

# Diversity of Wheat Phyllosphere Fungi in Different Agricultural Production Systems

With Special Reference to *Fusarium*

Ida Karlsson

*Faculty of Natural Resources and Agricultural Sciences  
Department of Crop Production Ecology  
Uppsala*

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# Diversity of Wheat Phyllosphere Fungi in Different Agricultural Production Systems. With Special Reference to *Fusarium*

## Abstract

*Fusarium* head blight (FHB) is a disease affecting cereals world-wide caused by *Fusarium* fungi. The disease is of great economic importance especially since it is associated with contamination by harmful mycotoxins produced by *Fusarium* spp. In recent years, several studies have reported lower mycotoxin contamination of organically produced cereals compared with those produced conventionally. An unexplored factor is differences in the microbial communities on cereal crops between the organic and conventional systems, which may exert a biocontrol effect on FHB. The aim of this thesis was to investigate the effect of different environmental and agricultural factors on *Fusarium* communities and fungal phyllosphere communities in wheat in different agricultural production systems.

New primers for amplifying *Fusarium* communities, in conjunction with 454 sequencing, were evaluated, and *Fusarium* communities associated with soil and wheat kernels were successfully characterised. Comparison of fungicide-treated and untreated wheat leaves from 18 fields in Sweden in 2011 revealed that fungicide use was associated with decreased evenness of fungal communities. Furthermore, organic farming was associated with increased fungal species richness on wheat leaves when pairs of 22 organically and conventionally managed fields in Sweden were compared in 2012. There was no difference in the abundance of leaf pathogens between cropping systems. Several fungal species were consistently found regardless of geographical location, year and cropping system, while fungal abundance was highly variable between fields. Both *Fusarium* and fungal communities were affected by agricultural intensity. However, there was no difference in *Fusarium* community composition in wheat kernels from organically and conventionally managed fields. The method evaluated in this thesis can be used to monitor *Fusarium* communities in the field, which is important in order to develop strategies for limiting FHB and mycotoxin contamination. The present thesis demonstrates that fungicide use and organic farming are associated with differential fungal communities in the wheat phyllosphere. This indicates that there is an opportunity for the farmer to manage these communities with the aim of enhancing their biocontrol potential against plant pathogens such as *Fusarium*.

**Keywords:** *Fusarium* head blight, phyllosphere, leaves, winter wheat, fungi, organic farming, fungicides, biodiversity, microbial communities, high-throughput sequencing

**Author's address:** Ida Karlsson, SLU, Department of Crop Production Ecology,  
P.O. Box 7043, 750 07 Uppsala, Sweden  
**E-mail:** [Ida.Karlsson@slu.se](mailto:Ida.Karlsson@slu.se)

# Dedication

Till min mormor Kerstin Persson

*Plants wear their guts on the outside*

D. H. Janzen, *The Biology of Mutualism*, 1985

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## List of Publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

- I **Karlsson I.**, Edel-Hermann V., Gautheron N., Brandström Durling M., Kolseth A-K., Steinberg C., Persson P., Friberg H. Evaluation of genus-specific primers for studying *Fusarium* communities in field samples (submitted manuscript).
- II **Karlsson I.**, Friberg H., Steinberg C., Persson P. (2014). Fungicide effects on fungal community composition in the wheat phyllosphere. *PLoS ONE* 9(11), e111786.
- III **Karlsson I.**, Friberg H., Steinberg C., Kolseth A-K, Persson P. Organic farming increases fungal species richness in the wheat phyllosphere (manuscript).
- IV **Karlsson I.**, Friberg H., Steinberg C., Kolseth A-K, Persson P. Factors affecting *Fusarium* community composition in wheat kernels (manuscript).

Paper II is reproduced with the permission of the publisher.

The contribution of Ida Karlsson to the papers included in this thesis was as follows:

- I Planned the experiments together with the co-authors. Performed parts of the experimental work and parts of the data analyses. Wrote a large part of the paper.
- II Planned the study together with the co-authors. Performed or supervised field work and laboratory analyses. Analysed the data and wrote the majority of the paper. Responsible for correspondence with the journal.
- III Planned the study together with the co-authors. Performed or supervised field work and laboratory analyses. Analysed the data and wrote the majority of the paper.
- IV Planned the study together with the co-authors. Performed or supervised field work and laboratory analyses. Analysed the data and wrote the majority of the paper.

## Abbreviations

BCA	biocontrol agent
CFU	colony forming units
DON	deoxynivalenol
EF-1 $\alpha$	transcription elongation factor 1 alpha
FHB	Fusarium head blight
GLM	generalised linear model
HTS	high-throughput sequencing
ITS	internal transcribed spacer
NIV	nivalenol
NMDS	non-metric multidimensional scaling
OTU	operational taxonomic unit
PCR	polymerase chain reaction
RDA	redundancy analysis
SH	species hypothesis
VBNC	viable but nonculturable
ZEA	zearalenone



## Svensk sammanfattning

Axfusarios är en sjukdom som drabbar stråsäd orsakad av svampar inom släktet *Fusarium*. Svamparna infekterar axen när grödan växer på fältet och kan där bilda mykotoxiner. Dessa mykotoxiner kan ha negativa hälsoeffekter på både människor och djur och därför finns gränsvärden för spannmål som säljs för livsmedelproduktion och foder i EU. De senaste åren har höga mykotoxinhalter uppmätts i spannmål i Sverige, framförallt av mykotoxinet deoxynivalenol (DON). Detta har medfört stora förluster för lantbrukare då spannmål som överskrider gränsvärdena får gå till förbränning. Det är viktigt att hitta åtgärder som kan begränsa infektionen och mykotoxinbildningen under odlings säsongen.

Flera europeiska studier har visat att halten av *Fusarium*-mykotoxiner är lägre i ekologiskt odlad spannmål jämfört med konventionellt odlad. Vad detta beror på är än så länge oklart. En faktor som inte är undersökt i sammanhanget är vilken betydelse andra mikroorganismer som lever på spannmål kan ha för infektion med *Fusarium*. Växter lever i samspel med många mikroorganismer både i jorden – rhizosfären, och ovan jord – fyllosfären. Det är troligt att *Fusarium*-svampar kommer i kontakt med andra mikroorganismer på t.ex. blad när de sprids i ett bestånd. Även ett grönt och till synes friskt blad bär på en mångfald av för ögat osynliga bakterier och svampar. Man vet att naturligt förekommande mikroorganismer kan motverka angrepp av olika sjukdomsframkallande svampar och bakterier genom t.ex. produktion av antibiotiska substanser, parasitism eller konkurrens om näring och utrymme.

Hypotesen bakom denna avhandling var att det finns en högre mångfald av mikroorganismer, och ett mer sjukdomshämmande mikrobiom i ekologisk odling, vilket ger upphov till lägre förekomst av *Fusarium* och lägre mykotoxinbildning jämfört med konventionell odling. Studier av andra organismgrupper som t.ex. insekter och växter pekar på att det ofta finns högre biologisk mångfald i ekologiskt odlade fält jämfört med konventionellt odlade, men det

finns inte så mycket kunskap om hur mikroorganismer påverkas av odlingsssystemet. Kunskapen om svampsamhällets sammansättning och ekologi i fyllösfären på spannmål är låg och det bredare syftet med denna avhandling var att med hjälp av ny sekvenseringsmetodik kartlägga svampsamhället på veteblad och undersöka hur det påverkas av olika odlingsåtgärder och miljöfaktorer.

Sekvensering av fylogenetiska markörer användes för att studera svampsamhällets artsammansättning. En fylogenetisk markör är en gen som varierar tillräckligt mycket för att kunna skilja olika arter åt. Beroende på vilken organismgrupp man studerar t.ex. bakterier, växter eller svampar behöver man använda olika gener som markörer. För att bestämma sammansättningen i ett svampsamhälle kopierar man först upp den valda markören med s.k. PCR och därefter sekvenseras kopiorna. De nya sekvenserna jämförs därefter med sekvenser från kända arter i databaser för artbestämning. De senaste åren har ny sekvenseringsmetodik, massiv parallellsekvensering ("next generation sequencing" eller "high-throughput sequencing") gjort det möjligt att skapa en mer detaljerad bild av vilka mikroorganismer som finns i olika miljöer och göra större och bättre replikerade studier.

I avhandlingen undersöktes svampsamhället på veteblad i två studier med hjälp av massiv parallellsekvensering. I den första studien kartlades svampsamhället på fungicid-behandlade och obehandlade blad från 18 fält i juni 2011. I den andra studien jämfördes blad från 11 par av ekologiskt och konventionellt odlade fält i juli 2012. År 2011 dominerades svampsamhället av olika jästsvampar av släktena *Sporobolomyces*, *Dioszegia* och *Cryptococcus*. År 2012 hade markant högre sjukdomstryck från bladfläcksvampar och släktet *Zymoseptoria* dominerade svampsamhället detta år. Detta släkte innefattar *Z. tritici* (*Mycosphaerella graminicola*) som orsakar svartpricksjuka på vete. Trots dessa skillnader var de vanligaste svamparterna relativt lika oavsett år och behandling och fyra svampar påträffades i alla fält under båda åren.

Två olika mått användes för att uppskatta den biologiska mångfalden av svampar - artrikedom (antal arter) och jämnhet ("evenness"). Jämnheten anger hur jämnt fördelade arterna är i ett samhälle och högre värden innebär ökad mångfald. Resultaten visade att fungicid-behandling minskade svampsamhällets jämnhet men inte hade någon märkbar effekt på artrikedomen. Ekologisk odling hade motsatt effekt, där antalet svamparter var signifikant högre i ekologiskt odlade fält än i konventionellt odlade, medan ingen skillnad i jämnhet kunde observeras. Undersökningarna visade också att både fungicid-behandling och odlingsystem påverkar svampsamhällets sammansättning, men effekten var relativt liten. Dessutom var det ingen skillnad på mängden av sjukdomsframkallande svampar på bladen i ekologisk respektive konventionell odling, men variationen var stor mellan olika fält.

Axfusarios orsakas av ett flertal olika *Fusarium*-arter och upp till 17 arter har förknippats med sjukdomen. Arterna har olika miljöpreferenser och är olika känsliga för kemiska bekämpningsmedel vilket gör det mer komplicerat att utveckla bekämpningsstrategier och göra prognoser för sjukdomen och mykotoxinbildningen. Det blir också mer krävande att studera sjukdomen då det är tidskrävande att ta reda på vilka *Fusarium*-arter som finns i ett prov med traditionella metoder som odling på näringsagar. Molekylära metoder för att kartlägga svampsamhällen använder markörer som inte ger information på art-nivå för *Fusarium*. I denna avhandling utvärderades och utvecklades en metod för att kartlägga alla *Fusarium*-svampar i ett prov med hjälp av massiv parallellsekvensering. Resultaten visade att metoden kunde identifiera vilka *Fusarium*-arter som fanns och också hur stor del varje art utgjorde, med god noggrannhet. Metoden tillämpades på vetekärnor från 18 fält i Sverige i ekologisk respektive konventionell odling. Resultaten visade att upp till åtta *Fusarium*-arter kunde finnas i ett och samma fält vid samma tidpunkt. Det var ingen skillnad mellan odlingssystem men däremot påverkade intensiteten i odlingen *Fusarium*-sambället. Odlingsintensiteten mättes som ett index bestående av antalet genomförda kemiska bekämpningar och mängden tillfört kväve. Detta pekar på att dessa faktorer är viktiga för *Fusarium*-förekomsten i vetekärnor.

Metoden som utvärderades i avhandlingen innebär att man inte på förhand måste bestämma vilka arter man letar efter, vilket underlättar övervakning av *Fusarium*-förekomsten i spannmål. Övervakningen är viktig för att t.ex. kunna upptäcka förändringar i *Fusarium*-samballets sammansättning över tid och spridning av *Fusarium*-arter till nya områden. En bättre kunskap om hela *Fusarium*-samballets ekologi är viktig för att förstå axfusariosens epidemiologi och för att kunna utveckla effektiva bekämpningsmetoder mot sjukdomen och mykotoxinbildningen.

En detaljerad kartläggning av mångfalden av bladsvampar i rådande veteproduktion i Sverige kunde göras tack vare ny metodik. Resultaten visar att både odlingssystem och fungicider påverkar de bladlevande svamparna vilket innebär att det finns möjlighet att påverka dessa svampsamhällen i odlingen. Den stora variationen i mängden svamp som uppmättes, även mellan näraliggande fält, tyder på att andra faktorer också är viktiga som t.ex. väderförhållanden och inflöde av mikroorganismer från atmosfären. Det innebär att det troligen är svårare att påverka mikroorganismer ovan än under jord. Men med ökad kunskap om bladlevande svampars ekologi och funktion kan odlingsstrategier utvecklas som gynnar naturligt förekommande sjukdomshämmande mikroorganismer mot *Fusarium* eller andra sjukdomsframkallande organismer.



# 1 Introduction

Fusarium head blight (FHB) is an important disease in cereal production world-wide caused by a range of *Fusarium* fungi. The most problematic aspect of the disease is contamination by a diverse set of *Fusarium* mycotoxins that can occur already when the crop is growing in the field. High mycotoxin levels, especially of the mycotoxin deoxynivalenol (DON), have been observed in Sweden in recent years (Lindblad *et al.*, 2013). This has led to severe economic losses for farmers as contaminated grain cannot be sold for food or feed.

Several European surveys have shown that the amount of *Fusarium* and mycotoxin contamination is lower in organic cereal production than in conventional production (Birzele *et al.*, 2002; Edwards, 2009; Gottschalk *et al.*, 2009; Meister, 2009; Bernhoft *et al.*, 2010). The cause of the lower mycotoxin contamination in organic production is poorly understood but certain farming practices differing between the conventional and organic systems have been proposed as explanations. These include pesticide use, high nitrogen application and cereal intense rotations in the conventional system (Edwards, 2009; Bernhoft *et al.*, 2010).

An unexplored factor in this context is that there might be differences in the indigenous microbial communities present on cereal crops between the organic and conventional systems. These microorganisms may have competitive interactions with *Fusarium* and could exert a biocontrol effect on FHB. Organic farming has often been associated with higher biodiversity of for example birds, plants and insects (Hole *et al.*, 2005; Tuck *et al.*, 2014). However, little is known about the effect of organic farming on the microorganisms inhabiting the aerial parts of cereal crops.

Many countries have devoted support and subsidies to organic production with the aim of reducing the environmental impact of agriculture. For example, in 2005 the European Union devoted 17% of the €3.83 billion spent on agri-environmental measures to organic agriculture (European Commission, 2010). Between 2000-2008 the area under organic production increased at an annual

rate of 7.4% in the EU and there is growing demand for organic products from consumers (European Commission, 2010). Organic agriculture is governed by a set of criteria that can differ between countries and certification schemes. In the EU, there is a common regulation for organic production (Commission regulation 889/2008/EC). Additionally, most organic producers in Sweden are certified by the Swedish certification association KRAV, which has set additional criteria, *e.g.* regarding animal welfare. However, two criteria central to organic farming are the exclusion of mineral fertilisers and chemical pesticides. Instead, farmers practising organic production rely on animal manure, nitrogen-fixing crops and by-products from the food industry as fertilisers. Weed control is performed by mechanical weeding and pests and diseases are managed by growing more resistant cultivars and crop rotation.

Wheat (*Triticum aestivum*) is grown on a larger proportion of land worldwide than any other crop (Food and Agriculture Organization of the United Nations, 2013) and is an important cash crop for farmers. In Sweden, two main types of wheat are grown, spring wheat and winter wheat, of which the latter is grown on the largest area (Statistics Sweden, 2014). Winter wheat is sown in the autumn and requires vernalisation (a sufficiently long cold period) to flower in the summer. In this thesis, winter wheat was used as a model to investigate differences in phyllosphere fungal communities between organic and conventional production.

## 2 Aim

The overall aim of this thesis was to characterise the *Fusarium* and fungal communities present in wheat and explore their response to different agricultural management practices.

The specific aims of the different studies were:

- To evaluate genus-specific primers for amplicon high-throughput sequencing of *Fusarium* communities in field samples (Paper I)
- To investigate how fungicides affect fungal diversity and community composition in wheat leaves in two different areas of Sweden (Paper II)
- To study how organic farming affects fungal diversity and community composition in wheat leaves (Paper III)
- To determine how agricultural and environmental factors affect *Fusarium* community composition in wheat kernels (Paper IV)



## 3 *Fusarium*

### 3.1 *Fusarium* – a Diverse Genus

*Fusarium* is a large and diverse fungal genus of great economic and agricultural importance. *Fusarium* fungi have world-wide distribution and are ubiquitous in many habitats including soil, water and in association with plants (Palmero *et al.*, 2009; Summerell *et al.*, 2010).

Currently the definition of *Fusarium* is polyphyletic and includes three teleomorph genera: *Gibberella*, *Haematonectria* and *Albonectria* (Summerell & Leslie, 2011; Geiser *et al.*, 2013). The genetic diversity within *Fusarium* is high and several species are regarded as species complexes (O'Donnell *et al.*, 2013). For example, it has been proposed that *F. graminearum* comprises 13 phylogenetically distinct species (O'Donnell *et al.*, 2004, 2008).

Many fusaria are important plant pathogens. In cereal production, *Fusarium* spp. cause root and crown rot (Smiley *et al.*, 2005) and FHB, which is caused by *F. graminearum* and a range of other species (Parry *et al.*, 1995). There are also several soil-borne pathogens within *Fusarium*. *Fusarium solani* (teleomorph *Haematonectria haematococca*) causes diseases on pea, bean, potato, citrus and other crops (Leslie & Summerell, 2006). The different *formae speciales* of *F. oxysporum* are responsible for severe vascular wilts and root rot diseases of a wide range of crops. For example, Fusarium wilt of banana, caused by *F. oxysporum* f. sp. *cubense*, has been a serious threat to the banana industry for decades (Ploetz, 2015). Recently, *F. graminearum* and *F. oxysporum* were selected among the top 10 fungal plant pathogens based on their scientific and agricultural importance (Dean *et al.*, 2012). Interestingly, some strains of *F. oxysporum* have an effect as biological control agents (BCAs) against Fusarium wilts (Alabouvette *et al.*, 2009).

Moreover, several *Fusarium* species are human pathogens causing for example corneal infections (Chang *et al.*, 2006) and infections of immunocom-

promised patients (Guarro, 2013). *Fusarium* species have also been reported as pathogens of several marine animals (Khoa *et al.*, 2004; Makkonen *et al.*, 2013).

One of the most prominent characteristics of *Fusarium* is the diverse range of secondary metabolites they produce, including many mycotoxins (Summerell & Leslie, 2011). Notably, *Fusarium* species produce three important classes of mycotoxins: trichothecenes, fumonisins and zearalenones (Desjardins, 2006).

### 3.2 *Fusarium* Head Blight

*Fusarium* head blight (FHB) (synonyms: ear blight, scab) is an important disease in cereal production world-wide. The disease affects the heads of small grain cereals: wheat, barley, oats, rye and triticale. Symptoms of FHB can include pink to red mycelial growth and/or bleached spikelets and grains can become shrunken (Parry *et al.*, 1995). *Fusarium* head blight is caused by several *Fusarium* species of which *F. graminearum*, *F. culmorum* and *F. avenaceum* predominate, but up to 17 species have been associated with the disease (Parry *et al.*, 1995).

*Fusarium* head blight can cause economically important yield reductions (Windels, 2000; Nganje *et al.*, 2004). However, it is often the mycotoxin contamination that makes the disease most problematic (D’Mello *et al.*, 1999; Reddy *et al.*, 2010). Many of the *Fusarium* species involved in FHB produce one or several mycotoxins. Important mycotoxins associated with FHB that have negative effects on human and animal health (D’Mello *et al.*, 1999; Reddy *et al.*, 2010) include DON, nivalenol (NIV), HT-2 and T-2, zearalenone (ZEA) and fumonisins. The type A trichothecenes are considered the most toxic, *e.g.* T-2 toxin (type A) has a higher toxicity than DON (type B) (Krska *et al.*, 2001). In addition, there are several so-called ‘emerging’ *Fusarium* toxins including enniatins, beauvericin and moniliformin that have received less attention (Jestoi, 2008). In Sweden, DON has been considered the most important contaminant in cereal production but other mycotoxins may also be prevalent (Lindblad *et al.*, 2013).

In farm animals, exposure to *Fusarium* toxins has been linked to reduced feed intake, oral- and gastrointestinal lesions and reproductive dysfunction (D’Mello *et al.*, 1999). Pigs have the highest sensitivity to DON among domestic animals (Miller, 2008). Historically, severe outbreaks of *Fusarium* toxicoses of humans have occurred, *e.g.* in Russia during the Second World War (Joffe, 1971; Miller, 2008). Nowadays, in many countries harvested grain is often tested for the presence of *Fusarium* toxins in order to protect human and

animal health. In the EU, there are maximum limits for DON, ZEA and fumonisins in cereals designated for human consumption (Commission Regulation 1881/2006/EC) and maximum recommendation levels for animal feed (Commission Recommendation 2006/576/EC). Recently, the Commission adopted indicative levels for HT-2 and T-2 (Commission Recommendation 2013/165/EU).

### 3.2.1 Disease Cycle and Epidemiology

The *Fusarium* species involved in FHB generally produce asexual conidia, but some have sexual stages producing ascospores such as *F. graminearum* (teleomorph: *Gibberella zeae*) and *F. avenaceum* (teleomorph: *G. avenacea*). *Fusarium culmorum* produces thick-walled chlamydospores (Leslie & Summerell, 2006).

*Fusarium* inoculum sources include infected crop residues, seed and soil, and the inoculum can be spread by air (Parry *et al.*, 1995; Champeil *et al.*, 2004). Infected seed may play a role as an inoculum source and in the spread of the disease to new areas (Champeil *et al.*, 2004; Persson & Bötter, 2014). The importance of alternative host plants such as weeds (Jenkinson & Parry, 1994) for FHB has also been discussed but their importance is unclear. *Fusarium* species can survive saprotrophically on crop residues and infect the following crop. Dispersal of conidia from crop residues and/or infected stem bases by rain splashing has been proposed as a major infection route (Champeil *et al.*, 2004). Wind dispersal can also be important, especially for ascospores (Keller *et al.*, 2014). Ascospores are formed in perithecia and discharged under favourable environmental conditions. However, the importance of locally present inoculum in the field compared to air-borne inoculum is not well understood (Keller *et al.*, 2014).

It appears that infection occurs directly at the head of the plant rather than by systemic growth, *e.g.* from infected stem-bases (Parry *et al.*, 1995). However, gradual upward movement of *Fusarium* infection towards the head throughout the season has been reported (Zinkernagel *et al.*, 1997) that could be the result of repeated splash-dispersed infections. The crop is most susceptible to *Fusarium* infection at flowering. Therefore, the weather conditions at flowering are one of the most important risk factors of FHB. Warm and wet weather conditions during this period have been shown to favour *F. graminearum* infection and subsequent DON contamination (Obst *et al.*, 1997).

### 3.2.2 Control of Fusarium Head Blight

*Fusarium* fungi can produce significant amounts of mycotoxins already when the crop is growing in the field, in contrast to other toxigenic fungi that develop

during storage, such as *Penicillium* and *Aspergillus* (Miller, 2008). Therefore, control methods must aim at reducing the spread of the pathogens and infection and disease development in the field.

Several agricultural practices are known to increase the risk of FHB (Champeil *et al.*, 2004; Edwards, 2004). Because crop residues can carry *Fusarium* inoculum to the following crop, both the type of preceding crop and the amount of crop residues are important. Preceding crops that are susceptible hosts for *Fusarium* increase the risk of FHB. Maize has been associated with the highest risk of *Fusarium* and DON contamination (Obst *et al.*, 1997; Dill-Macky & Jones, 2000; Schaafsma *et al.*, 2001). Continuous cropping of small grain cereals has also been associated with increased *Fusarium* and mycotoxin contamination (Bernhoft *et al.*, 2012). Ploughing is a way of reducing the amount of crop residues on the surface and will also increase decomposition of residues (Leplat *et al.*, 2012). Ploughing is generally considered to decrease *Fusarium* infection and DON contamination (Obst *et al.*, 1997; Beyer *et al.*, 2006).

Furthermore, high nitrogen application through mineral fertilisers has been associated with increased *Fusarium* infection (Martin, 1991; Bernhoft *et al.*, 2012). Nitrogen fertilisation may have indirect effects on FHB through changes in the microclimate in the canopy, *e.g.* humidity (Tompkins *et al.*, 1993) as well as physiological changes of the plant cell wall (Van Arendonk *et al.*, 1997).

Chemical control of *Fusarium* infection and mycotoxin contamination has been limited and inconsistent (Parry *et al.*, 1995). For example, in field trials inoculated with *F. culmorum* or *F. graminearum*, FHB disease severity was reduced by 25-77% depending on cultivar, location and type of fungicide (Haidukowski *et al.*, 2005). Fungicide application at flowering appears to be most efficient in reducing *Fusarium* incidence and DON contamination (Chala *et al.*, 2003). Application of fungicides before anthesis targeting leaf pathogens has even been reported to increase *Fusarium* infection in cereals (Henriksen & Elen, 2005). The type of fungicide is also of importance, where the triazole fungicides have been most efficient, but with large variation between different triazoles (Paul *et al.*, 2008; Edwards & Godley, 2010). Several fungal and bacterial BCAs have been tested against *F. graminearum* (Gilbert & Fernando, 2004). To date, no biocontrol products against FHB are commercially available in Europe.

Genetic resistance could be an effective means of controlling the disease and much effort has been invested in breeding FHB-resistant wheat cultivars (Bai & Shaner, 2004; Buerstmayr *et al.*, 2009). Although FHB-resistant wheat lines have been developed in breeding programmes world-wide, it has proven

difficult to combine resistance with other desired characters such as high yield (Bai & Shaner, 2004).



## 4 The Phyllosphere

### 4.1 The Phyllosphere – a microenvironment of interest

The phyllosphere is the above-ground parts of plants as a habitat for microorganisms. The term phyllosphere was coined in analogy to the rhizosphere (Last, 1955; Ruinen, 1956) and is often used referring only to the leaves. Sometimes the leaf surface is specifically identified as the ‘phylloplane’, whereas other areal plant parts are described with other names, *e.g.* the anthersphere (flowers), carposphere (fruits) and caulosphere (stems) (Leveau, 2015). In this thesis, I will use the term to define total above-ground plant parts (Newton *et al.*, 2010). The phyllosphere is the habitat for many bacteria, yeasts, filamentous fungi and sometimes other eukaryotes (Lindow & Brandl, 2003; Fonseca & Inacio, 2006; Vorholt, 2012). Some organisms live on the surface of plants as epiphytes, while the endophytes live inside the plant tissue, but rather than being distinct these two categories might be more of a continuum (Beattie & Lindow, 1999).

The phyllosphere is interesting from several human perspectives, which calls for a better understanding of the ecology of phyllosphere microorganisms. Many pathogens attack the leaves and fruits of crops, but the phyllosphere can also harbour microorganisms with biocontrol activity (Enya *et al.*, 2007; Janisiewicz *et al.*, 2010). In the wine-making industry, microorganisms naturally present on grape berries can affect the fermentation process and the product quality (Fleet, 2003). Due to outbreaks of *Salmonella* and *Escherichia coli* linked to contaminated fresh vegetables, there has been an interest in understanding the interactions of these human pathogens with the other inhabitants of the phyllosphere (Klerks *et al.*, 2007; Heaton & Jones, 2008; Ottesen *et al.*, 2013). Phyllosphere microorganisms may also provide an ecosystem service to human health. It has been proposed that exposure to environmental microbiota

in the air, soil and on plants, is essential for regulating the human immune system (Rook, 2013).

Bacteria are considered the most numerous inhabitants often with population sizes of  $10^6$  to  $10^7$  cells/cm<sup>2</sup> (Lindow & Brandl, 2003). Yeast populations on different plant leaves have been estimated to range between  $10^1$  and  $10^5$  colony forming units (CFUs)/cm<sup>2</sup> (Fonseca & Inacio, 2006). Whereas many studies of the phyllosphere have focused on bacteria, the focus of this thesis is on phyllosphere fungi.

## 4.2 Life in the Phyllosphere

The phyllosphere is considered a harsh environment for microorganisms with low nutrient and water availability, exposure to high temperatures and UV radiation (Lindow, 2006). It is believed that phyllosphere microorganisms have developed adaptations to survive in this habitat, for example pigmentation to protect against UV radiation. Sundin & Jacobs (1999) found that pink- and orange-pigmented bacteria isolated from the phyllosphere of groundnut were less sensitive to UV-B radiation compared to non-pigmented bacteria. The presence of pigmented yeasts in the phyllosphere of many plants has often been reported (Fonseca & Inacio, 2006). Another character often encountered in the phyllosphere is the production of forcefully discharged ballistoconidia by many basidiomycete yeasts, which is regarded an important trait for efficient dispersal (Fonseca & Inacio, 2006).

Patchy distribution of microorganisms on leaves has often been described (Kinkel, 1997). In a study by Andrews *et al.* (2002), *Aureobasidium pullulans* exhibited a patchy distribution on leaves with more fungal growth in the veinal areas. *A. pullulans* colonised leaves as groups of cells or single cells while hyphae were infrequently observed (Andrews *et al.*, 2002).

The availability of nutrients is a limiting factor for phyllosphere organisms (Bashi & Fokkema, 1977; Mercier & Lindow, 2000). The availability of sugar exudates to phyllosphere bacteria has been shown to be both spatially and temporally heterogeneous, which may explain the spatial distribution of microorganisms on leaves (Leveau & Lindow, 2001). In addition to leaf exudates, exogenous nutrient sources such as pollen or aphid honeydew may also be consumed by phyllosphere microorganisms (Dik *et al.*, 1992).

### 4.2.1 Factors affecting Phyllosphere Communities

The dynamic nature of the phyllosphere has often been emphasised (Kinkel, 1997; Vorholt, 2012). Leaves of both annual plants and perennial deciduous plants are colonised by microorganisms each year. Microbial colonisers of the

phyllosphere can originate from different sources including soil, air, seed and other plants (Vorholt, 2012). The atmosphere is important both as a source and a sink of phyllosphere microorganisms (Kinkel, 1997). However, the contribution of different sources is not well understood.

Phyllosphere populations generally increase during the season (Magan & Lacey, 1986; Thompson *et al.*, 1993; Legard, 1994; Williams *et al.*, 2013). On wheat leaves, the abundance of the yeast *Sporobolomyces roseus* has been observed to increase over the growing season, and few yeast colonies were observed during the first half of a leaf's lifespan (Last, 1955). Successional patterns throughout the growing season have also been reported generally with initial colonisation by bacteria followed by yeasts and finally filamentous fungi (Kinkel, 1997). Climate factors appear to be important for phyllosphere populations, especially relative humidity (Bashi & Fokkema, 1977; Bokulich *et al.*, 2014).

The biogeography of microorganisms is less well known than for macroorganisms, but microorganisms also exhibit biogeographical patterns (Martiny *et al.*, 2006). Several studies have reported negative correlations between microbial community similarity and geographical distance (Arnold *et al.*, 2003; Finkel *et al.*, 2012; Rastogi *et al.*, 2012). A recent meta-analysis of soil and phyllosphere fungal communities found that fungal richness was negatively correlated to latitude (Meiser *et al.*, 2014).

There is evidence suggesting that the plant genotype is important for phyllosphere communities (Whipps *et al.*, 2008). Redford *et al.* (2010) reported a correlation between tree species phylogeny and the phylogenetic composition of bacterial communities. Some studies have revealed cultivar-specific phyllosphere communities on different crops such as potato, lettuce and grapes (Sesitsch *et al.*, 2002; Hunter *et al.*, 2010; Bokulich *et al.*, 2014), while no differences among cultivars was observed for endophytes in wheat (Larran *et al.*, 2002). Moreover, agricultural management practices such as pesticide application (Fokkema, 1988; Gu *et al.*, 2010; Čadež *et al.*, 2010) and cropping system (Granado *et al.*, 2008; Ottesen *et al.*, 2009; Gasser & Berg, 2011) can influence phyllosphere communities.

Nevertheless, many questions regarding the ecology of phyllosphere microorganisms remain unanswered, including the most important drivers of community structure in the phyllosphere, their functional roles and interactions with their host plants.

#### 4.2.2 Implications for Plant Health

Phyllosphere microorganisms influence their host plants in a number of different ways and may have both positive and negative effects on plant health and productivity (Newton *et al.*, 2010; Friesen *et al.*, 2011).

One important positive effect of phyllosphere microorganisms is biocontrol of plant diseases. Some microorganisms have antagonistic properties against pathogens. Disease suppression by antagonistic microorganisms involves different mechanisms including resource competition, antibiosis, parasitism and induced resistance (Blakeman & Fokkema, 1982). Biocontrol of foliar diseases can be achieved either by adding antagonistic microorganisms (BCAs) to the phyllosphere (augmentative biocontrol) or by stimulating naturally occurring antagonists (conservation biocontrol). Several studies have isolated antagonistic microorganisms from the phyllosphere of different plants (Perello *et al.*, 2001; Enya *et al.*, 2007; Janisiewicz *et al.*, 2010; Schmid *et al.*, 2011).

More diverse microbial communities have in theory a higher probability of containing antagonists to pathogens or a higher antagonistic co-evolutionary potential (Kinkel *et al.*, 2011). For example, disease suppression of corky root of tomato has been correlated to microbial activity and the abundance and diversity of several microbial groups (Van Bruggen, 1995). Inoculation with an assemblage of fungal endophytes reduced damage by *Phytophthora* sp. in the cacao tree (Arnold *et al.*, 2003).

Phyllosphere microorganisms may also have negative effects on plant health. An example is the observation of so-called ‘helper bacteria’ associated with *Parastagonospora nodorum* (*Phaeosphaeria nodorum*) that can facilitate infection by the fungus (Dewey *et al.*, 1999). However, most phyllosphere microorganisms are thought to have neutral interactions with the plant. But even the presence of saprotrophic species may come with a cost to the plant. Some of the yield increase after fungicide treatment not explained by pathogen attack could be attributed to delayed leaf senescence in the absence of saprotrophic fungi (Dickinson, 1973; Bertelsen *et al.*, 2001).

Several fungal pathogens attack the leaves and heads of wheat. Fusarium head blight is an important disease affecting heads and was treated in Chapter 3. Common fungal foliar diseases of wheat in Sweden are different types of rusts (*Puccinia* spp.), powdery mildew (*Blumeria graminis*) and leaf blotch diseases including septoria tritici blotch (*Zymoseptoria tritici*), tan spot (*Pyrenophora tritici-repentis*) and stagonospora nodorum blotch (*Parastagonospora nodorum*).

In cereals, inoculation with saprotrophic fungi has been shown to reduce infection by pathogens attacking leaves or heads (Fokkema & Nooij, 1981; Liggitt *et al.*, 1997; Perello *et al.*, 2001) indicating that indigenous phyllosphere

fungi may exert a biocontrol effect in cereal crops. It is important to investigate the effect of agricultural practices on phyllosphere fungal communities in order to be able to identify practices that can stimulate the biocontrol potential of these communities. This knowledge is very likely also important for improving the effectiveness and consistency of BCAs, which are often reported to produce variable results under field conditions (Nicot *et al.*, 2012).



## 5 Studying Microbial Diversity

### 5.1 Methods for Studying Phyllosphere Communities

Studying microbial communities is challenging due to their small size and still largely undescribed diversity. Traditionally, microbial communities have been investigated by isolation on nutrient media. However, culture-dependent methods are time-consuming and only a portion of all microorganisms can be grown in the laboratory. Estimates of the culturable portion of phyllosphere bacteria compared to PCR or microscopy have ranged from 0.1-50% (Müller & Ruppel, 2014). This is also the case for fungi. Density of *A. pullulans* on apple leaves was greater when assessed by fluorescent in situ hybridisation compared to plate counts (Andrews *et al.*, 2002). It is well-known that bacteria can enter a 'viable but nonculturable state' (VBNC), meaning that although the cell is metabolically active, it no longer grows on laboratory media (Oliver, 1993). The existence of VBNC in yeast has also been documented (Divol & Lonvaud-Funel, 2005; Serpaggi *et al.*, 2012).

Culture-independent methods are based on the extraction and analysis of nucleic acids. Although culture-independent methods can provide a more comprehensive view of microbial communities, they also have drawbacks such as the detection of DNA from dead cells (Müller & Ruppel, 2014). Regardless of whether culture-dependent or culture-independent methods are used, sample preparation has an impact on the phyllosphere community recovered. Commonly, epiphytes are recovered by washing leaves and using the wash solution for DNA extraction or plating, while endophytic species are recovered from surface-sterilised leaf tissue. The fungal community recovered from barley has been reported to differ between these two methods (Dickinson, 1973). It is also known that the lower and upper leaf surfaces may differ in population sizes (Southwell *et al.*, 1999; Andrews *et al.*, 2002).

Phyllosphere fungal communities have been explored using DNA fingerprinting methods such as terminal restriction fragment length polymorphism (t-

RFLP), single strand conformation polymorphism (SSCP) or denaturing gradient gel electrophoresis (DGGE) and/or construction of clone libraries and sequencing for species identification (Blixt *et al.*, 2010; Moulas *et al.*, 2013; Martins *et al.*, 2014). In recent years, the application of high-throughput sequencing (HTS) has led to a better understanding of the diversity and ecology of phyllosphere microorganisms (Rastogi *et al.*, 2013). To date, most culture-independent methods depend on the amplification of a marker gene with PCR. The development of HTS has facilitated the use of shotgun metagenomics where total DNA is sequenced, which can provide insights about both the identity and function of phyllosphere communities (*e.g.* Delmotte *et al.*, 2009).

## 5.2 Marker Genes for Microbial Community Profiling

A suitable marker gene should capture as large a diversity as possible of the target group and yet exclude other organisms. Moreover, the marker should be variable enough to distinguish between species. The design of group-specific primers is therefore a crucial step. Previous studies have revealed biases of ‘universal’ primers targeting different organism groups (Turnbaugh *et al.*, 2010; Ihrmark *et al.*, 2012; Bokulich & Mills, 2013; Highlander, 2014). In addition to primer mismatches, there are also other potential problems associated with PCR targeting a mixed template, *e.g.* variations in GC content (Benita *et al.*, 2003) and length of PCR product between species (Ihrmark *et al.*, 2012). Such biases may skew the community composition during PCR.

### 5.2.1 Markers for Fungi

In fungi, the internal transcribed spacer (ITS) region is widely used for species identification and the ITS was recently proposed as a universal barcode for fungi (Schoch *et al.*, 2012). In eukaryotes, the ITS is the non-coding DNA located between the genes coding for the small (18S) and large (28S) subunit of the ribosomal RNA. The ITS1 is located between the 18S and the 5.8S genes and the ITS2 between the 5.8S and the 28S genes. Due to the short read lengths resulting from HTS, either the ITS1 or the ITS2 have mostly been used in fungal community studies (Bazzicalupo *et al.*, 2013; Monard *et al.*, 2013). The small subunit gene, which is widely used as a marker for bacterial communities, does not provide enough phylogenetic resolution for fungi (Schoch *et al.*, 2012; Lindahl *et al.*, 2013).

There are about 100 000 described fungal species but the total diversity is estimated to be between 1.5 and 5.1 million species (Blackwell, 2011). The application of HTS has led to an increasing discovery of unnamed operational taxonomic units (OTUs) inferred only from sequence data (Hibbett *et al.*, 2011).

Sequence-based species identification is dependent on well-annotated databases. However, it has been reported that up to 20% of fungal sequences in major databases such as GenBank may be misidentified (Bridge *et al.*, 2003; Nilsson *et al.*, 2006). The coverage of already described species is also known to be incomplete (Brock *et al.*, 2009). Thus, a major challenge for mycologists is to name and track fungal diversity discovered in environmental studies. However, some attempts exist to facilitate sequence-based identification and communication of fungal OTUs, such as the ‘species hypotheses’ in the UNITE database (Kõljalg *et al.*, 2013) or the ‘virtual taxa’ in the MaarjAM database for the Glomeromycota (Öpik *et al.*, 2010).

### 5.2.2 Markers for *Fusarium*

For *Fusarium*, the ITS region does not provide species-level resolution and many fusaria have non-orthologous copies of the ITS2 (O’Donnell & Cigelnik, 1997; O’Donnell *et al.*, 1998a; Watanabe *et al.*, 2011). Common phylogenetic markers for *Fusarium* include the genes for the RNA polymerase subunits II (RPB), the  $\beta$ -tubulin gene and the transcription elongation factor 1 alpha gene (EF-1 $\alpha$ ) (Gräfenhan *et al.*, 2011; Watanabe *et al.*, 2011; O’Donnell *et al.*, 2013). The EF-1 $\alpha$  gene is a useful marker for species identification of *Fusarium* and it appears to be single-copy in *Fusarium* (Geiser *et al.*, 2004). The latter characteristic is particularly useful for community studies allowing for better quantitative comparisons between species. Furthermore, many EF-1 $\alpha$  sequences are available in databases such as FUSARIUM-ID (<http://isolate.fusariumdb.org>) and Fusarium MLST (<http://www.cbs.knaw.nl/fusarium>) that facilitate sequence-based identification of *Fusarium* species.



## 6 Materials and Methods

### 6.1 Evaluation of *Fusarium*-Specific Primers (Paper I)

The application of HTS to study *Fusarium* communities has been limited by the lack of specific primers for the genus. Recently, Edel-Hermann *et al.* (2015) developed a nested PCR assay to study *Fusarium* diversity in soil. The nested PCR included primers EF1 and EF2 (O'Donnell *et al.*, 1998b) and a new primer pair: Fa and Ra. Nested PCR is necessary to amplify *Fusarium* EF-1 $\alpha$  from soil, since the gene is single copy and present in a complex substrate. In Paper I, we evaluated and adapted the method developed by Edel-Hermann *et al.*, (2015) to describe *Fusarium* communities using HTS.

Mock communities were assembled to evaluate how well the primers reflect the relative abundances of the different species throughout PCR and sequencing. Mock communities are mixtures of known microbes or their DNA used to mimic natural communities and can be used to evaluate the performance of primers targeting specific taxonomic groups (Ihrmark *et al.*, 2012; Highlander, 2014; Nguyen *et al.*, 2015). Mock communities also serve as a positive control in HTS (Nguyen *et al.*, 2015).

Ten species representing the phylogenetic diversity of *Fusarium* including important plant pathogens were included in the study: *F. avenaceum*, *F. culmorum*, *F. graminearum*, *F. langsethiae*, *F. oxysporum*, *F. proliferatum*, *F. redolens*, *F. sambucinum*, *F. solani* and *F. tricinctum*. Mock communities consisting of DNA from 10 or two *Fusarium* species were prepared as described in Paper I. Two sets of mock communities were prepared in order to test for biases of the primer pairs of both PCR steps. Field samples of soil and wheat kernels were included to test the method on more complex material.

## 6.2 Sampling of Wheat Fields (Paper II-IV)

Winter wheat leaves were sampled from farmers' fields in Sweden in June 2011 (Paper II) and July 2012 (Paper III).

In 2011, sampling was carried out in the Västergötland and Skåne regions (Fig. 1). Fungicide-treated and untreated leaves were sampled in pest surveillance plots, disease control and variety testing trials placed in conventionally managed fields. The pest surveillance plots are used for monitoring the incidence of pests and diseases. Fungicides or insecticides are not applied within these plots. The fields surrounding the plots had received 1-3 fungicide applications prior to sampling. Leaf samples representing seven different winter wheat cultivars were collected from a total of 18 fields. One fungicide-treated sample and one untreated sample were taken from each field.

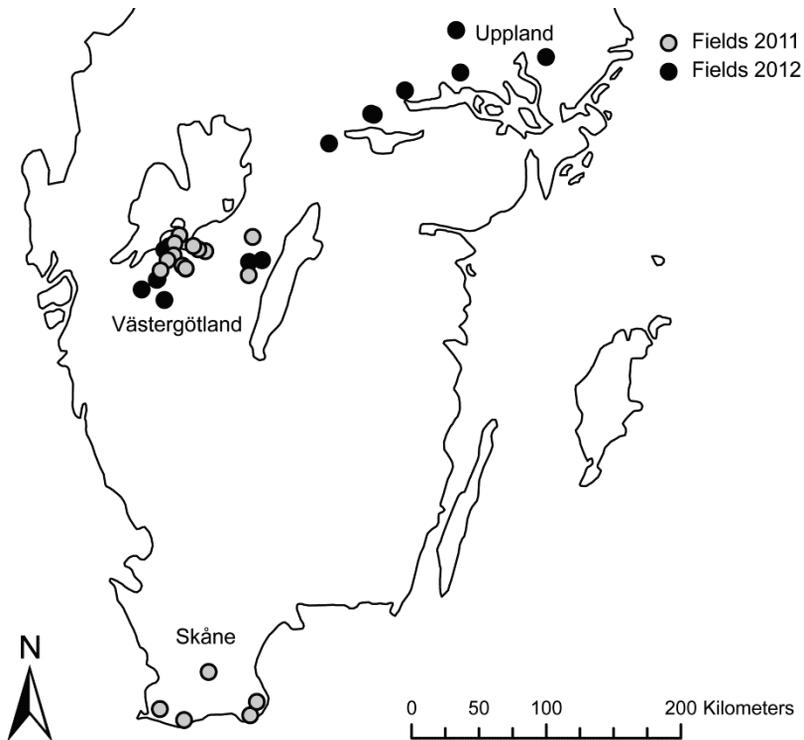


Figure 1. Map of 18 wheat fields sampled in 2011 (grey dots, study II) and 24 fields sampled in 2012 (black dots, study III). Samples were taken from the Västergötland region in both sampling years. In 2012, closely situated fields were paired, fields within a pair are not always distinguishable on the map.

In 2012, leaves were collected from 12 pairs of organically and conventionally managed fields. Fields were located on a gradient starting in Uppland in the north-east stretching down to Västergötland in the south-west (Fig. 1). Comparison of biodiversity in organic and conventional cropping systems is complex as there can be large variation between farms and there are several possible confounding factors. Pairing of farms based on different criteria is often used to limit variation due to for example climate conditions, soil type and geographical location (Yeates *et al.*, 1997; Chamberlain *et al.*, 1999; Hyvönen *et al.*, 2003; Granado *et al.*, 2008). Fields were paired based on geographical distance and wheat cultivar to minimise variation due to these factors. Paired fields were adjacent or located between 0.5 and 9.5 kilometres from each other and on which either winter wheat cultivar ‘Olivin’ or ‘Stava’ were grown.

At sampling, a number of additional data were collected from each field. Farmers were interviewed about agricultural practices and the amount of weeds and crop biomass was estimated in each field (Fig. 2). These data were also used to calculate an agricultural intensification index for each field based on the index proposed by Herzog *et al.* (2006), including the amount of nitrogen applied and the number of pesticide applications.



*Figure 2.* Sampling in an organically (left) and conventionally (right) cultivated wheat field. A 0.25 m<sup>2</sup> large frame (in orange) was used when sampling the wheat crop and weeds for biomass estimation.

### 6.2.1 Leaf Samples (Papers II-III)

The leaf below the flag leaf was collected from 10 randomly chosen plants in each plot or field and was pooled into one sample. Ten halved leaves, including the middle vein were used for DNA extraction. In order to capture both endophytic and epiphytic fungi, the whole leaf tissue was included. The leaf materi-

al was frozen in liquid nitrogen and homogenised with a pestle. One (Paper II) or three (Paper III) 100 mg subsamples were taken from each homogenised leaf sample and DNA was extracted using the DNeasy Plant Mini Kit (Qiagen, Germany).

#### 6.2.2 Kernel Samples (Paper I and IV)

At harvest in 2012, farmers were asked to send a sample of harvested kernels from the same fields where leaves were sampled earlier in the summer. From each sample of about 1 kg wheat kernels, 100 g were milled and DNA was extracted from two 100 mg subsamples using the DNeasy Plant Mini Kit (Qiagen).

### 6.3 PCR Amplification of Marker Genes (Papers I-IV)

The ITS2 was used as a marker to characterise fungal communities in Papers II and III. The ITS2 region was amplified with primers fITS7 (Ihrmark *et al.*, 2012) and ITS4 (White *et al.*, 1990), which mainly target the basidiomycete and ascomycete fungal phyla. The fITS7 primer was designed to minimise amplification of plant DNA but may exclude some fungi in the Mucorales and *Pezizomycotina* including *Penicillium* spp. (Ihrmark *et al.*, 2012).

*Fusarium* communities were analysed by amplification of the EF-1 $\alpha$  gene (Paper I and IV) with nested PCR using primer pairs EF1 and EF2 (O'Donnell *et al.*, 1998b) or Fa+7 and Ra+6 (Paper I) in the first step and Fa and Ra (Edel-Hermann *et al.*, 2015) in the second step. Mock communities were amplified with both nested and single PCR, soil samples with nested PCR and wheat kernel samples with single PCR.

#### 6.3.1 Real-Time PCR Estimation of Fungal Biomass (Papers III-IV)

High-throughput sequencing is at best semi-quantitative, providing the relative abundance of each species in a community (Lindahl *et al.*, 2013). Therefore, 454 sequencing data were complemented with real-time PCR to obtain an estimation of fungal (Paper III) and *Fusarium* (Paper IV) biomass respectively. The number of ITS copies has been reported to vary by an order of magnitude among different fungal species (Rodland & Russell, 1982; Maleszka & Clark-Walker, 1993) and within the same fungal species (Liti *et al.*, 2009) while the EF-1 $\alpha$  is thought to be single-copy in *Fusarium* (Geiser *et al.*, 2004). For the ITS, this introduces a bias. When communities differ greatly in species composition, variation in ITS copy numbers among species can compromise comparisons of fungal biomass among samples (Baldrian *et al.*, 2013).

Fungal ITS copies were quantified using the fITS7 and ITS4 primers as for 454 sequencing. A dilution series of plasmid containing a fungal ITS fragment was used as standard. *Fusarium* DNA was quantified using the Fa and Ra primers used for 454 sequencing. A dilution series of a mix of DNA from 10 *Fusarium* species was used as standard.

Fresh leaf and kernel material were transferred to the freezer directly after sampling and DNA was extracted from frozen material. The plant material may have varied in dry matter content and the efficiency of the DNA extraction can also be variable among samples. Therefore, fungal biomass estimations were normalised to the amount of wheat DNA, which was quantified by amplification of a portion of the EF-1 $\alpha$  gene with primers Hor1 and Hor2 (Nicolaisen *et al.*, 2009). Wheat DNA was quantified using a standard curve of DNA from healthy wheat leaves grown in a greenhouse or steam-treated wheat kernels (ThermoSeed, BioAgri, Sweden), respectively.

## 6.4 454 Sequencing and Bioinformatics (Papers I-IV)

Samples were purified using AMPure (Beckman Coulter, CA, USA), pooled and sent for adaptor ligation and sequencing. Amplicon pools were sequenced on a GS FLX Titanium sequencer (Paper I and II), a GS Junior 454 sequencer (Paper III) or a GS FLX++ 454 sequencer (Roche, Switzerland) (Paper I and IV).

Raw sequence data were quality filtered and clustered using the SCATA pipeline (Brandström Durling *et al.*, [<http://scata.mykopat.slu.se>]). Sequences passing the quality control were clustered at 1.5% dissimilarity cut-off into operational taxonomic units (OTUs) approximating fungal or *Fusarium* species. SCATA implements single linkage clustering (nearest neighbour), which is useful for the highly variable ITS region and is efficient in handling sequencing errors (Lindahl *et al.*, 2013). Global singletons were removed from the datasets as many of them were considered to represent sequencing errors (Tedesoo *et al.*, 2010). Singletons were also removed on a per-sample basis in order to control for primer cross-contamination and tag switching (Carlsen *et al.*, 2012).

Several methods were used to identify OTUs in this thesis. Species identification was first attempted in SCATA by including reference sequences, but could not identify all OTUs. In Papers I, II and IV, identification of OTUs was refined by constructing neighbour-joining trees including reference sequences. In Paper III, OTUs were identified using the RDP classifier (Wang *et al.*, 2007) with the UNITE ITS dataset. Species hypothesis (SH) accessions in UNITE (Ködljalg *et al.*, 2013) were assigned to OTUs when possible.

## 6.5 Statistical Analyses

Biodiversity can be measured in a number of different ways. In this thesis, species richness and evenness were used to describe fungal biodiversity in the wheat phyllosphere (Papers II and III). Species richness is the number of species present. However, total DNA was extracted from leaf tissue meaning that sequences cannot be linked to individual fungi. Instead, sequences were grouped into OTUs based on sequence similarity and the number of OTUs was considered as an approximation of fungal species richness. Evenness measures how equal a community is numerically. A community where species are more equal in abundance has a higher evenness and is considered more diverse. In this thesis, Pielou's evenness index was calculated (Pielou, 1966).

High-throughput sequencing is often associated with variable read numbers although equal amounts of DNA from each sample are pooled. This technical bias introduced during sequencing can affect ecological patterns in the dataset (Goodrich *et al.*, 2014). There are several methods to overcome this problem. In Paper II, data were rarefied to the lowest number of reads per sample which has been a common practice for HTS data. However, this method has been criticised as valid data are omitted (McMurdie & Holmes, 2014). In Papers III and IV read numbers were included as a factor in the statistical models instead.

In Paper II, the effect of fungicide treatment and geographical area on OTU richness and community evenness was tested using linear mixed models where field and the interaction between field and treatment were modelled as random factors. In Paper III, the effect of cropping system and agricultural intensity on OTU richness and evenness was evaluated using generalised linear models (GLM) and linear models, respectively. Pair and the number of 454 reads were included as variables in the models.

In Papers II to IV, multivariate GLMs were used to test the effect of fungicides, cropping system, and agricultural intensity on community composition, providing a multivariate test for the overall community composition and univariate tests for each OTU (Wang *et al.*, 2012). The use of GLMs is particularly suited for count data which normally exhibit a positive mean-variance relationship which can be accounted for by GLMs (Warton *et al.*, 2012). Community composition was visualised using the non-metric multidimensional scaling (NMDS) ordination technique in Papers II to IV. NMDS was performed on Bray-Curtis dissimilarities. Rare OTUs only present in a few samples may represent transient 'visitors' to the phyllosphere and are not likely to provide information on the effect of agricultural practices on phyllosphere fungi. Therefore, only the most common OTUs were included in the GLM and NMDS analyses (Paper II: n=67, Paper III: n=57).

In Papers III and IV, the effect of selected environmental and agricultural variables on fungal and *Fusarium* community composition and abundance was evaluated with redundancy analysis (RDA). Model selection started from a model including the number of 454 reads and pair as conditional variables and stepwise forward model selection was performed.



## 7 Results and Discussion

### 7.1 Evaluation of *Fusarium*-Specific Primers

In Paper I, *Fusarium*-specific primers for HTS sequencing were evaluated. The mock communities revealed that when the EF1 and EF2 primers were used in nested PCR, the original abundances of the *Fusarium* species were not accurately reflected (Fig. 3a), while the Fa and Ra primers performed better (Figs. 3b and d). This suggests that the EF1/EF2 primers are biased against some species and it was found that the EF1 primer has a mismatch to at least *F. graminearum* (Paper I). The EF1/EF2 primer pair is widely used to identify newly isolated *Fusarium* strains and most EF-1 $\alpha$  sequences in public databases are generated with these primers. However, these results indicate that they are not suitable to amplify *Fusarium* communities.

New primers were designed that better preserved the mock communities throughout the nested PCR (Fig. 3c). Some variation between species remained though, which could be partly attributed to variation in amplicon length and possibly also genome size (Paper I).

This method can be useful for studying *Fusarium* ecology in different habitats. In Paper IV the method was applied to describe *Fusarium* communities associated with wheat kernels. The method enables the detection of shifts in species composition and spread of species to new areas, as the species monitored do not have to be set beforehand as is the case with real-time PCR with species-specific primers. On the other hand, the major advantage of real-time PCR is that quantification of total abundance is possible in contrast to amplicon sequencing, which is limited to relative abundances in a community. However, amplicon sequencing can be complemented with real-time PCR, which was done in Paper IV.

As modern sequencers produce ever increasing amount of data at lower costs, shotgun metagenomics is likely to replace amplicon-based sequencing in

the future. However, the processing and analysis of these large datasets is challenging and the coverage of complex communities such as those in soil may be insufficient (Lindahl & Kuske, 2013).

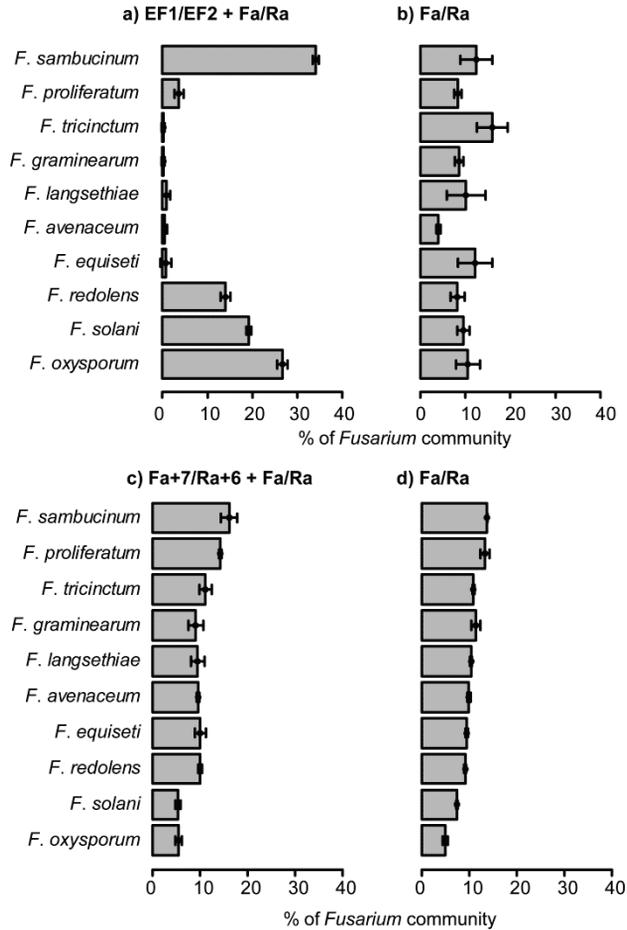


Figure 3. Species composition of mock *Fusarium* communities amplified with a) nested PCR with EF1/EF2 and Fa/Ra primers, b) Fa/Ra on a mix of EF1/EF2 amplicons, c) nested PCR with Fa+7/Ra+6 and Fa/Ra primers and d) single PCR with Fa/Ra. Error bars represent the standard deviation among three PCR replicates in a) and b) and among three mock community mixes in c) and d).

## 7.2 The Fungal Phyllosphere Community of Wheat

The fungal community on winter wheat leaves from farmers' fields in Sweden was described in 2011 (Paper II) and 2012 (Paper III). The leaf under the flag leaf was collected and the generation of ITS sequences using HTS was similar

in both years. The main difference was that three subsamples were taken from each leaf pool in Paper II, while only one subsample was taken in Paper III. In 2011, 235 fungal OTUs were identified in the pool of 420 wheat leaves from 18 fields. In 2012, 284 fungal OTUs were identified from 220 wheat leaves from 22 fields. The mean number of OTUs per extraction replicate from each sample of 10 leaves was 27 in 2011 and 30 in 2012, and from each field, 42 in 2011 and 47 in 2012. In a previous study in Sweden, Blixt *et al.* (2010) identified 1-10 fungal species in individual wheat leaves using t-RFLP in combination with cloning and sequencing.

The proportions of ascomycetes and basidiomycetes were almost equal in 2011 (54% and 46%, respectively), while the ascomycetes were more abundant in 2012 with 72% of the OTUs compared to 25% for the basidiomycetes. In 2012, the fungal community was dominated by the ascomycete *Zymoseptoria* including the leaf blotch pathogen *Z. tritici* (*Mycosphaerella graminicola*). The incidence of leaf blotch diseases was above average this year (Swedish Board of Agriculture, 2012) which could explain the observed difference in the proportion of ascomycetes. Leaves were sampled about 1-2 weeks later in 2012 which could also contribute to the higher proportion of ascomycetes, as ascomycetes such as *Cladosporium* and *Alternaria* have been reported to become more abundant throughout the growing season on cereal leaves (Magan & Lacey, 1986).

Out of the 67 and 57 most common OTUs identified in Paper II and III respectively, 35 OTUs were shared between the two years (Fig. 4). The shared basidiomycete OTUs were all yeasts. Three fungal pathogens on wheat leaves were also shared among the most common OTUs (Fig. 4). In previous studies using culture-dependent methods, the wheat phyllosphere fungal community has been described as consisting of 'pink' yeasts (*Sporobolomyces* and *Rhodotorula*), 'white' yeasts (*Cryptococcus*) and ascomycete saprotrophs such as *Cladosporium* and *Alternaria* (Fokkema & Nooij, 1981; Magan & Lacey, 1986; Legard, 1994). This description seems to apply also for the fungal community on wheat leaves revealed by HTS (Fig. 4, Papers II and III). However, there were also some unidentifiable OTUs among the most common, *e.g.* in the order Helotiales (Fig. 4).

In both years, fields were sampled in the Västergötland region, and included other locations that were not shared between the two years (Fig. 1). In both studies a 'core' set of OTUs present across all fields was identified: six OTUs in 2011 and seven OTUs in 2012. Four of these OTUs were identical between the two years: *Cladosporium* (OTU 3), two *Dioszegia* (OTUs 1 and 2) and one *Cryptococcus* (OTU 6) OTU (Fig. 4). This indicates that the fungal community on wheat leaves includes some cosmopolitan members regardless of geograph-

ical location, year and cropping system. In a recent study in Denmark, it was found that many phyllosphere fungi were present on all cereal species investigated (Sapkota *et al.*, 2015). Several fungal genera identified in the wheat phyllosphere, *e.g.* *Sporobolomyces* and *Cryptococcus*, which have also been found in the phyllosphere of a range of different plants (Fonseca & Inacio, 2006).

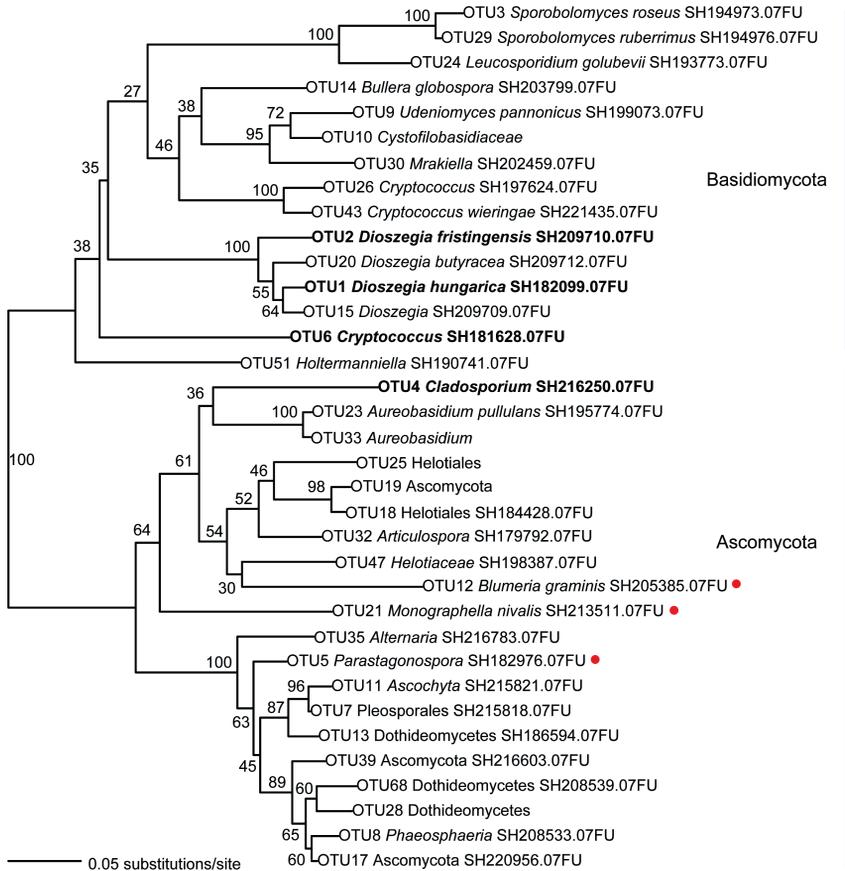


Figure 4. Neighbour-joining tree of shared operational taxonomic units (OTUs) among wheat leaves sampled in 2011 (Paper II) and 2012 (Paper III). OTUs marked in bold were present in all fields in both studies. Major wheat pathogens are marked with red dots. Species hypotheses accessions in the UNITE database (version 7.0, <http://unite.ut.ee>) are indicated when available.

Although the same fungal species and genera were often observed in Papers II and III, there were some indications of biogeographical patterns. In Paper II it was found that community composition differed between the two areas sampled. For example, *Sporobolomyces roseus* was the most abundant OTU in

Skåne while *Dioszegia fristingensis* was the most abundant in Västergötland (Paper II). Furthermore, fungal OTU richness was also significantly lower in southern Sweden (Fig 5, Paper II).

Paper II compared fungicide-treated and untreated wheat leaves from the same fields. The statistical analyses revealed significant differences in fungal community composition between fields. On the other hand, in Paper III where fields were paired within 10 km distance, no effect of pair was observed in the statistical analyses. This could indicate that fungi are effectively dispersed within a field but that dispersal limitation exists between fields. However, other factors may also have varied between fields in a pair, e.g. soil type. In the study by Blixt *et al.* (2010), low variation in phyllosphere fungal communities of wheat was observed within fields but with larger variation between different regions in Sweden.

### 7.3 Effect of Agricultural Practices on Wheat Phyllosphere Fungi

The effect of fungicide-treatment and organic farming on biodiversity of fungi was investigated in Papers II and III, respectively. Species richness and evenness were calculated to reflect two different dimensions of biodiversity (Purvis & Hector, 2000; Magurran, 2004). As the use of fungicides is prohibited in organic farming, similar effects in the two studies could be expected to some degree. However, opposite patterns were observed for species richness and evenness in the two studies. Fungicide-treatment did not have a significant impact on fungal species richness, but evenness was significantly lower in fungicide-treated leaves (Fig. 5, Paper II). On the other hand, fungal species richness was found to be significantly higher in organically grown wheat leaves compared to those grown conventionally, but evenness was unaffected by the cropping system (Fig. 6, Paper III). Evenness was overall lower in 2012 than in 2011 (Figs. 5 and 6) probably reflecting the dominance of *Zymoseptoria*. Fungal biomass estimated with real-time PCR in Paper III was highly variable among fields and also between closely located fields in a pair, but no difference between cropping systems was found (Fig. 6, Paper III).

Negative effects of fungicides on both fungal species richness and evenness could be expected as fungicide sensitivity can vary among fungi (Southwell *et al.*, 1999; Müllenborn *et al.*, 2008) which could lead to the elimination of some species and increased dominance of others. Overall, the effects of fungicides on fungal phyllosphere communities were surprisingly small (Paper II). No estimation of fungal biomass was performed in Paper II, which might have revealed larger differences between fungicide-treated and untreated leaves. Previous studies examining fungicide effects on non-target fungi in the wheat

phyllosphere using culture-dependent methods have reported reduced population sizes by some fungicides (Dickinson & Wallace, 1976; Fokkema & Nooij, 1981; Magan & Lacey, 1986; Fokkema, 1988; Southwell *et al.*, 1999; Wachowska, 2009). However, most fungicides used in these studies are now prohibited in Sweden, and there may be differences in the effect of the newer fungicides compared with the older ones.

Nevertheless, it was found that fungicide treatment had a negative effect on a few fungi including *A. pullulans* (Paper II), a yeast-like fungus of which several isolates are known antagonists against necrotrophic pathogens (De Curtis *et al.*, 2012; Sylla *et al.*, 2013). It has been suggested that reduction in yeast and saprotroph populations by fungicide spraying may result in reduced competition with pathogens (Dickinson, 1973; Fokkema, 1988). A recent study however reported little effect of fungicide treatment on fungal and bacterial communities on grapevine leaves and their biocontrol effect against downy mildew on sterilised leaf discs (Perazzolli *et al.*, 2014).

Organic farming has previously been associated with higher evenness (apple: Glenn *et al.*, 2015), richness (apple: Granado *et al.*, 2008; grapes: Martins *et al.*, 2014) and abundance (Granado *et al.*, 2008; Martins *et al.*, 2014) of phyllosphere fungi and different community structure (grapes: Schmid *et al.*, 2011) than in conventional production. Most studies have focused on describing the effect of cropping systems on phyllosphere communities while fewer have tried to relate differences in microbial communities to ecosystem services such as biological control. However, Schmid *et al.*, (2011) reported distinct fungal communities and a higher proportion of antagonistic fungal isolates against *Botrytis cinerea* on organically managed grapes than on those conventionally managed, and Workneh and Van Bruggen (1994a; 1994b) reported higher suppression of corky root of tomato in organic systems that was related to higher activity and abundance of several microbial groups. It would be of interest to investigate whether the increased fungal diversity in organic farming reported in Paper III is associated with any positive or negative effects on the plant.

Surprisingly, no effect of either fungicides or cropping system was detected on any of the common wheat leaf pathogens identified in the two studies. In 2011, some samples were collected in fungicide trials and fungicide treatment was reported to reduce the leaf area attacked by septoria leaf blotch and powdery mildew in these trials (trials L15-1050 and L15-1071, <http://www.skaneforskoken.nu>). But neither the relative abundance of *Z. tritici* or *Blumeria graminis* was altered by fungicide use in Paper II. However, *Puccinia striiformis* dominated the community in untreated samples while it was almost absent from fungicide-treated samples, but this pathogen only occurred

in two fields. Total fungal abundance was not estimated in Paper II, so it is possible that fungicide use reduced the total abundance of fungi (including pathogens) but did not affect the relative abundance of most pathogens in the community.

The case is more complex when comparing disease occurrence in organic and conventional farming. In the conventional system, fungicides can be used to reduce fungal diseases. In the organic system there are also factors that may have protecting effects such as lower nitrogen application (Thomas *et al.*, 1989; Simón *et al.*, 2003) and more diverse crop rotation (Bailey *et al.*, 2001). Previous studies have reported lower severity of foliar diseases in organic production compared with conventional or no difference, with the exception of potato late blight (Van Bruggen, 1995). In a recent study in France, the occurrence of fungal leaf blotch disease was reported to be lower in organic wheat production (Gosme *et al.*, 2012). In Paper III, no effect of cropping system on *Zymoseptoria* was found, which was the most abundant OTU. The high abundance of this potential leaf blotch pathogen in 2012 probably obscured the effects of any measures that could have had a protective effect in both systems.

Pairing of organic and conventional farms can reduce variability due to other factors than the cropping system but at the same time there is a risk of excluding the very differences between the two systems (Hole *et al.*, 2005). Different wheat cultivars are usually grown in organic and conventional production respectively. Therefore, the pairing based on wheat cultivar may to some extent have selected non-representative farms in Papers III and IV. In order to control for this potential bias, an agricultural intensity index including the number of pesticide applications and the amount of nitrogen applied was used. It was found that the agricultural intensity was lower in the organically managed fields than in the conventionally managed ones and that agricultural intensity was associated with lower fungal OTU richness and a different community composition (Paper III).

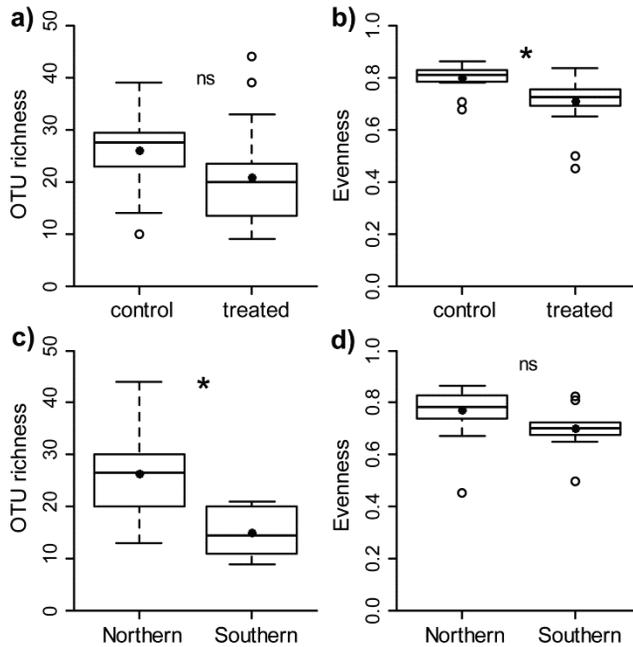


Figure 5. Richness of fungal operational taxonomic units (OTUs) and evenness of fungal communities in wheat leaves depending on fungicide-treatment and geographical area. Samples grouped by fungicide treatment (a and b) and area (c and d). The Northern area is located in the Västergötland region and the Southern in the Skåne region. Boxes represent interquartile ranges and circles values more than 1.5 times the interquartile range above the third quartile or below the first quartile. Horizontal lines represent medians and dots mean values. \* $p < 0.05$ , ns=non-significant.

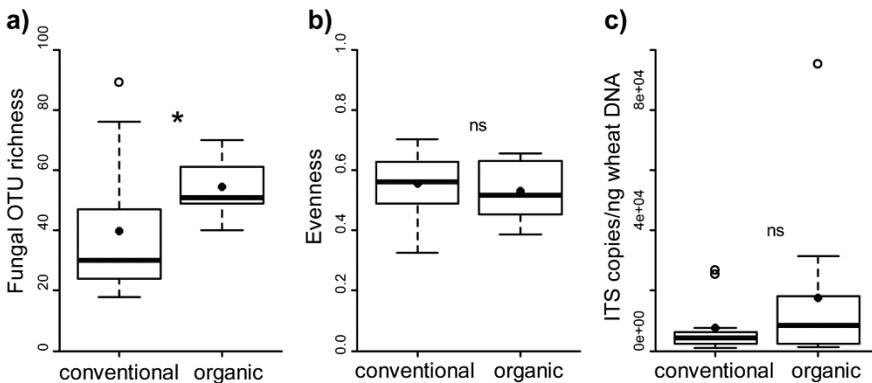


Figure 6. Richness of fungal operational taxonomic units (OTUs), evenness of fungal communities and estimated fungal biomass in wheat leaves in different cropping systems. Distribution of fields regarding a) OTU richness, b) evenness and c) estimated fungal biomass (ITS copy numbers) grouped by cropping system. Boxes represent interquartile ranges and circles values more than 1.5 times the interquartile range above the third quartile. Horizontal lines represent medians and dots mean values. \* $p < 0.05$ , ns=non-significant.

## 7.4 *Fusarium* Communities in Wheat Kernels

In Paper IV, HTS of EF-1 $\alpha$  amplicons (method described in Paper I) was applied to characterise *Fusarium* communities in wheat kernels from the same fields as in Paper III. *Fusarium* spp. were identified in all fields investigated with two to eight OTUs present per field and the amount of *Fusarium* DNA varying by two orders of magnitude between fields. *Fusarium avenaceum* was the most frequently recovered species, while *F. graminearum* was the most abundant.

There were differences in *Fusarium* community composition between pairs of fields and *F. graminearum* was more prevalent in the western part of the sampling area (Paper IV, Fig. 7). This area has been particularly affected by high DON levels in recent years and high levels of *F. graminearum* have been observed simultaneously (Fredlund *et al.*, 2013; Lindblad *et al.*, 2013). The reason for this pattern is unclear, but favourable climate conditions for *F. graminearum* in this region has been proposed. In a recent study, several variables related to flowering date, varietal resistance and climate conditions including relative humidity, temperature and rainfall were found to explain DON contamination of wheat in north-western Europe (van der Fels-Klerx *et al.*, 2012). However, an attempt to predict the risk of DON contamination of oats in the Nordic countries failed to identify any agronomical or climate factors that could explain variation in DON levels between regions (Lindblad *et al.*, 2012). It is unclear to what extent *F. graminearum* is currently restricted by climate or other local conditions and if it could spread to other regions of Sweden with the risk of increased mycotoxin problems.

No difference in *Fusarium* communities between wheat kernels from organic and conventional production could be detected, but an effect of agricultural intensity was observed, where *F. tricinctum* was associated with higher agricultural intensity (Paper IV, Fig. 7). Lower levels of *Fusarium* contamination in organic cereal production than in conventional production has been reported previously (Birzele *et al.*, 2002; Bernhoft *et al.*, 2010). However, agricultural intensity, which included the number of pesticide applications and the amount of nitrogen applied, reflects some of the important differences between the organic and conventional systems. Trying to understand the cause of the difference in *Fusarium* and mycotoxin contamination that has been observed between organic and conventional production presents a significant challenge, since many factors differ between the two systems and it is difficult to test system-level differences in controlled experiments.

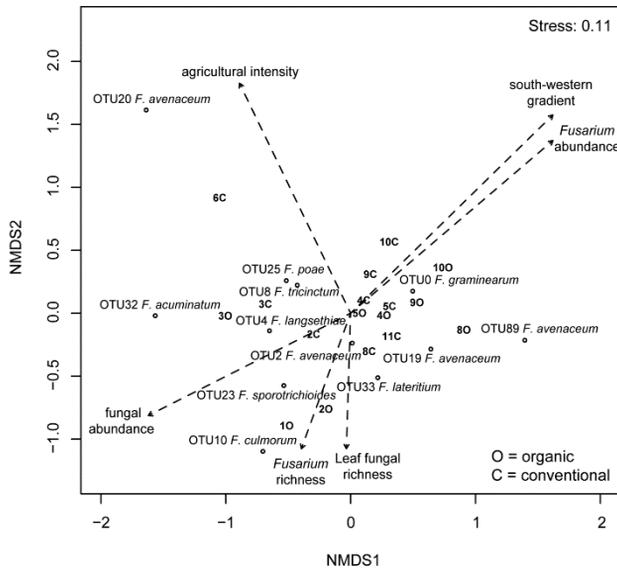


Figure 7. Non-metric multidimensional scaling (NMDS) ordination of *Fusarium* community composition in harvested wheat kernels from organically and conventionally managed fields in Sweden. Ordination showing fields (numbered letters) and species (circles) with fitted environmental variables. Fields are paired and numbered on a south-western gradient starting from the north (Fig. 1). 17 fields were included in the analysis.

## 7.5 *Fusarium* and Leaf Fungal Communities

The leaves and heads of cereals are exposed to similar inoculum sources so their fungal communities can be expected to overlap. For example, Vujanovic *et al.* (2012) reported that many fungal species were shared between different plant organs of durum wheat. Leaves may act as an intermediate step in *Fusarium* infection towards the head (Zinkernagel *et al.*, 1997) where interactions with leaf fungi can occur. *Fusarium* spp. have previously been identified on green leaves (Köhl *et al.*, 2007; Vujanovic *et al.*, 2012), but no *Fusarium* species were identified among the most common fungal OTUs in this thesis (Papers II and III). Leaves were collected around flowering so later leaf infections by *Fusarium* cannot be excluded.

Procrustes analysis was used to test whether the composition of the *Fusarium* kernel community could be predicted from the composition of the leaf fungal community in the same fields earlier in the season. It was found that there was an agreement between the two NMDS ordinations compared (Procrustes correlation 0.46,  $p < 0.05$ ). Moreover, the *Fusarium* abundance and the abundance of leaf fungi appeared to be negatively correlated (Fig. 7). This may reflect the preference for opposing environmental conditions, biogeographical

patterns or negative interactions between *Fusarium* and leaf fungi. It would be interesting to investigate this pattern further and compare *Fusarium* and fungal abundance and community composition on leaves to that on heads throughout the growing season. This knowledge could provide another piece of the puzzle to understand the occurrence of FHB in cereals and the associated mycotoxin contamination.



## 8 Concluding Remarks

The present thesis demonstrates that different agricultural practices are associated with differential fungal communities in the wheat phyllosphere. Using modern sequencing methods, the fungal community could be described in more detail, revealing previously undescribed diversity. The results provided insights into the effects of fungicides and organic farming on fungal communities in farmers' fields in Sweden. However, the changes associated with agricultural practices were rather limited, which could have been due to variability in other factors such as climate conditions. It is probably more difficult to manage phyllosphere microbial communities than their counterparts in soil since the phyllosphere is more directly affected by climate conditions and there is continuous interchange with the air microbiota. Nevertheless, these results indicate that there is an opportunity for the farmer to influence these communities through agricultural management. Such management could aim at enhancing the bio-control potential of phyllosphere communities against *Fusarium* and other pathogens, or other functions beneficial for the crop plants. Future studies could focus on functional characterisation of phyllosphere fungal communities using, for example, metatranscriptomics or shotgun metagenomics. A better understanding of the importance of phyllosphere microorganisms for plant health and physiology is required in order to be able to exploit the phyllosphere microbiota in crop production under variable environmental conditions.

The method for characterising *Fusarium* communities evaluated in this thesis can be useful for monitoring *Fusarium* occurrence in cereals and studying *Fusarium* communities associated with FHB, gaining a better understanding of their ecology. Increased focus on the effects of environmental and agricultural factors on *Fusarium* as a community could facilitate the development of strategies to control FHB and limit mycotoxin contamination.



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