and results evaluated in respect to mycotoxin content and region. Results indicated that *F. graminearum, F. culmorum, F. avenaceum, F. poae* and *F. tricinctum* are frequently occurring species, making further studies of these fungi interesting.

1.1 Objective

The purpose of this study was to assess the prevalence of *Fusarium* species and common mycotoxins in Swedish Spring wheat, by examining 28 kernel samples and 10 ear samples obtained from farms located in the southern and middle parts of Sweden. The survey was based on the following research questions:

- What quantity of *Fusarium graminearum*, *Fusarium culmorum*, *Fusarium avenaceum, Fusarium poae* and *Fusarium tricinctum* and corresponding mycotoxins, were present in Spring wheat samples from Swedish farms in 2010?
- Is there a relationship between the incidence of *Fusarium* and the presence of common mycotoxins in Swedish Spring wheat?
- Are ear samples picked pre-harvest good indicators of toxin contamination?
- How does the occurrence of *Fusarium* and corresponding toxins vary between different geographical areas in Sweden?

2 Background

2.1 Infection of wheat by *Fusarium*

Fusarium is a mould within the phylum ascomycota, a group of fungi that produce sexual ascospores and asexual spores called conidia. Vast numbers of spores are spread by wind, rain and insects to host plants where they germinate given appropriate moist and temperature conditions (Parry *et al.*, 1995). In colder climates, growth is limited to the spring and summer, but mycelia can survive over winter on infected plant material in the soil and inoculate crops during following seasons (Goswami and Kistler, 2004).

Fusarium impact several aspects of the crop, corrupting starch granules, storage proteins and cell walls (Bechtel *et al*., 1985). Growth proceeds primarily in the aleurone (the endosperm cell wall) and pericarp tissues (the tissue surrounding the seed), although prevalent throughout the endosperm, and in cases of severe infection in the germ, with decreased germination as a consequence (Bechtel *et al*., 1985). Accumulation of toxin has been demonstrated to occur predominately in the outer parts of the wheat kernels, indicating that they remain at the site of production rather than being transported to the other parts of the seed (Schollenberger *et al*., 2002). Kernels heavily contaminated by *Fusarium* can show visual signs of infection, such as discolouring and shrivelling, and to a certain extent appearance works well as an indicator of the severity of fungal and toxin contamination. In general, shrunken and discoloured seeds have been found to contain the highest levels of mycotoxin (Bechtel *et al*., 1985).

2.2 *Fusarium* Mycotoxins

The most prevalent *Fusarium* mycotoxins in cereals are trichothecenes, zearalenone and fumonisins. The latter is foremost associated with contamination of maize cultivated in warm climates and is rarely found in grains grown in temperate zones (Shephard, 2005). The trichothecene group consists of approximately 148 toxin subtypes, but only a small fraction are classified as food contaminants (Peraica *et al*., 1999). In wheat and other small grains, deoxynivalenol (DON), nivalenol (NIV), T-2 toxin and HT-2 toxin are frequently occurring members of this group. DON is the most prevalent toxin and has been found to contaminate a large number of test samples. In a survey of wheat flour collected from mills and grocery stores in the area of south west Germany, DON were detected in 98% of analysed samples (Schollenberger *et al*., 2002) and reports from Asia indicate that DON contaminate approximately 60 % of cultivated wheat (Creppy, 2002), however, these observations are not rare. Several extensive reviews presenting similar findings from most parts of the world are available (Bottalico and Perrone, 2002; Placinta *et al.*, 1999; McMullen *et al*., 1997).

In addition to the trichothecenes and zearalenone, the presence of moniliformin (MON), beauvericin (BEA) and enniatins (ENNs), a group of frequently occurring toxic compounds, have been reported in wheat, particularly in crops from northern Europe. In the Nordic countries, ENNs and MON have recently attracted considerable attention as a result of several surveys reporting high levels in wheat and barley (Uhlig *et al*., 2007). ENN B, ENN B1 and ENN A1were detected at levels frequently above 1 mg/kg in all or in a majority of samples assessed in Finland and Norway and MON has been reported to contaminate approximately 75% of samples in these countries (Jestoi *et al*., 2004; Uhlig *et al*., 2006; Uhlig *et al*., 2004). The natural occurrence of BEA is widespread, but reported levels are generally quite low in comparison to MON and ENNs.

NIV and ZEN are frequently found to co-occur with DON in cereals and all three substances are associated with infection by *F. graminearum* (Bottalico and Perrone, 2002). Furthermore, *F. culmorum* is a potent producer of DON and ZEN and *F. poae* is known to produce NIV (Bottalico and Perrone, 2002). *F. poae* has been reported as the most important producer of T-2 and HT-2 toxin (Pitt and Hocking, 1997), in addition to other *Fusarium* species including *F. sporotrichioides* and *F. langsethiae* (Thrane *et al*., 2004). ENNs and MON are primarily produced by *F. avenaceum* (Chelkowski *et al*., 1990; Jestoi *et al*., 2004; Uhlig *et al*., 2007) and to a lesser extent correlation to other species such as *F. tricinctum* has been established (Bottalico and Perrone, 2002). A study of MON contaminated grains in Norway indicated that the MON concentration is significantly correlated to infection by *F. culmorum* (a non-MON producing species) as well as *F. avenaceum* content, which is proposed to be a result of fungal interaction (Uhlig *et al*., 2004). BEA is primarily related to *F. poae* (Bottalico and Perrone, 2002). In addition, a survey of Finnish wheat samples from 2002 indicated a connection between *F. avenaceum* and both BEA and ENNS (Logrieco *et al*., 2002). Table 1 summarises important *Fusarium* fungi and mycotoxins frequently associated with the respective species.

Table 1 *Fusarium* species and commonly associated mycotoxins.

***Fusarium* species**	**Commonly associated mycotoxins**	**References**
F. graminearum	Deoxynivalenol, Nivalenol, Zearalenone	Bottalico and Perrone, 2002
F. culmorum	Deoxynivalenol, Zearalenone	Bottalico and Perrone, 2002
F. avenaceum	Moniliformin, Beauvericin, Enniatins	Bottalico and Perrone, 2002 Uhlig *et al*., 2007
F. poae	Nivalenol, Beauvericin, T-2 toxin, HT-2 toxin	Bottalico and Perrone, 2002 Pitt and Hocking, 1997 Thrane *et al*., 2004
F. tricinctum	Moniliformin	Bottalico and Perrone, 2002

2.3 Mycotoxins and human and animal health

Fusarium mycotoxins are capable of causing a wide range of diseases in humans. Such disorders, caused by exposure to mycotoxins, are called mycotoxicoses. A review by Bryden (2007) shows that chronic exposure to low levels of trichothecenes and ZEN potentially have a damaging impact on fetal development, the immune system and may cause birth defects, while symptoms of acute toxicoses, are primarily related to stomach disease, like nausea, vomiting, diarrhea and dizziness (Creppy, 2002; Pitt, 2000; Peraica *et al*., 1999). The toxic effects are closely linked to the toxicity of the compound, dose and duration of exposure (Steyn, 1995). T-2 and HT-2 toxin are poisonous at very low levels, whereas DON is less toxic, but represents a bigger health concern due to its widespread nature and prevalence (Placinta *et al*., 1999). The worst recorded case of mycotoxicoses is alimentary toxic aleukia in the USSR in 1932, caused by trichothecenes (Peraica *et al*. 1999). No records of the exact number of people affected are available, however, tens of thousands of people were exposed to infected grains and the mortality rate was 60%. In this case, approximately 40% of grain samples showed signs of *Fusarium* infection, compared to 2±8% in areas where no disease was observed. The duration of exposure to mycotoxins contributed to the severity of this particular outbreak, but there are records of cases where a single ingestion of contaminated bread resulted in trichothecene mycotoxicoses. In India, 1987, a substantial part of the population in Kashmir Valley were affected by gastrointestinal disease, from what turned out to be caused by bread made from wheat infected by *Fusarium* and *Aspergillus* (Bhat *et al*., 1989). No outbreaks of this magnitude have been reported since, however, several cases of human mycotoxicoses caused by *Fusarium* toxins have been reported from India, China, Japan and Korea (Pitt, 2000).

As an indication of the toxicity of mycotoxins, tolerable daily intake (TDI) can provide a benchmark. TDI is a measure based on the toxicity of the substance in combination with the estimated health impact of consumption. In addition to information regarding consumer exposure it is used to calculate acceptable maximum levels of naturally occurring contaminants in certain foods, however, determining acceptable levels of mycotoxins is not a simple process due to lack of available data and the sensitive nature of changing conditions for producers concerned (Tritscher and Page, 2004). When available data is insufficient to conclude whether dietary intake of a substance is safe over a longer period of time, but adequate information about short term exposure is available, a temporary TDI (t-TDI) is set (JECFA, 2011). This is the case for several frequently occurring mycotoxins. Since risk assessment relies to a great extent on the use animal data the purpose of a t-TDI value is to make sure limits are not set too high for safe human exposure until further information is available. Limits for daily intake and maximum levels for foods intended for human consumption are presented in Table 2.

Table 2 Tolerable daily intake (TDI) for common *Fusarium* mycotoxins and maximum levels set for mycotoxin content in wheat. TDI levels are given in µg mycotoxin/kg bodyweight and maximum levels for unprocessed cereals/cereals for direct human consumption in µg mycotoxin/kg grain. Reference: European Commission (2006).

Mycotoxin	**Tolerable daily intake** (TDI)	**Maximum levels**
Deoxynivalenol	1 µg/kg TDI	1250/750 µg/kg
Zearalenone	0,2 µg/kg TDI	100/75 µg/kg
Nivalenol	0,7 µg/kg t-TDI	Under evaluation
T-2-toxin and HT-2-toxin	0,06 µg/kg t-TDI	Under evaluation

2.4 Mycotoxin regulation

The potentially harmful effects of *Fusarium* mycotoxins require good practice standards and regulations to secure safe food and feedstuff for consumers, but until recently no legislation existed within this field and strategies are rarely established on a national level (Brera *et al*., 1998). For the development of food standards and risk assessments, two main international actors exist, the joint collaboration between the World Health Organisation (WHO) and the Food and Agriculture Organisation of the United Nations (FAO); FAO/WHO Expert Committee on Food Additives (JECFA) and the European Food Safety Authority (EFSA). EFSA perform risk assessments in response to different food safety matters in collaboration with experts within the field of concern. The results are published as scientific opinions on which the EU bases their recommendations and legislation. In 2006 the EU published *Commission Recommendation 2006/583/EC on the prevention and reduction of Fusarium toxins in cereals and cereal products* and *Commission Regulation 1881/2006/EC Setting maximum levels for certain contaminants in foodstuff*, outlining recommendations and preventive measures for reducing toxins in food. Furthermore, the *Commission Regulation 1881/2006/EC* includes legislation regarding maximum acceptable levels of certain *Fusarium* mycotoxins in products intended for consumption. According to the regulatory framework, governmental bodies within the member states are responsible for the implementation of EU regulations, and within Sweden, the Swedish Food Administration establish these codes in order to reduce the dietary intake and prevalence of mycotoxins.

2.5 Factors influencing *Fusarium* growth

In response to the major economic impact and health aspect of *Fusarium* growth, prevention and prediction of infection has naturally become an area of great interest. It has, however, proven to be a very complicated task due to the complex growth pattern of the fungi. The two single most important factors influencing *Fusarium* growth are temperature and humidity, although these are not

independent from other climatic, agricultural and environmental conditions (Doohan *et al*., 2003). Significant agricultural factors include crop rotation, the use of fertilizers, chemical controls and soil cultivation, primarily dependent on how old crop debris is mixed with the soil before seeding (Edwards, 2004). In addition, climatic factors such as aeration and light impact the capability of *Fusarium* inoculum and growth (Doohan *et al*., 2003). Amongst agronomic variables, wheat variety account for the greatest degree of variation in DON content (Schaafsma and Hooker, 2007). Some cereal species are naturally more resistant to *Fusarium* infection then others, however these inherent susceptibility features have been observed to vary between countries (Edwards, 2004).

Another aspect that further complicates the matter is interactions between *Fusarium* species. *Fusarium* fungi can infect host plants either individually, or in a complex of synergistic interactions between two or more species that respond differently to environmental conditions (Xu *et al*., 2005). Since these interactions affect growth patterns and consequently mycotoxin production, knowledge about the effects of environmental conditions for both single and mixed pathogen species is required to predict the spread of *Fusarium*. As an example of the complicated nature of these types of assessments, Bernhoft *et al*. (2010) found that wet conditions in July favours the total amount of *Fusarium* growth in Norwegian cereals, but on the contrary, *F. graminearum* infestation increased when precipitation in July was low.

2.6 Prevention of *Fusarium* growth

2.6.1 Prediction strategies

As a result of the connection between *Fusarium* growth and climatic factors, there is a strong reason to believe that climate change will have an effect on the spreading of moulds, and several reports put emphasis on potential consequences (FAO, 2008; Miller, 2008; Doohan *et al*., 2003; McMullen *et al*., 1997). In response to these reports, forecasting models are becoming an increasingly interesting tool to predict the prevalence of *Fusarium*. FAO (2008) suggest in the report *Climate Change: Implications for Food Safety* the use of predictive modelling, in addition to prevention, monitoring and agricultural policies, as a mean of reducing the incidence of *Fusarium* and Miller (2008) emphasise the importance of proactive action, by changing agronomic practices when the risk of an outbreak is high, rather than applying fungicides on a general basis. However, the development of forecasting systems has proven to be a very complicated task primarily due to the background discussed above. To date there is only one forecasting system commercially available, DONcast, with successful application in Canada, the United States, Uruguay and France (Schaafsma and Hooker, 2007). In order to develop successful models for Sweden, access to comprehensive data about the prevalence of *Fusarium* and mycotoxins is essential.

2.6.2 Prevention strategies

McMullen *et al*. (1997) suggest incorporating *Fusarium* resistance in wheat varieties as a possible way to preventing spreading, in addition to crop rotation, tillage and the use of protective chemicals. The importance of international collaborations and more funding for research purposes is also noted. Research and development of *Fusarium* resistant wheat crops are in progress and several loci contributing to less infestation have been identified, however the course of action is still poorly understood. Trials with the resistant wheat line Sumai 3 have been ongoing for more than two decades, but low yields and inadequate protection in certain environments make cultivation commercially unfavourable (Bai and Shaner, 2004). Several studies (Bernhoft *et al*., 2010; Schollenberger *et al*., 2002), including a recently performed study of *Fusarium* content in Norwegian grains (Bernhoft *et al*., 2010), indicate less contamination by *Fusarium* and lower mycotoxin levels in organically produced wheat, barley and oats. *Fusarium* infestation was found to increase when fungicides and mineral fertilisers were used. The opposite effect, reduced infection for crops treated with fungicides, has also been demonstrated, but the cost of treatment in relation to effectiveness has led to limited use (Bai and Shaner, 2004). Furthermore, there is evidence regarding the effect of crop rotation on mycotoxin content that wheat following wheat and wheat following maize implies a greater risk of contamination (Edwards, 2004).

2.7 Quantification and identification of *Fusarium*

Traditionally, determination of *Fusarium* infection has relied on morphological analyses, such as enumeration and isolation, and it is not until recently methods based on real-time PCR has attracted more attention (Fredlund *et al*., 2010; Brunner *et al*., 2009; Yli-Mattila *et al*., 2008; Kulik, 2008; Waalwijk *et al*., 2004). Since *Fusarium* species differ in sensitivity to fungicides and respond differently to climatic and agricultural factors, accurate identification of individual species is of great importance. Enumeration and isolation is an approach where crop material such as kernels are placed on growth media and incubated to allow fungal growth. Colonies are analysed based on morphology and species of interest are isolated and identified by microscopy. The use of real-time PCR allows absolute quantification of infection, rather than a percentage of infected kernels, in addition to being an accurate tool when identifying morphologically similar species, a task that would otherwise require extensive expertise. Waalwijk *et al*., (2004) compared results obtained using the two approaches and found that plating may result in outgrowth of individual species, while quantitative PCR is capable of determining the mixture of species in a FHB complex. Moreover, PCR overcomes several other drawbacks of traditional methods; enumeration and isolation is time consuming, cannot be used to quantify levels of *Fusarium* in crops during the growing season and fast growing species may be biased (Waalwijk *et al*., 2004). Previous research has also shown better correlation

between toxin content and *Fusarium* infection using quantitative PCR as the determining method, rather than visual scoring (Brunner *et al*., 2009).

2.7.1 Real-time PCR

In order to obtain assays capable of detecting specific species of *Fusarium*, PCR gene targets need to be well characterised and contain substantial sequence variability between species. Numerous genes that possess these features have been identified and can be used to design primers for *Fusarium* detection and quantification. Among these, the intergenic spacer (IGS) 1 region rDNA, tri5 and translation elongation factor 1α gene (EF1α) are frequently used to target *Fusarium* species and has demonstrated to be both specific and provide accurate detection (Yli-Mattila *et al*., 2008; Kulik, 2008; Nicolaisen *et al*., 2009; Fredlund *et al*., 2010).

In addition to specific probes and primers, a well designed PCR setup is essential for precise quantification. The absolute quantification method is a real-time PCR approach frequently applied for detection of *Fusarium* in wheat and other grains. The technique is based on amplification of a known concentration of starting material, which in the case of this project is DNA isolated from mycelia, obtained from reference strains of specific species of *Fusarium*. A standard curve is created by determining the OD_{260} of the defined reference template and amplifying a series of dilutions with known template concentration. The result is used as a reference to determine the quantity of the same target in a sample of unknown concentration. In addition, it is possible to assess the efficiency of the assay by analysing the standard curve, that is, how well the target template is amplified when running the PCR. In an optimal assay, molecules are amplified in an exponential manner with one new molecule synthesized from every template in each cycle, until reagents are consumed and the reaction reaches a plateau. To generate a standard curve the Ct value for each sample in the dilution series are plotted against the logarithm of the starting quantity, and fitted by linear regression. The efficiency is calculated from the slope of the regression line by the following formula:

$$Efficiency = \left(10^{\left(-\frac{1}{slope}\right)}\right) - 1$$

A slope of approximately -3.32 represents an efficiency of 100%, as shown in Figure 1. This infers that if dilution series are accurately made and the master mix components and concentrations are optimal, the spacing between two amplification curves in a series of 10-fold dilutions should be approximately 3.3 cycles. It is also important to note the R^2 value and y-intercept for the standard curve to determine whether the assay can be used for reliable quantification. If the y-intercept is too high, degradation of the template might be a problem, and a low R^2 value indicate bad precision when creating dilution series. Another drawback

with an increasing y-intercept, is decreasing quantification sensitivity, since more reaction cycles are required to detect the same amount of starting material (Dorak, 2006).

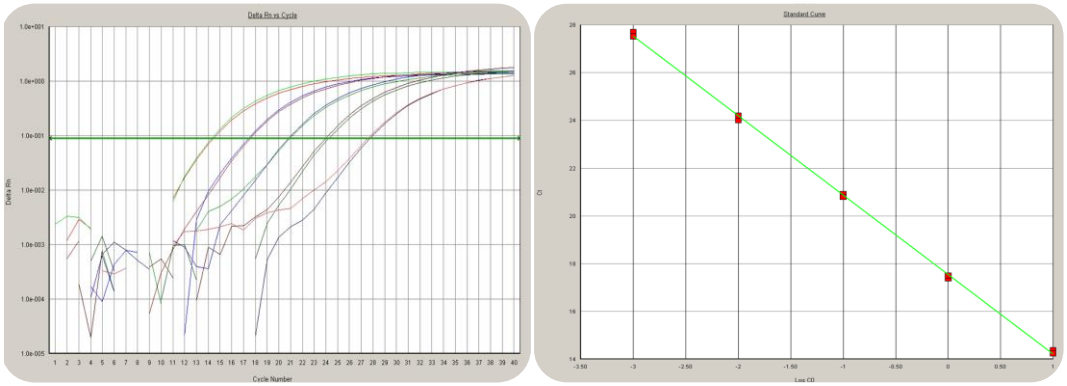

Figure 1 Amplification plot (left) and corresponding standard curve (right) for real-time PCR analysis of *F. poae* performed on an ABI 7500 Instrument (Applied Biosystems). The amplified DNA was diluted 10-fold, ranging from 10^{-1} -10^{-5} and the standard curve created with the 7500 System SDS Software. The slope is -3.328 representing an assay with an efficiency of 100%.

When analysing samples with an unknown content of a template gene, a standard curve always has to be run simultaneously, in order to quantify the starting material using the Ct value of the samples. Since the standard curve and the samples are run under the same conditions, quantification is possible.

3 Materials and methods

3.1 Collection of wheat samples

28 kernel samples of spring wheat were collected for *Fusarium* quantification and mycotoxin analysis from 28 different cultivators in the southern and middle parts of Sweden. Farms were divided into 6 regions (Figure 2) based on geographical distribution and the number of participating cultivators in each area. In addition, 10 ear samples were collected pre harvest, intended for evaluation of ear samples as potential indicators of toxin contamination. Ears were sampled from field trials during the late stages of growth and kernel samples were harvested in August 2010 and collected from either field or farm trials.

Figure 2 Location of farm and field trials. Circles represent the 6 geographical regions used for the analysis of results.

3.1.1 Field trials

Field trials were performed by Hushållningssällskapet; a regionally based knowledge organisation with extensive experience of farming and field trials. Sample grains were grown under monitored conditions, making data collections well suited for construction of prediction models. Cereals were cultivated in defined plots and information such as weather data and previously grown crops are available for all samples collected from field trials. Wheat kernels were taken from several plots in each selected field, and an equal fraction of the harvest from each plot combined into one sample, to get a characteristic sample for the total acreage.

3.1.2 Farm trials

Growth conditions for farm trails were less controlled than for field trials, but represent wheat intended for production. Kernels were randomly picked from the full harvest by Lantmännen at delivery of grain transports to their production facilities. 14 samples were received from participating cultivators in the regions of Uppsala, Kvänum and Köping.

3.2 Sample treatment

Kernel samples (300-500g) and ear samples (approximately 20 ears per sample) were sent to the Swedish National Food Administration for analysis. Upon retrieval, samples with water content above 14% were dried in 40°C for two hours to prevent fugal growth during storage. Dry wheat samples were milled to a fine powder in a RasMill® (Romer Labs). Approximately 200 g of each sample were further milled to fine granules using a coffee grinder (De'Longhi). All samples were stored in -20°C prior to analysis.

3.3 Experimental setup

Quantification of *Fusarium* by real-time PCR was carried out for *F. graminearum*, *F. culmorum*, *F. avenaceum*, *F. poae* and *F. tricinctum*, in a five step procedure, as illustrated in Figure 3. The method is based on previously described probes and primer sequences (Waalwijk *et al.*, 2004; Yli-Mattila *et al.*, 2008; Kulik, 2008; Brunner *et al.*, 2009) and optimisation studies of DNA extraction and PCR procedures by Fredlund *et al.* (2008 and 2010).

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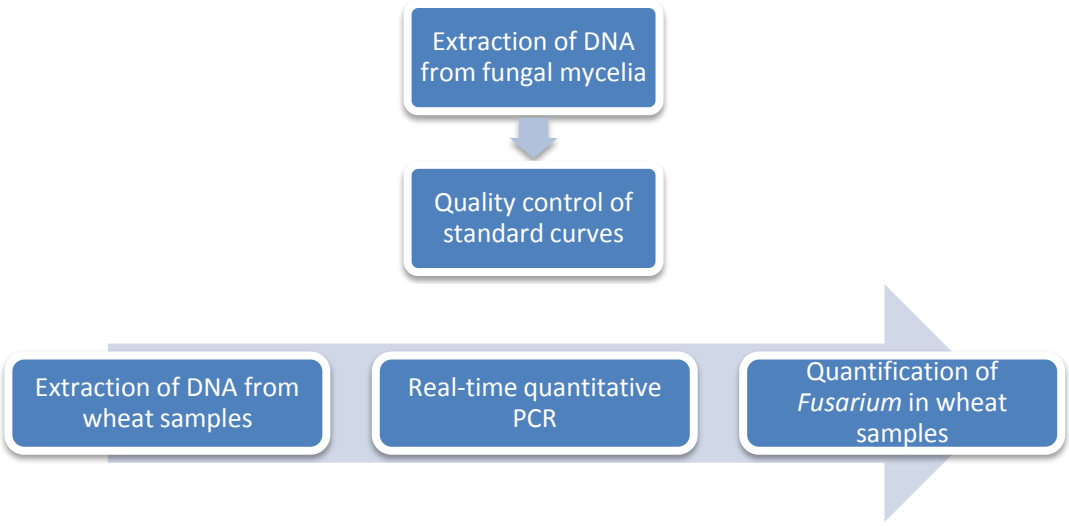

Figure 3 Experimental setup for the identification and quantification of *Fusarium* species by real-time PCR.

DNA was extracted from frozen reference mycelia and standard curves were generated for all five species of *Fusarium* in order to control the quality and the amplification efficiency of the assay. DNA was extracted from wheat samples and the amount of species specific DNA in each sample were quantified using real-time PCR. In addition, the percentage of infected kernels was determined by enumeration on *Fusarium* selective media. Kernels were plated and incubated to allow fungal growth and *Fusarium* colonies isolated and re-streaked on growth media (Figure 4).

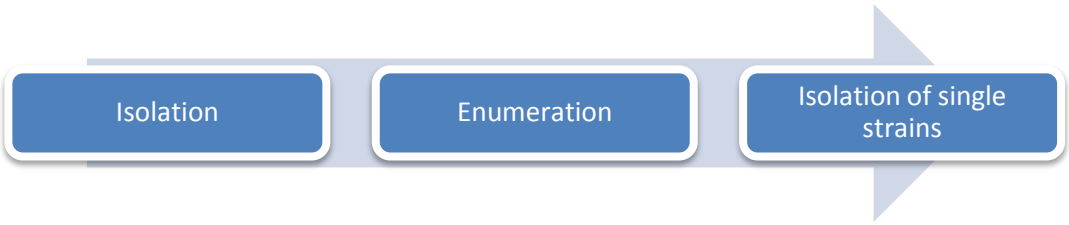

Figure 4 Workflow for mycological identification and isolation of *Fusarium* species.

3.4 Extraction of DNA from freeze dried *Fusarium* mycelia

DNA from freeze dried *Fusarium* mycelia was extracted using a modified version of Qiagen DNeasy Plant kit (Fredlund *et al*., 2008). DNA was extracted from all 5 species of *Fusarium* for the production of standard curves and positive controls for real-time PCR. Mycelium isolates were grown in ME-broth for five days, freeze dried and stored in -20°C. 10 mg of freeze dried mycelia was placed in 4 Lysing Matrix A tubes (MP Biomedicals), containing small garnet particles and one ceramic sphere for mechanical disruption. 200 µl of S3 lysis buffer, 200 µl of lysis Buffer AP1, 4 µl of RNase A and 5 µl of protease K was added to degrade the mycelium and digest RNA and protein. The samples were homogenized by FastPrep® (Qbiogene) for 1 min at 5 m/s and incubated at 65°C for 1 h.

Samples were treated according to the Qiagen DNeasy Plant kit protocol. 130 µl of Buffer AP2 was added, the samples incubated on ice for 5 min and centrifuged at 13000 rpm for 5 min. Cell debris and precipitates were removed by running the supernatant through a QIAshredder Mini Spin Column (Qiagen) in a centrifuge at maximum speed for 2 min. The lysate was transferred into a new tube and impurities were precipitated by phenol extraction using a mixture of phenol:chloroform:isoamylalcohol (25:24:1). After vortexing the samples for 1 min and centrifuging at maximum speed for 5 min the upper phase was transferred into a new tube and the procedure was repeated twice, with the phenol mixture replaced by one volume of chloroform:isoamylalcohol (24:1) . The upper DNA phase was pipetted into a new tube and 1.5 volumes of binding Buffer AP3 (Qiagen) were added to promote binding of DNA to the membrane of the column. The samples were transferred into the DNeasy® Mini Spin Columns and applied onto the membrane by centrifuging at 8000 rpm for 1 min. The membrane was subsequently washed twice with 500 µl of Buffer AW (Qiagen) by centrifuging at 8000 rpm for 1 min, to remove contaminants. The column was transferred to an eppendorf tube and the DNA eluated by adding 100 µl of Buffer AE (Qiagen), a buffer consisting of 10 mM Tris·Cl, 0.5 mM EDTA, and incubating in room temperature for 5 min before centrifuging at 8000 rpm for 1 min. Tris is a buffering agent (titrated to pH 9 with HCl) and EDTA protect against DNA degradation by chelating metal ions essential for nucleases and other enzymes.

The purity and concentration of the DNA samples were determined using a Nanodrop ND-1000 spectrometer (Thermo Scientific). Concentrated DNA solution were aliquoted (12 µl samples) for use in the construction of standard curves and DNA for positive control samples was diluted to approximately 1 ng/µl in Buffer AE (Qiagen) and aliquoted (15 µ samples) to avoid damage from freezing and thawing. All samples were stored until further analysis at -20°C.

3.5 Quality control of standards curves for real-time PCR

Since the quality of real-time PCR quantification is based on standard curves it is important that the assay is efficient enough to provide accurate results. Therefore, standard curves were analysed prior to analysis of the wheat samples, to make sure efficiency was within the acceptable range of 80%-110%. Earlier research has demonstrated efficient detection of *Fusarium* species using TaqMan and SYBRGreen methods with species specific primers and probes (Fredlund *et al.*, 2010). Analysis of DNA samples extracted from *F. tricinctum* were performed using SYBR® Green (Applied Biosystems) and analysis of *F. avenaceum*, *F. poae*, *F. culmorum* and *F. graminearum* was performed using TaqMan® (Applied Biosystems). 10-fold dilution series (10^{-1}-10^{-5}) were prepared from aliquoted reference DNA. In addition, standard curves were compared to standard curves of reference DNA extracted in 2009 according to the same procedure. All dilutions were analysed in duplicates on 96-well plates with the ABI 7500 Instrument (Applied Biosystems) and standard curves were generated by the 7500 System SDS Software (Applied Biosystems). Slope, y-intercept and R^2-value were noted and efficiency calculated.

3.6 Extraction of DNA from wheat samples

DNA from wheat samples were extracted using a method similar to the procedure for mycelium DNA extraction, but with a few alterations. A comparative study of different DNA extraction methods for wheat (Fredlund *et al.*, 2008) show that the highest yield of DNA can be obtained using a method based on Qiagen DNeasy Plant Mini Kit. Samples of 200 mg were extracted in duplicates and suspended in 400 µl of S3 lysis buffer and homogenized by sonication for 30 seconds on full amplitude (UP 100H, Dr Hielscher). 400 µl of Buffer AP1 (Qiagen), 8 µl of RNase A and 10 µl of Protease K was added and the mixture incubated at 65°C for 30 minutes. Following incubation, 260 µl of Buffer AP2 (Qiagen) was added, the samples placed on ice for 5 min and centrifuged at 14000 rpm for 5 min. Thereafter, the extraction procedure was performed according to the DNeasy Plant Mini Kit Handbook (Qiagen, 2006), following the same principals as described above for the mycelium. The purity and concentration of DNA eluates were determined using a Nanodrop ND-1000 spectrometer (Thermo Scientific) and aliquoted in 40 µl fractions. Samples were stored in -20°C until further analysis.

3.7 Real-time PCR analysis

In addition to the five species of *Fusarium*, the wheat gene for translation elongation factor EF-G was quantified in all samples as an internal control. This procedure has previously been described by Brunner *et al*., (2009) in a study were the importance of equalising PCR results from different runs, by using the wheat gene as a reference to obtain more accurate results, were demonstrated. The use of different units was evaluated by regression analysis to check for extraction biases. Correlation between the unit pg target DNA/mg grain and the references units target DNA/total DNA (determined by nanodrop) and target DNA/wheat gene DNA were controlled. Aliquotes for positive controls and standard curves were previously prepared as described above and stored at -20°C. Analyses were performed in duplex for the wheat gene and *F. culmorum* and for *F. poae* and *F .avenaceum*, whereas *F. graminearum* and *F. tricinctum* were amplified in singleplex. DNA from wheat samples were diluted 5 times before mixed with reaction components on 96-well plates.

Duplex reactions were carried out in PerfeCTa® MultiPlex qPCR SuperMix (Quanta Biosciences) with low ROX dye as a passive reference and species specific probes and primers (Table 3). 5'-FAM (6-carboxyfluorescein) labelled probes were used for *F. culmorum* and *F. poae* and 5'-HEX labelled probes for *F. avenaceum* and translation elongation factor EF-G, both with non fluorescent quenchers. Quantification of *F. graminearum* was performed in singleplex with TaqMan® Universal Master Mix (Applied Biosystems) and a 5'-FAM probe. FAM and HEX probes are well suited for duplex reactions due to their different emission and adsorption wavelengths. *F. tricinctum* was quantified using SYBR® Green universal Master Mix (Applied Biosystems).

Probes and primers for *Fusarium* were previously developed by Waalwijk *et al*. (2004), Yli-Mattila *et al*. (2008) and Kulik (2008) in species specific studies, and validated for this specific purpose by Fredlund *et al*. (2010). Primer pairs and probes for *F. poae* and *F. tricinctum* were developed based on sequence alignment of the intergenic spacer (IGS) 1 region and designed to detect a rDNA fragment within this span (Yli-Mattila *et al*., 2008; Kulik, 2008). For the remaining Taqman assays primers and probes were generated by alignment of sequenced amplicons created with species specific primers (Waalwijk *et al*., 2004). The set of primers and probes for *F. avenaceum* are not fully specific and detect *F. tricinctum* as well. Analysis of the primer specificity for *F. tricinctum* performed at the Swedish National Food Administration present similar observations; *F. avenaceum* is detected in addition but with a different T_m. All probes and primers are presented in Table 3.

Table 3 Primers and probes used for real time PCR detection of *Fusarium* species. The primer and probe design is described by [a] Waalwijk *et al*. (2004), [b] Yli-Mattila *et al*. (2008) and [c] Kulik (2008).

Target	Primer/probe name	Sequences (5'-3')
F. avenaceum[a]	*avenaceum* MGB- F	CCA TCG CCG TGG CTT TC
	avenaceum MGB-R	CAA GCC CAC AGA CAC GTT GT
	avenaceum MGB-P	VIC-ACG CAA TTG ACT ATT GC
F. culmorum[a]	*Culmorum* MGB-F	TCA CCC AAG ACG GGA ATG A
	Culmorum MGB-R	GAA CGC TGC CCT CAA GCT T
	Culmorum MGB probe	FAM-CAC TTG GAT ATA TTT CC
F. graminearum[a]	*Graminearum* MGB-F	GGC GCT TCT CGT GAA CAC A
	Graminearum MGB-R	TGG CTA AAC AGC AGC AAT GC
	Graminearum MGB probe	FAM-AGA TAT GTC TCT TCA AGT CT
F. poae[b]	TMpoaef	GCT GAG GGT AAG CCG TCC TT
	TMpoaer	TCT GTC CCC CCT ACC AAG CT
	TMpoaep	FAM-ATT TCC CCA ACT TCG ACT CTC CGA GGA
F. tricinctum[c]	TRI1	CGT GTC CCT CTG TAC AGC TTT GA
	TRI2	GTG GTT ACC TCC CGA TAC TCT A
Wheat gene[a]	EF-G-fw	AGG TAT TAA GCA GTA CAT TTT CTC
	EF-G-rev	GGA CTA GAC TCA AAA TTA GTA TTT G
	EF-G-probe	HEX-CCA GCC TTC TCC ACT ACT AAT AC

A standard curve with five dilutions ranging from 10^{-1}-10^{-5} was included in all runs. For *F. tricinctum* amplification of the first dilution was detected at a very early stage (cycle 8) and the dilution series was therefore adjusted to start at 10^{-2}. All samples, including standard curves and positive controls, were analysed in duplicates and an additional four negative controls were added per plate. Total reaction volume was 25 µl for each well.

3.8 Isolation and enumeration of *Fusarium* in wheat kernels

To quantify the total mould infection in the wheat kernels, 50 kernels from each farm trial were placed on czapek-dox iprodione dichloran agar (CZID), and dichloran 18% glycerol (DG18), respectively. The seeds were surface disinfected in 10% sodium hypochlorite, dried on filter paper and distributed evenly in groups of 10 on plates with the two media. CZID is a selective media which has been recognized as the best media for detection of *Fusarium* species in a comparative study (Thrane, 1996), with its main advantage being easier recognition of *Fusarium* and differentiation between species by pigmentation and appearance of the cultures. It contains the fungicide iprodione, allowing only certain fungi to grow. DG18 is a general purpose medium suitable for xerophilic moulds, such as the mycoflora of dry foods like wheat. The a_w of the media is lower than for those traditionally used for moist foods, which makes it appropriate for enumeration of xerophilic fungi. Dichloran limits the growth of fast developing fungi and thereby

allow identification of a wider range of species (Hocking and Pitt, 1980). On CZID, *Fusarium* primarily grows in different shades of purple, and though visual identification of different species within the genus is complex, it is possible to determine colonies of *Fusarium* from other types of mould. The total amount of mould infected seeds and the amount of visual *Fusarium* colonies were counted after 7 days incubation at 25°C. Isolates of colonies that showed signs of *Fusarium* infection were picked and re-streaked on potato dextrose agar (PDA) and incubated for 7 days at 25°C. PDA is a medium rich in nutrients which promote sporulation and facilitate identification of species, recommended for identification of *Fusarium* by colony characteristics such as colour (Pitt and Hocking, 1997). Pure isolates were stored at 4°C.

3.9 Mycotoxin analysis

Toxin analysis were carried out by IFA-Tulln (Department of agrobiotechnology, University of Natural Resources and Applied Life Sciences, Vienna) using an HPLC/ESI-MS/MS method capable of detecting and quantifying 186 mycotoxins and fungal metabolites in cereals (Vishwanath *et al*., 2009).

3.10 Statistical analysis

Statistical analyses were performed using Minitab 14 Statistical Software and Microsoft Excel 2007. Regional differences of detected *Fusarium* and mycotoxin levels were evaluated by General Linear Model (GLM) and yearly fluctuations compared by 2-Sample t-Tests. Regression analyses were applied to examine the correlation between *Fusarium* infection and mycotoxin content and the predictive ability of ear samples picked pre harvest on mycotoxin content in kernel samples post harvest.

4 Results

4.1 Evaluation of units

The unit *pg target DNA/mg grain* correlated well with both *target DNA/wheat gene DNA* and *target DNA/total DNA* (coefficient of determination ranged from R^2=0.87 to R^2=0.99 for the different species). Consequently, the unit pg target DNA/mg grain was used for presentation of results.

4.2 Real-time PCR studies of *Fusarium*

Fusarium was detected in all 38 wheat samples analysed. *F. poae* was detected in 100% of kernel samples while *F. culmorum*, *F. avenaceum* and *F. tricinctum* were above the limit of detection (LOD) in approximately 97-99% of the samples. 90% of samples contained *F. graminearum.* The total incidence of *Fusarium* infection varied considerably between samples, however, *F. avenaceum*, *F. culmorum* and *F. graminearum* were the most predominant fungi from the perspective of mean content (Figure 5a).

Ear samples were generally less contaminated then kernel samples, with *F. culmorum* detected only in 8% of the 40 extracts. Remaining fungi ranged from 65% (*F. graminearum*) to 100% infection (*F. poae*). The highest level of mean infection in ear samples was found to be caused by *F. avenaceum* (Figure 5b). Total *Fusarium* infection in kernel samples is illustrated in Figure 6.

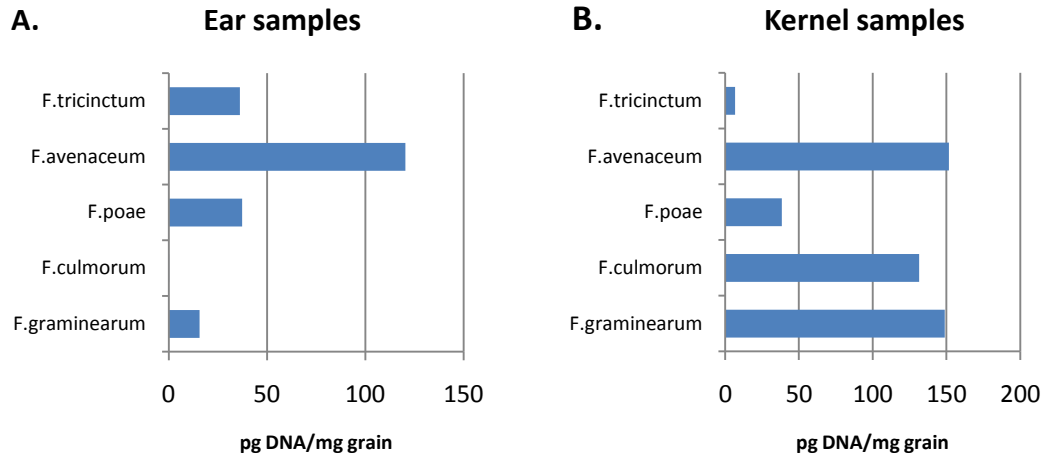

Figure 5 Mean levels of *Fusarium* contamination in ear (A) and kernel (B) samples. X-axes show mean *Fusarium* DNA content detected by real-time PCR.

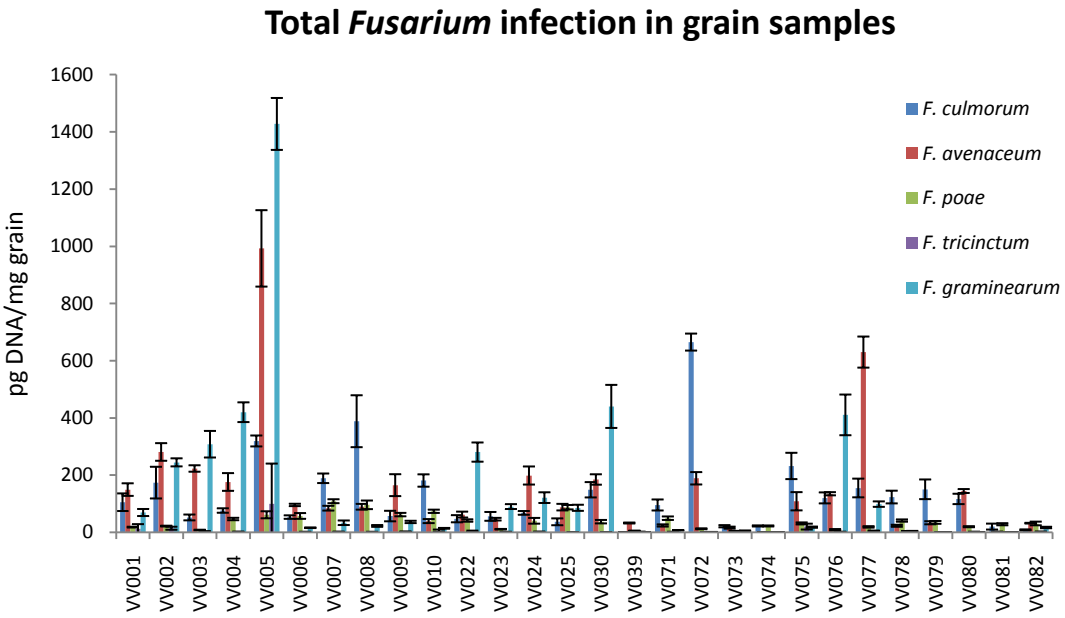

Figure 6 The mean content of *F. culmorum*, *F. avenaceum*, *F. poae*, *F. tricinctum* and *F. graminearum* in the 28 kernel samples analysed by real-time PCR. Each denotation on the x-axis includes levels for A and B samples analysed in duplex and error bars represent the standard deviation between the four samples.

Mean levels of *F. graminearum*, *F. culmorum* and *F. avenaceum* were substantially higher in 2010 than for samples from the same regions harvested and analysed in 2009 (Fredlund *et al*., 2010, unpublished results). The results indicate a 2-fold, 5-fold and 20-fold increase of *F. avenaceum*, *F. graminearum* and *F. culmorum*, respectively. In 2010 the incidence of *F. poae*, however, was reduced to approximately a third of levels in 2009 and 99% less *F. tricinctum* was detected (Figure 7). The yearly variation is statistically significant for all *Fusarium* species analysed (Table 4).

Table 4 Comparison of variation in *Fusarium* content (pg DNA/mg grain) recorded in the southern and middle parts of Sweden in 2009 (Fredlund *et al.*, 2010, unpublished results) and 2010. Analyses were performed in MInitab with 2-sample t-tests.

Species	Year	Mean	Min	Max	P-value
F. graminearum	2009	29,2	0	165.2	
	2010	149	0	1515.0	0,000
F. culmorum	2009	7,3	0	23.3	
	2010	132	0	705.0	0,000
F. poae	2009	131	13.5	729.1	
	2010	38,5	3.2	118.5	0,001
F. avenaceum	2009	71	0.4	384.5	
	2010	152	0	1120.0	0,002
F. tricinctum	2009	634	13.8	4880.3	
	2010	6,8	0	304.5	0,001

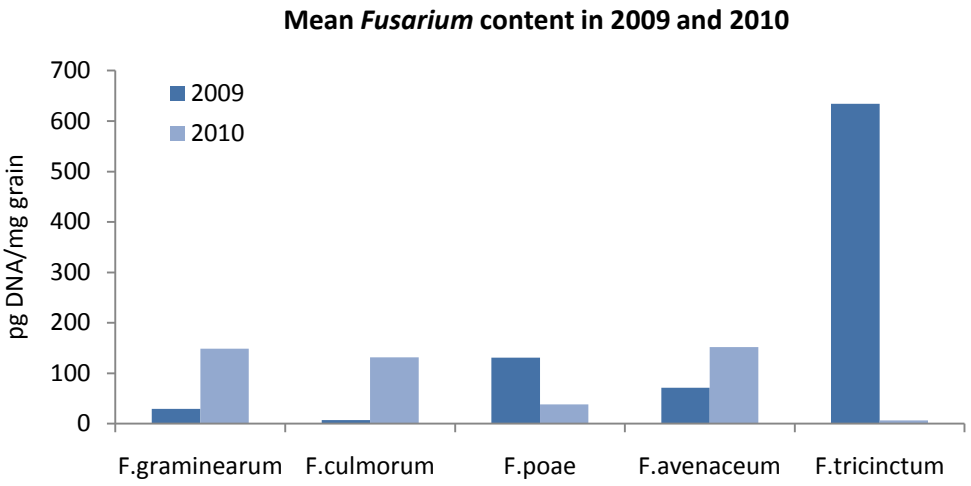

Figure 7 Comparison of mean *Fusarium* infection in wheat kernels in 2009 (Fredlund *et al.*, 2010, unpublished results) and 2010.

4.3 Morphological studies

Results from plating studies showed high levels of total mould infection. Fungal growth occurred in 100% of the kernels of all samples (obtained from farm trials). The highest incidence of *Fusarium* contamination was 78% (VV030) and the lowest 12% (VV006). Overgrowth was a common problem which made enumeration of *Fusarium* infected kernels and isolation of individual *Fusarium* isolates difficult. The incidence of *Fusarium* as a percentage of infected kernels is presented in Figure 8.

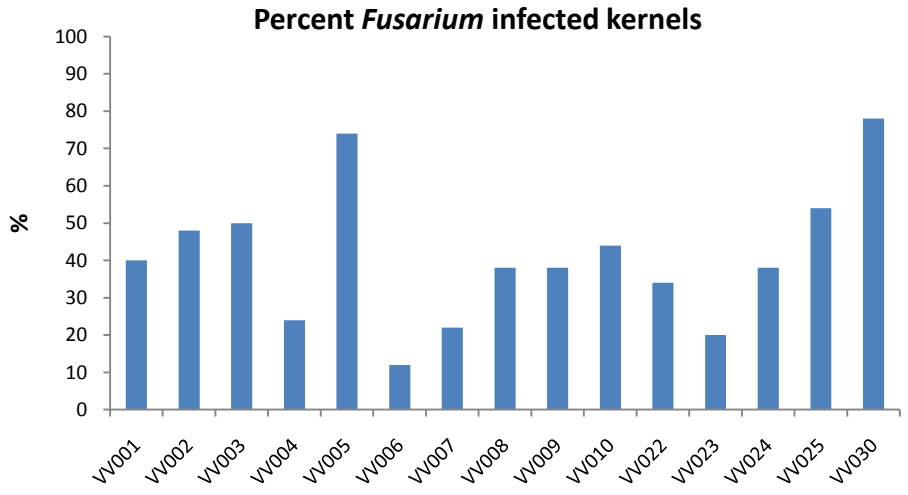

Figure 8 Percent *Fusarium* infected kernels determined by enumeration. 50 kernels from each farm trial were placed on CZID and colonies with visual signs of *Fusarium* infection counted. Denotations on the x-axis represent participating farms.

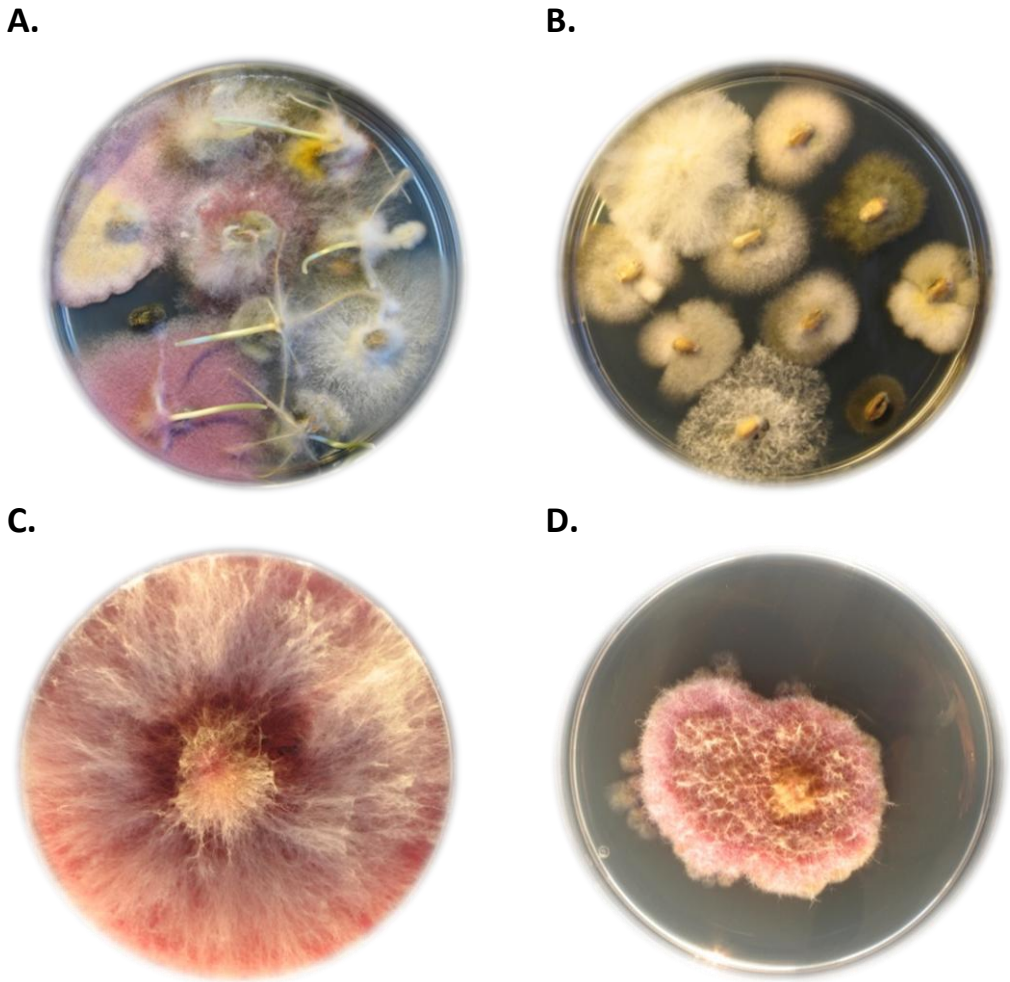

Figure 9 Plated kernels from farm trials on CZID (A), DG18 (B) and re-streaked isolates of *Fusarium* on PDA (C, D) after 7 days incubation in 25°C. Purple colonies on CZID represent *Fusarium* species.

4.4 Mycotoxin analysis

DON, ENN B, ENN B1 and MON represented the most frequently detected mycotoxins in the samples analysed. All kernel samples contained levels of ENN B and ENN B1 ranging from trace amounts to above 2 mg/kg grain. DON and MON were present in 93% and 72% of samples, respectively. In addition, ZEN, ENN A, ENN A1, ENN B2, ENN B3 and BEA were detected in a majority of the samples though at substantially lower levels. Traces of T-2 and HT-2 toxin were detected in 32% and 14% of samples, respectively, and at levels consistently below 20 µg/kg grain. Mean infection levels of ear samples were approximately in the same range as mean infection of the kernel samples, with exception of T-2 and HT-2 toxin and DON, including its derivatives. T-2 and HT-2 toxin were detected primarily in ear samples and DON primarily in kernel samples. None of the ear samples contained any 3-acetyl-DON or ZEN.

The highest level recorded for each toxin were 1130 µg/kg (DON), 299 µg/kg (DON-3-Glucoside), 74,1 µg/kg (NIV), 136 µg/kg (T2 toxin), 50,5 µg/kg (HT-2 toxin), 29,2 µg/kg (ZEN), 2090 µg/kg (ENN B), 1690 µg/kg (ENN B1), 291 µg/kg (ENN A1), 24,5 µg/kg (ENN A), 140 µg/kg (ENN B2), 0,7 µg/kg (ENN B3), 23,4 µg/kg (BEA) and 990 µg/kg (MON). The DON content was just below the maximum legal levels set for unprocessed cereals (1250 µg/kg), but exceeded the limit if combined with its derivative DON-3-Glucoside. Noteworthy is also that DON-3-Glucoside was detected in all kernel samples, and a majority contained several other *Fusarium* metabolites such as Apicidin, Butenolide, Equisetin, Culmorin, Aurofusarin, Avenacein Y and Chlamydosporol.

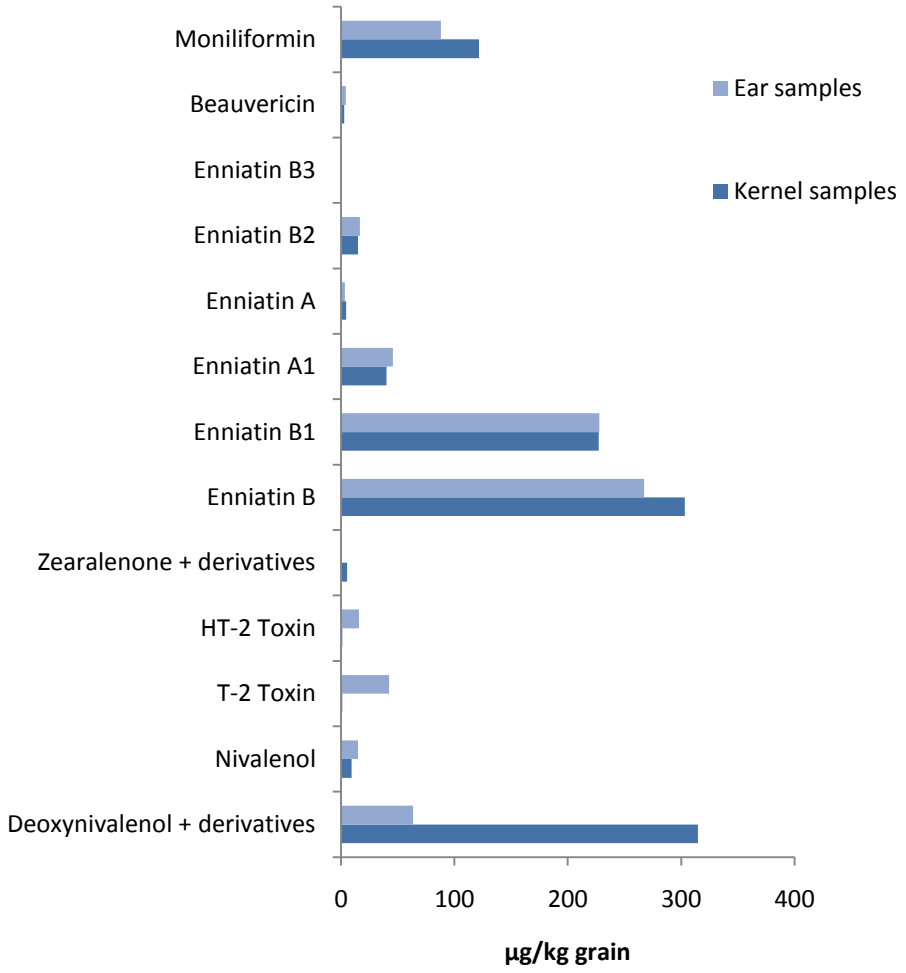

Figure 10 Comparison of mean content of mycotoxins in ear and kernel samples from 2010.

Mean toxin levels were substantially higher in 2010, compared to results from the trials in 2009, for a majority of the toxins analysed (Figure 11), however, only variation in DON, DON-3-Glucoside and NIV content are statistically significant (Table 5). A 4-fold and 8-fold increase in infection was noted for DON and DON-3-Glucoside, respectively. As a point of reference, the highest reported level of DON in 2009 was 323 µg/kg compared to 1130 µg/kg in 2010, and a third of samples did not contain any detectable levels of DON-3-Glucoside in 2009, whereas the level of contamination was 100% in 2010. Furthermore, results indicate rising levels of several ENNs and MON, but these results are not statistically significant. Wheat samples were less contaminated by NIV (P=0,000) and lower mean levels of ENN A1, ENN A and BEA were noted compared to results from 2009.

Table 5 Comparison of variation in mycotoxin content (µg toxin/kg grain) recorded in the southern and middle parts of Sweden in 2009 (Fredlund *et al.,* 2010, unpublished results) and 2010. Analyses were performed in MInitab with 2-sample t-tests.

Mycotoxin	Year	Mean	Min	Max	P-value
Deoxynivalenol	2009	63,9	0	323.2	
	2010	256	0	1130	0,001
DON-3-Glucoside	2009	7,1	0	37.6	
	2010	57,4	7.55	299	0,000
Nivalenol	2009	26,2	0	86.4	
	2010	9,16	0	74.1	0,000
Enniatin B	2009	125	0	639.2	
	2010	303	0.4	2090	0,060
Enniatin B1	2009	153	0	1190	
	2010	227	0.5	1690	0,342
Enniatin A1	2009	77	0	694.4	
	2010	40	0.4	291	0,137
Enniatin A	2009	10	0	89.6	
	2010	4,39	0	24.5	0,078
Enniatin B2	2009	4,3	0	27.7	
	2010	14,9	0	140	0,063
Beauvericin	2009	3,82	0	13.0	
	2010	2,8	0	23.4	0,118
Moniliformin	2009	65,9	0	384	
	2010	122	0	990	0,217

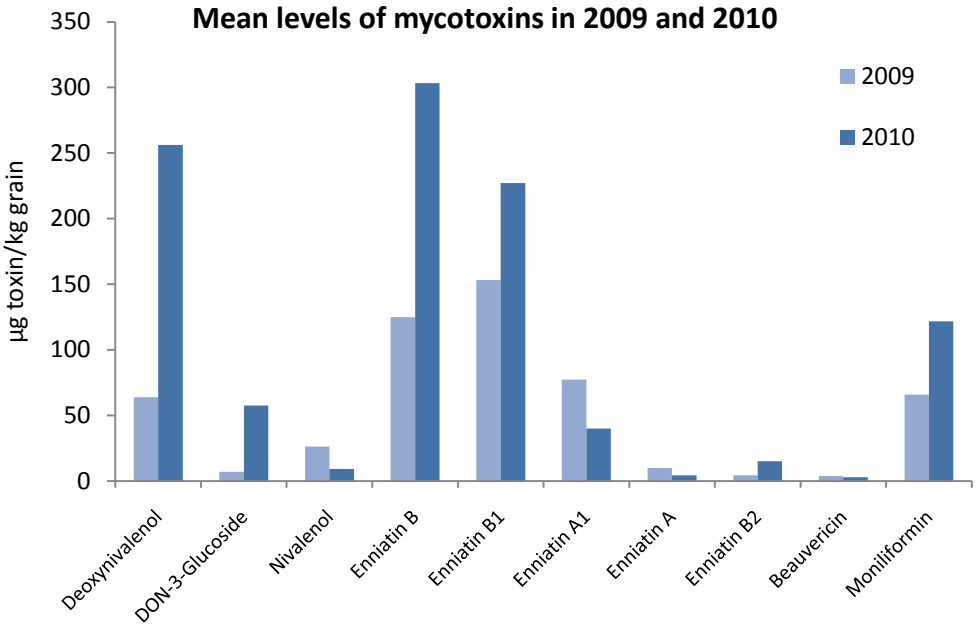

Figure 11 Comparison of mean content of common mycotoxins in wheat kernels in 2009 (Fredlund *et al.*, 2010, unpublished results) and 2010.

4.5 Regression analysis

Correlation between mycotoxin content and *Fusarium* species indicated several significant relationships (Figure 12), however, no connection was observed between the total incidence of *Fusarium* and the overall level of toxin. T2 and HT-2 toxin were excluded from the regression analysis due to recorded levels below the LOD for a majority of samples, resulting in determination coefficients without significance. The content of DON including DON derivatives were strongly linked to the presence of *F. graminearum* and *F. culmorum* (R^2=0.86) in the wheat samples. *F. graminearum* accounted for the highest individual coefficient of determination (R^2=0.69), whereas *F. culmorum* indicated a weaker connection (R^2=0.32). MON showed a very strong correlation to *F. avenaceum* (R^2=0.96). No considerable connection between NIV and any of the analysed fungal species were determined, however, results indicate a limited relationship to *F. poae* (R^2=0.17). ZEN contamination was best explained by combined *F. graminearum* and *F. culmorum* content (R^2=0.62). Findings indicate a strong relationship between levels of *F. avenaceum* and ENNs (R^2=0.88) and individual analysis of ENNs pointed out ENN B and ENN B1 as the single most predictive factors. The coefficients of determination for BEA and potent *Fusarium* producers were R^2=0.7 for *F. poae* and below R^2=0.01 for *F. avenaceum*, suggesting the likelihood of infection with BEA is foremost related to the presence of *F. poae*.

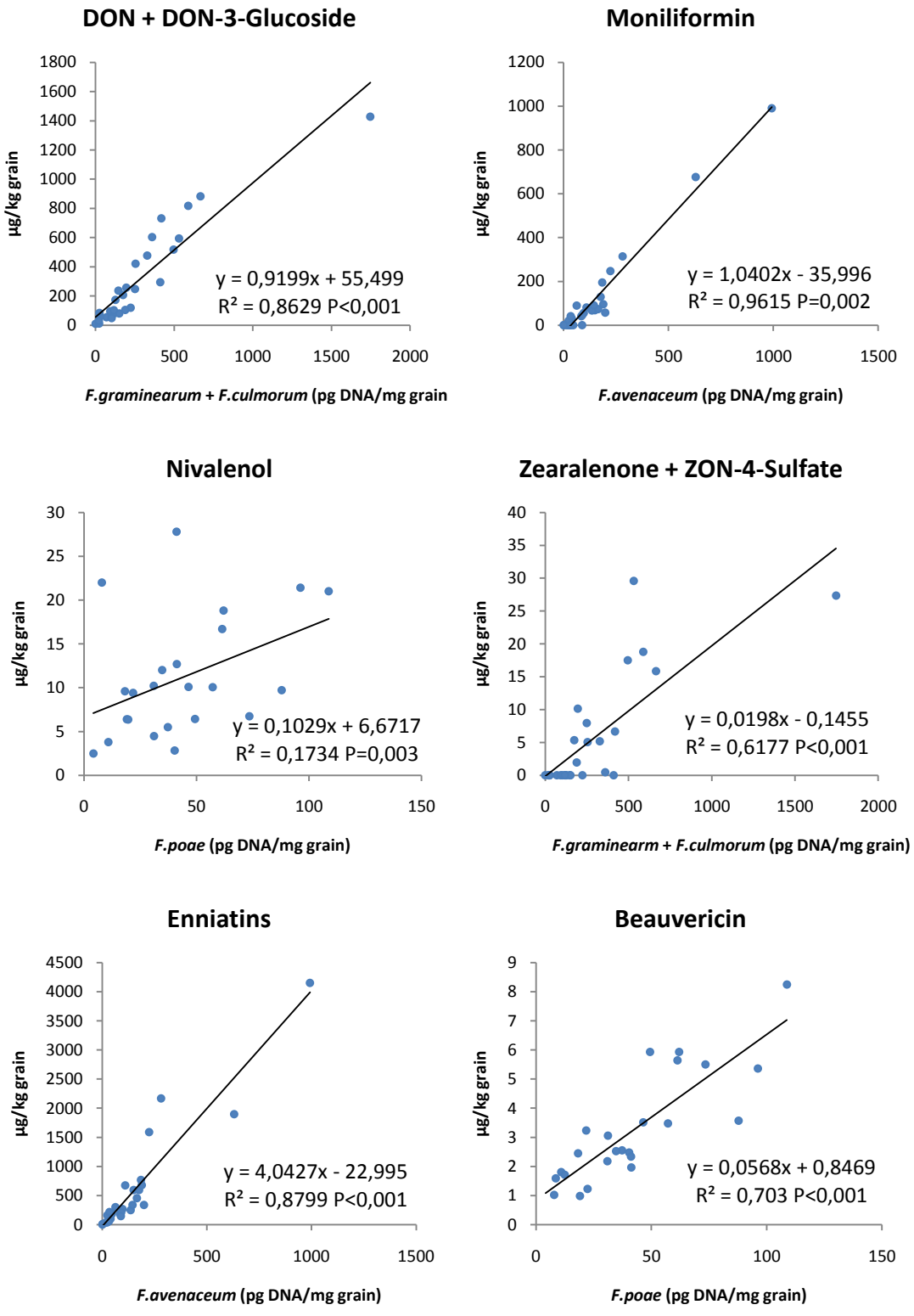

Figure 12 Correlation between mycotoxins and *Fusarium*. Plots represent the *Fusarium* species with the highest determination coefficient for each mycotoxin included in the regression analysis. P-value calculations were performed in Minitab.

4.6 Analysis of geographical differences

All statistical analyses were performed on log-transformed data and the factors Gotland and Halland were excluded due to the low number of observations within these regions. GLM studies indicated that the prevalence of *Fusarium* is related to region only in some instances. Significant variations were observed for Skåne in relation to the three remaining regions for all *Fusarium* species excluding *F. poae*. In the instance of *F. poae*, differences were established only between Skåne and Uppland. Levels of *Fusarium* were significantly lower in Skåne compared to remaining regions included in the analysis. In addition, regional variation was evaluated for combined levels of *F. culmorum* and *F. graminearum*, due to the relation established between these fungi and mycotoxin production in the correlation analysis, but without any further observations.

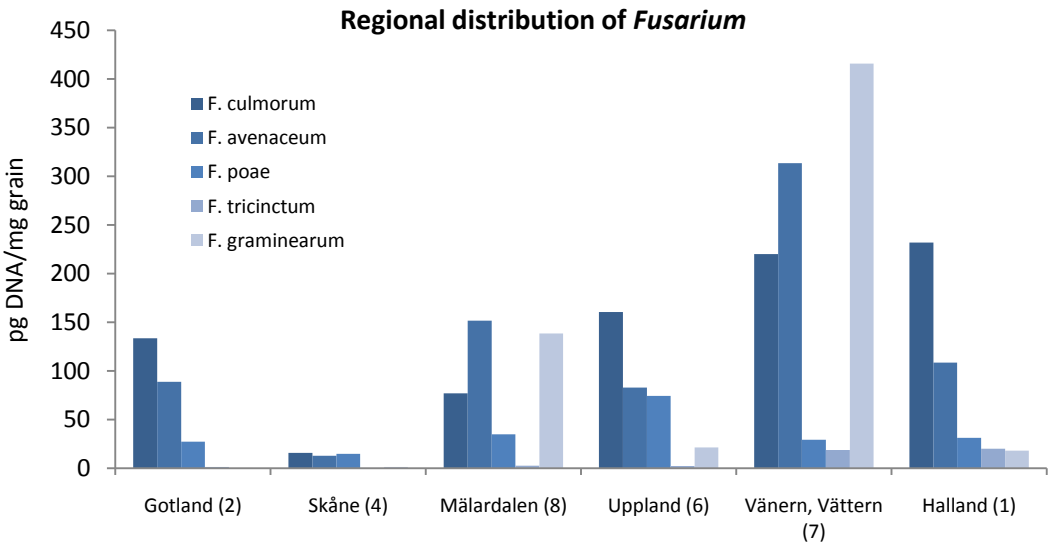

Figure 13 Regional distribution of mean *Fusarium* content. Numbers in brackets represent number of samples from each region included in the analysis.

Significant regional differences were observed for ENNs, BEA and combined levels of DON and derivatives, whereas no variation was noted for ZEN and NIV. Mean levels of DON were significantly higher in the Vänern, Vättern area compared to the incidence in Uppland, Mälardalen and Skåne, whereas wheat samples from Skåne contained significantly less DON then wheat from remaining regions. Similar results were observed for ENNs, with mean levels three times as high in the Vänern, Vättern area as detected for Mälardalen, the area with the second highest incidence. Occurrence of toxin in Skåne was once more significantly below mean levels noted for remaining regions. Pair wise comparisons among regions revealed significant differences for both DON and ENNs between all regions except Mälardalen and Uppland, were the 95% confidence interval was breached (P=0.469 and P=0.902, respectively). For BEA, only the contamination levels in Uppland could be significantly separated from

remaining regions with Tukey simultaneous tests. In addition, indications of significant variations between regions were noted for MON (P=0.051) including significantly higher levels of toxin in the area of Vänern, Vättern than in Uppland (P=0.045).

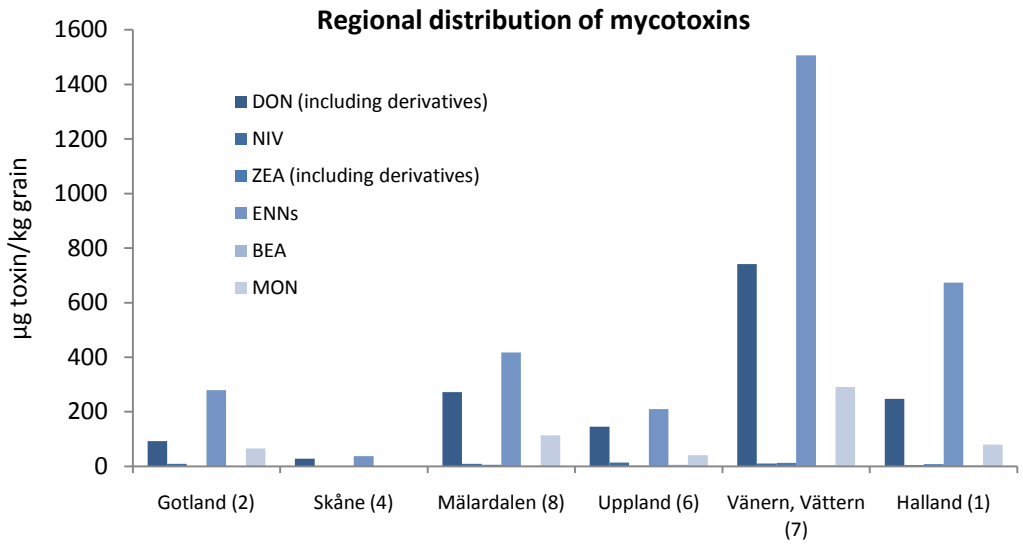

Figure 14 Regional distribution of mean mycotoxin content. Numbers in brackets represent number of samples from each region included in the analysis.

4.7 Ear samples as indicators of toxin contamination

The predictive ability of ear samples picked pre harvest on the resulting levels of mycotoxin in kernels picked post harvest were evaluated by regression analysis. All data were log-transformed prior to analysis and mycotoxin content post harvest compared with both toxin and species specific *Fusarium* levels in the ear samples. A significant correlation between *F. avenaceum* in the ear samples pre harvest and the level of ENNs in the corresponding kernel samples post harvest was established (P=0.042, R^2=0.47) and a similar relationship was indicated for MON and *F. avenaceum* (P=0.054, R^2=0.90). Moreover, the correlation between the sum of all types of toxin and total level of Fusarium infection was evaluated, but no significant relationship was found.

Regression analysis of toxin content in ears picked pre harvest and corresponding kernels post harvest was only feasible for DON, BEA and ENNs. Remaining toxins were excluded due to the low number of contaminated samples. No significant correlation could be established between mycotoxin levels pre and post harvest for any of the toxins.

5 Discussion

Results from a survey in 2009 (Fredlund *et al*., 2010, unpublished results) indicated that *Fusarium* and corresponding mycotoxins are common in Swedish wheat. Results presented in this study establish the presence of high levels of *Fusarium* and several common mycotoxins in wheat harvested in 2010, from areas in the southern and middle parts of Sweden. Mean levels of approximately 150 pg DNA/mg grain of *F. graminearum*, *F. culmorum* and *F. avenaceum* were detected by real-time PCR in a majority of samples. These three species have previously been recognised as the most predominant in Europe (Bottalico and Perrone, 2002). Only limited data is available on the specific levels of *Fusarium* fungi in wheat since a majority of studies rely on morphological analyses and several of the research papers available where real-time PCR was applied either focus on artificially inoculated grain samples, other species of *Fusarium* or other types of grain than wheat. More PCR based research is of prime importance to identify patterns in the prevalence of *Fusarium*. Compared to 2009, levels of *F. graminearum*, *F. culmorum* and *F. avenaceum* were significantly higher in 2010. The mean level of *F. avenaceum* was higher than for any of the other species. This is in line with reports from Nordic countries presenting evidence of high levels of particularly *F. avenaceum* and related mycotoxins in several grains (Uhlig *et al*., 2007). As a result of the considerable yearly variation, observations made in this study provide valuable data in the perspective of predictive modelling for *Fusarium* infection. Records of climatic and agricultural conditions are available for samples collected from field trials and of prime importance to evaluate the differences observed between 2009 and 2010. Evaluation of these data is however not covered by the scope of this study, but an interesting area for further research.

DON was found to be the most prevalent trichothecene in Swedish Spring wheat. Similar results have been reported from several other countries (Bottalico and Perrone, 2002) and mean levels (313 µg/kg for DON including derivatives) were substantially higher compared to results from 2009. Langseth and Rundberget (1999) reported mean levels of 16 µg/kg for DON in Norwegian wheat harvested in 1996-1998, suggesting an alarming increase, however, levels well above 300 µg/kg have been reported from several European countries (Bottalico and Perrone, 2002). Altogether, the varying levels reported in published literature emphasise the great fluctuations in detected levels of mycotoxins between both regions and seasons. These observations stress the importance of thorough control to prevent heavily infected wheat from reaching consumers. Higher levels of DON in 2010 than 2009 could be accounted for by climatic differences; hence weather data available for samples collected from field trials may provide valuable insights. Another important agricultural aspect is that samples from 2009 were Fall wheat, a variety less sensitive to infection.

Detected levels of ENNs, MON and BEA correspond well with observations from Norway and Finland, where levels of ENN B and ENN B1 over 1000 µg/kg are commonly reported and evidence point at ENNs being the most frequently occurring mycotoxin in Nordic wheat (Uhlig *et al*., 2006; Uhlig *et al*., 2004; Jestoi *et al*., 2004). The prevalence of MON and BEA is also consistent with previous reports. BEA is only sporadically detected, and when present, levels are usually just above the LOD.

Levels of T-2 and HT-2 toxin were notably high in a number of ear samples, however, important producers of these toxins *F. langsethiae* and *F. sporotrichioides* (Bottalico and Perrone, 2002; Thrane *et al*., 2004) were not analysed and no correlation to *Fusarium* species included in this study could therefore be established. Rising levels of T-2 and HT-2 toxin has recently been reported for European cereals (Edwards *et al*., 2009) and information about producing species is therefore of increasing concern and interest. Neither of these two species are known to be heavy contaminators of wheat, however, they have both been detected in several mapping studies. *F. langsethiae* were isolated from wheat kernels by Torp and Nirenberg (2004) and *F. sporotrichioides* were detected in 4 % of Norwegian wheat samples in 2002 (Uhlig *et al.* 2006). In a Polish study of winter wheat from 2006 and 2007 both *F. langsethiae* and *F. sporotrichioides* were detected in several samples, however, at relatively low levels (Lukanowski and Sadowski, 2008). In addition, Langseth and Rundberget (1999) detected mean levels of 108 µg/kg of T-2 and HT-2 toxin during 1996-1998 in Norweigan oats, a result they suggested might be explained by the presence of *F. sporotrichioides*, and similar observations were more recently made in Lithuanian trials (Mankevičienė *et al.*, 2007). In the light of these results it may be relevant to analyse the prevalence of *F. sporotrichioides* and *F. langsethiae* in wheat, to investigate whether a significant correlation to T-2 and HT-2 content could be established.

MON showed a very strong correlation to *F. avenaceum* (R^2=0.96), in line with results reported by Uhlig *et al.* (2004) from a survey of Norwegian wheat and Brunner *et al*. (2009) presented a strong relationships between DON and *F. graminearum* and *F. culmorum*. Fredlund *et al*. (2008) obtained similar results in a study of Swedish Spring wheat harvested in 2006, where both DON and ZEN were significantly correlated with *F. graminearum*, but with the exception that no connection to *F. culmorum* was noted. While a majority of the regression studies corresponded well with what was expected based on previous research, a few noteworthy differences were observed. In the 2009 study of wheat samples from the same regions regression studies suggested that *F. tricinctum* was likely to be the most potent producer of ENNs, whereas only a very weak connection to *F. avenaceum* content (R^2 =0,13) could be demonstrated. Findings from 2010 conversely indicate a strong relationship between levels of *F. avenaceum* and

ENNs (R^2=0.88), in accordance with results presented by Uhlig *et al.* (2006). Evidence of the natural co-occurrence between BEA and *F. avenaceum* and BEA and *F. tricinctum* has previously been presented by Logrieco *et al*. (2002), but no such connection could be established in this study. Jestoi *et al.* (2004) found a weak relationship between BEA and *F. sporotrichioides* and *F. tricinctum*, but noted the lack of correlation to *F. avenaceum* and suggested contradicting results could possibly be explained by the different sensitivities of the methods (HPLC and LC-MS/MS) applied for mycotoxin analysis.

Contradicting results is seemingly due to the application of different detection and extraction methods of both the *Fusarium* fungi and mycotoxins. Brunner *et al*. (2009) pointed out differences in results obtained when using visual scoring in comparison to PCR based detection. Correlation between visually identified infection and mycotoxin content was fairly modest in relation to detection of *Fusarium* by PCR. In addition, the influence of the choice of DNA extraction method on obtained DNA yield has been demonstrated by Fredlund *et al*. (2008). Altogether, the inconsistency in method choice makes comparison of results very difficult. Noteworthy is also that very low or no correlation was observed between mycotoxins and species that are known to be potent producers. This emphasise the need of better knowledge regarding factors that influence the natural production of different mycotoxins.

Analysis of ear samples is an interesting area for further research. In this study the number of observations was too small to draw any significant conclusions, but regression analyses indicate that there might be a connection between infection of *F. avenaceum* in ear samples pre-harvest and the resulting level of ENNs and BEA in kernels post-harvest. If a connection exists, it would serve as a useful tool for predicting the mycotoxin levels early during the season. For most of the toxins, however, no relationship could be established. This could be a result of *Fusarium* development and toxin accumulation during the late stages of cereal growth. In order to evaluate the impact of sampling time, optimisation studies are essential. Moreover, results from the PCR analysis suggest that some species grow predominately late during the season, for instance, levels of *F. graminearum* and *F. culmorum* were substantially lower in ear samples than in kernel samples. In contrast to these findings, T-2 and HT-2 toxin were found predominantly in ear samples pre harvest. This could indicate that toxin production occurred early in the growth season and were thereafter degraded gradually.

This thesis has identified *Fusarium* and corresponding mycotoxins in Swedish Spring wheat. Results on the presence of *Fusarium* species were in accordance with results published from other Nordic countries but differences were identified from a similar study of Swedish Fall wheat carried out in 2009. Standardised methods would ease comparison of results obtained in different studies and

facilitate international mapping of the current prevalence of *Fusarium*. Accurate and comparable results are of great importance in the process of constructing prediction models, an important tool in response to implications of climate change. Furthermore, extensive data is needed to obtain a comprehensive picture of the spread and growth pattern of *Fusarium*.

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