On *Phaeosphaeria nodorum* in Wheat

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Cover: Winter wheat field on a bright summer's day
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Abstract

The fungus *Phaeosphaeria nodorum* (anamorph *Stagonospora nodorum*) causes stagonospora nodorum blotch on wheat. Besides environmental conditions the biology of the pathogen is an important parameter that determines the epidemiology of the disease. The dispersal pattern of the pathogen depends on the mode of reproduction. The studies reported in this thesis show that *P. nodorum* not only reproduces asexually but also sexually. This conclusion is based on the random distribution of the two mating types and the diverse genetic structure of the population. The sexual ascospores are windborne and able to initiate epidemics in new wheat fields, whereas asexual spores, conidia, give rise to new lesions when spread upwards in the canopy and to adjacent plants through splash water droplets.

Isolates of *P. nodorum* vary in aggressiveness towards wheat in terms of latency period and production of pycnidia. The wheat cultivars commonly grown in Sweden have similar susceptibility to *P. nodorum*. Use of low inoculum concentrations could be useful in cultivar screening in breeding programs due to the comparability to natural infection.

In agricultural practice, fungicides are used as a control of leaf spot pathogens. This thesis includes the first report of isolates of *P. nodorum* that have lost their sensitivity to the strobilurin substance azoxystrobin. The majority (79% of 227 isolates) of the *P. nodorum* isolates collected in 2003-2005 had the amino acid substitution G143A in the gene encoding cytochrome *b*, which is associated with loss of sensitivity to strobilurins in other pathogenic fungi. The substances propiconazole, prothioconazole and cyprodinil were efficient in reducing the fungal growth.

Wheat leaves are colonised by other fungal species besides pathogenic fungi. The fungal community on wheat leaves also contains saprotrophic fungi and yeasts. *P. nodorum* was common in fields sampled in 2003 and 2004 but in 2005 the pathogenic fungi *Pyrenophora tritici-repentis* and *Mycosphaerella graminicola,* causing tan spot and septoria tritici blotch respectively, were more common in the sampled fields.

Keywords: aggressiveness, cultivar, fungal community, fungicide, genetic diversity, sexual recombination, stagonospora nodorum blotch, *Phaeosphaeria nodorum*, wheat

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Till Mor, Bror och min älskade Make

Livet är den sköraste av gåvor
Fröet som gror, trotsar vind, torka och patogener
Blir böljande säd, ett under att skåda
Ljuvlig är doften av mognad
Fröet blir mat till oss hungriga
Men ack, ska det räcka?
Ta det inte för givet
Hon lider i tysthet
Hon suckar. Hon skälver: Gör något!
Näktergalen manar oss mangrant:
Tillsammans kan vi rädda Moder Jord
E.B.

Contents

List of Publications

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

I Blixt E., Olson Å., Lindahl B., Djurle A., Yuen J. Spatiotemporal variation in the fungal community associated with wheat leaves showing necrotic leaf spots. (Manuscript).

II Blixt E., Olson Å., Högberg N., Djurle A., Yuen J (2008). Mating type distribution and genetic structure are consistent with sexual recombination in the Swedish population of *Phaeosphaeria nodorum*. *Plant Pathology* 57, 634–641.

III Blixt E., Djurle A., Olson Å, Yuen J. Variation in aggressiveness of *Phaeosphaeria nodorum* to six Swedish winter wheat cultivars. (Manuscript).

IV Blixt E., Djurle A., Yuen J., Olson Å. Fungicide sensitivity in Swedish isolates of *Phaeosphaeria nodorum*. (Accepted for publication in *Plant Pathology*).

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1 Introduction

1.1 “Eat or be eaten”

Our planet Tellus is a complex environment with a tremendous range of species; plants, animals, microbes *etc.* of which the majority live under the concept “eat or be eaten”. All species on the planet are part of the food web: You either have enough nourishment to live and reproduce or someone else will have you for breakfast. We humans belong to the category that eat and don’t have to worry so much about predators. Some of us are unfortunate enough to live under food shortage. Wheat is one of the main staple foods nowadays in many parts of the world together with maize and rice. In Scandinavia we have been growing wheat for centuries but it is not until the middle of the twentieth century that wheat became common on everyman’s table. The agricultural practices have changed drastically during this period of time as well. The fields have become larger, cereal crops are often frequent in the crop rotation and the struggle for profitability has improved the mechanical equipment used in modern agriculture. The wheat cultivars have changed from long straws with short spikes, often with awns, to short straws with long spikes and large flag leaves. These morphological changes and the more dense wheat stands have probably improved the environmental conditions for those organisms that feed on wheat. Fungi and insects are the two largest groups of species nourishing on wheat, but viruses, bacteria and nematodes may also cause large damage to the wheat crop.

Socioeconomical aspects of plant pathogens are of importance, not least for the people in developing countries. Pathogenic fungi caused 10% yield loss in average worldwide during 2001-2003, which is a third of the total yield loss caused by various pests (Oerke, 2006). Yield loss, often in the form of reduced grain weight, is in general due to reduction in assimilate as a consequence of leaf senescence and hastened maturity (Rees & Platz, 1983). Once harvested, storage pests such as rodents, insects and microbes may reduce the available amount of food even more.

2 Aims of Thesis

The aim of this thesis was to investigate biological aspects of *Phaeosphaeria nodorum*, the causal agent of stagonospora nodorum blotch in wheat, in order to better understand the epidemiology of the fungus. The main focus is on the reproduction system and dispersal of the pathogen. Another important issue is how the fungus can be controlled in agricultural practice, through choice of cultivar and fungicide treatment.

3 Background

3.1 Plant pathology: some stepping stones

There are many pathogenic fungi that cause yield losses on wheat and have done so for a very long time. The oldest written report of crop loss due to plant diseases were made by the Greek philosopher Homer (*c.* 1000 B.C.). Diseased plants are also mentioned in several books of the Old testament (*c.* 750 B.C.) (Agrios, 2005). Crop losses of wheat by rust fungi were reported by the ancient Romans who feared famine due to yield loss caused by rust fungi so much that they sacrificed dogs to please the gods (Zadoks, 1985). After those reports there is a large gap in the documentation of plant diseases. Plant pathology got a large upswing after the invention of the microscope in the mid 1600s when filamentous fungi and bacteria suddenly became visible. The technique was further developed and in 1729 the Italian Pier Antonio Micheli made a new breakthrough for plant pathology by describing many genera of fungi and their reproductive structures. In April 1728 the Frenchman Henry-Lois DuHamel du Monceau reported to the Royal Academy of Science of his findings of the disease, called *la Mort*, destroying the saffron crocus (Zadoks, 1981). This was one of the first epidemiological descriptions of a disease and its causal agent (Zadoks, 1981). Unfortunately his findings were left in darkness and it took about a century until causal agents were discussed scientifically again (Zadoks & Koster, 1976). During the nineteenth century more pathogenic fungi were described. In 1845 reverend Miles J. Berkeley described a pathogen on wheat glumes and called it *Depazea nodorum* which was the asexual stage of the fungus, nowadays called *Stagonospora nodorum* (*vide* 3.3.1). The Swedish mycologist Jakob Eriksson (1848-1931) contributed much to fungal taxonomy during the late 1890s. Eriksson and Henning described the pathogenicity of several rust fungi, among those *Puccinia graminis*. This pathogen was found to be of various biological races depending on which host was infected; wheat, rye or oats (Eriksson & Henning, 1894). Another important concept was the theory of the disease triangle where the interaction between pathogen, host and environment was presented as an epidemic (McNew, 1960). Epidemics develop as a joint venture of the three corners of the triangle and if at least one of them is changed the epidemic will develop differently.

The first book of plant pathology was written by the German Julius Kühn in 1858 with the translated title: *Diseases of cultivated crops, their causes and their control* (Kühn, 1858). The book was well received by the scientists and extension workers at that time and it helped to understand the etiology and prevention of diseases. The first journal of plant pathology was *Zeitschrift für Pflanzenkrankheiten* which started in 1891. Another stepping stone in plant pathology was the book in epidemiology written by James E. Vanderplank: *Plant Diseases: Epidemics and Control* (Vanderplank, 1963). The intention of the book was to, in a simple way, explain the theories of resistance inheritance and mathematical formulas of spread of diesease. The theories of epidemiology are used to evaluate dispersal patterns and the biology of plant pathogens are still used for prevention and control of plant diseases.

Another breakthrough in plant pathology was the development of molecular methods based on polymerase chain reaction, (PCR) (Saiki *et al.*, 1988). Unique DNA sequences in the genome can be used for detection and identification of species. The most frequent sequence used is the internal transcribed spacer (ITS) in the ribosomal DNA (Gardes *et al.*, 1991). Still, there are many questions about plant pathogens to be answered.

3.2 The host: *Triticum aestivum*, Bread wheat,

Bread wheat, *Triticum aestivum*, is one of the most commonly grown crops worldwide with an estimated production of approximately 600 million tonnes per year, including other species of wheat such as durum wheat and spelt wheat (*T. durum* and *T. spelta*) (FAO, 2005). In Sweden the wheat yield of year 2000 was 2.5 million tonnes, which is just 0.4% of the global wheat production (FAO, 2008).

The ancestors of bread wheat were domesticated in the Fertile Crescent in the Near East 12,000 years ago according to archaeological and genetic studies (Salamini *et al.*, 2002). The genome of *T. aestivum* is hexaploid, *i.e.* there are six sets of the seven chromosomes, (AABBDD, 42 in total). The genome originates from a crossing between jointed goatgrass (*Aegilops tauschii*, syn *A. squarrosa*) and a tetraploid wheat ancestor, plausibly emmer (*T. dicoccoides*), rivet wheat (*T. turgidum*) or durum wheat (*T. durum*) (Dvorak *et al.*, 1998; Salamini *et al.*, 2002). Wheat breeding may also include crossbreeding with the hexaploid spelt wheat (*T. spelta*) (Salamini *et al.*, 2002).

The crossing that ended up with the hexaploid wheat genome most likely occurred in the area of South-western Caspian Iran, Armenia and Transcaucasia, the land tongue between the Black Sea and the Caspian Sea, since the D genome of native *A. tauschi* ssp. *strangulata* of today is closely related to the D genome in *T. aestivum* (Dvorak *et al.*, 1998; Zohary & Hopf, 2000). The history of bread wheat may have to be rewritten since new archaeological findings and refined molecular methods have suggested that domestication of the ancestors of *T. aestivum* could be older than previously reported (Balter, 2007).

The positive qualities of bread wheat are a combination of the flour quality of the tetraploid wheat species and the adaptation to winter climate of *A. tauschi*. (Zohary & Hopf, 2000). Bread wheat is therefore a crop with good baking properties together with good winter hardiness and adaptation to humid summers, which made the crop popular. The spread of *T. aestivum* is correlated to the human migration, first to Asia and Europe, and then to the New world (Zohary & Hopf, 2000).

There are two types of *T. aestivum* grown today, spring wheat and winter wheat. Winter wheat needs a vernalisation period of low temperature, often in combination with short day length, in order to flower. Vernalisation triggers the genes encoding flowering (Trevaskis *et al.*, 2007). Spring and winter wheat are divided into cultivars, where each has properties adjusted for regional climate and purpose of the crop. Spring and winter wheat have different baking properties since the protein content and the activity of α-amylase varies between the two types of wheat (Weidow, 1998).

3.3 Pathogenic fungi on wheat leaves

Pathogenic fungi are divided into groups depending on the mechanisms of infection, effect on the host and nutrient uptake (Agrios, 2005). The fungi dealt with in this thesis belong to the group which are necrotophic and produce enzymes or toxins in order to kill the host cells to acquire nutrients. The number of dead host cells increase during disease progress, leading to expanding necrotic lesions on leaves or sometimes even on stems. Three common necrotrophic fungal pathogens on wheat, *Phaeosphaeria nodorum*, *Pyrenophora tritici-repentis* and *Mycosphaerella graminicola*, are presented below.

3.3.1 Phaeosphaeria nodorum

Phaeosphaeria nodorum (E. Müll.) Hedjar. (anamorph *Stagonospora nodorum* (Berk.) E. Castell. & Germano) is the causal agent of stagonospora nodorum blotch on wheat, also known as leaf and glume blotch. The early symptoms on the leaves are dark brown lesions, often surrounded by chlorosis that later expand into irregular lesions (Figure 1a) (Eyal *et al.*, 1987). When the fungus reaches the spikes, generally at the end of the growing season, brown stripes that later merge into brown spots develop on the glumes (Figure 1b-c).

The life cycle of the fungus consists of two cycles, one with asexual reproduction and one with sexual reproduction (Figure 2). The fungus overwinters on straw debris. During moist and warm conditions pycnidia develop (Figure 1c-d), which is the start of the asexual cycle. Under damp conditions the asexual spores, conidia (Figure 1e & 3a), emerge from the pycnidium surrounded by pinkish sticky secretion, cirrhus. The conidia are dispersed upwards in the canopy or to adjacent plants, by splashing water droplets. The conidia germinate when the leaf is moist or wet and the temperature is between 5-37°C; the optimum temperature for germination being 20-25°C. The fungal hyphae penetrate directly through the leaf cell walls and grow intercellularly in the epidermis and necrosis starts to develop (Karjalainen & Lounatmaa, 1986). Production of pycnidia begins typically in the centre of the necrotic lesion (Shearer & Zadoks, 1974). The fungus can complete several asexual cycles during a growing season where new conidia are splashed upwards in the canopy. Finally the fungus reaches the spikes and infects the glumes where the mycelium grows on to the kernels that become infested. To close the life cycle, the fungus needs to infect new plants with conidia produced on infected straw debris or by infested seed brought to other fields. When an infested seed germinates the fungus can follow the coleoptile up to the soil surface and cause disease or kill the seedling.

The sexual cycle has been known to exist in Europe (Mittelstadt & Fehrmann, 1987), Australia (Bathgate & Loughman, 2001) and North America (Cowger & Silva-Rojas, 2006). Analyses of the genetic structure have revealed that populations of *P. nodorum* are genetically diverse within fields, which is consistent with sexual recombination (Caten & Newton, 2000; Keller *et al.*, 1997; McDonald *et al.*, 1994; Sommerhalder *et al.*, 2007). The sexual stage has occasionally been found in Sweden (L. Johansson, Mase laboratories, personal communication) but no thorough investigation has been made since the 1960s (Leijerstam, 1962). *P. nodorum* has two mating types, and individuals with one mating type each have to be in close contact for sexual reproduction (Halama & Lacoste, 1991). When individuals of different mating type meet, one of them forms an antheridium and the other one forms an ascogonium, whereafter these two structures fuse. Later, a fruiting body containing ascospores, pseudothecium, is developed. The ascospores are wind borne and may be dispersed long distances within fields or to new fields (Bathgate & Loughman, 2001; Eyal *et al.*, 1987). After germination and infection a new disease cycle begins.

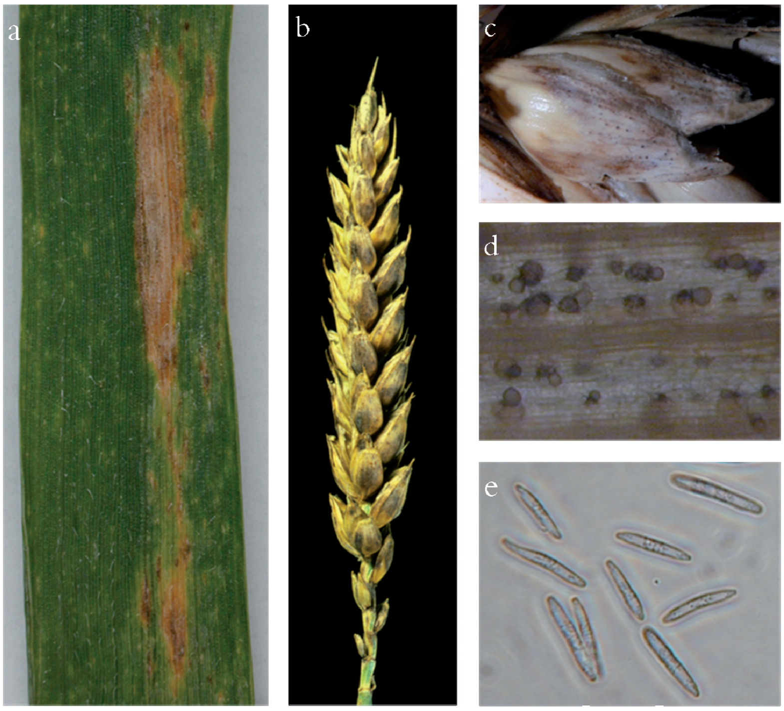

Figure 1. Symptoms of stagonospora nodorum blotch on a: leaf, b: spike, c: pycnidia on glumes, d: pycnidia on leaf with cirrhus containing conidia and e: conidia (photo: E. Blixt).

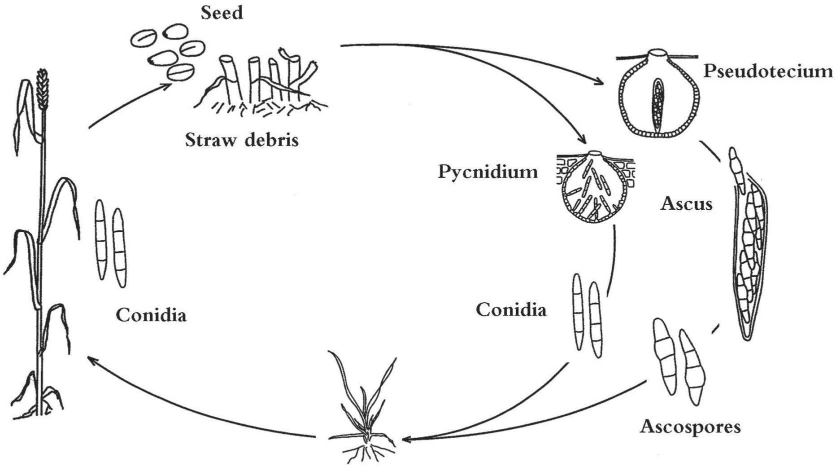

Figure 2. Life cycle of *Phaeosphaeria nodorum* with a sexual (right) and an asexual (middle) cycle (graphics: A. Djurle).

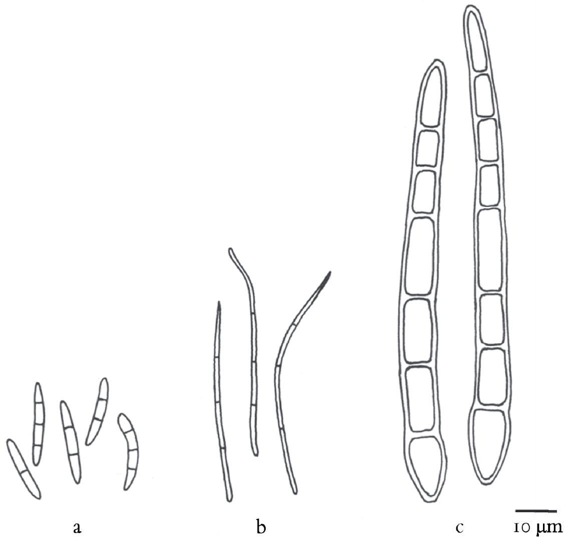

Figure 3. Conidia of a: *Phaeosphaeria nodorum*, b: *Mycosphaerella graminicola* and c: *Pyrenophora tritici-repentis* (graphics: K. Göransson, details from Twengström & Svensson, 2001).

3.3.2 *Pyrenophora tritici-repentis*

Pyrenophora tritici-repentis (Died.) Drechsler (anamorph *Drechslera tritici-repentis* (Died.) Shoemaker) is the causal agent of tan spot on wheat. The early symptoms are small dark brown lesions, often surrounded by chlorosis, and often thickly scattered on the leaves (Figure 4a). In the centre of the lesion there is a greyish spot which is a character used to distinguish the lesions of tan spot from those of stagonospora nodorum blotch (Figure 4b). Later in the season the symptoms become very similar to the leaf lesions of stagonospora nodorum blotch since the lesion widens to a brown, sometimes irregular lesion. The typical lesion still has the grey spot in the centre of the lesion but often the entire lesion has different nuances of brown, while the lesions of stagonospora nodorum blotch become yellow around the edges of the lesion (Dreschler, 1923; Eyal *et al.*, 1987). Variations in symptoms occur within the pathosystem of wheat and *P. tritici-repentis*. Eight races of the pathogen have been identified, each associated with host specific toxins leading to different symptoms on the differential cultivars (Strelkov & Lamari, 2003).

Besides early symptoms, the best way to distinguish between *P. tritici-repentis* and *P. nodorum* is the formation of conidia (Figure 3). The conidia of *P. tritici-repentis* are produced on the leaf surface with the conidia attached directly on conidiophores grown out from the lesion. The formation of conidia takes place during damp conditions at night but the conidia are dispersed by wind when the leaf has dried or are splashed through rain drops (Wiese, 1987). Otherwise the life cycle of *P. tritici-repentis* is similar to the life cycle of *P. nodorum*, including seed transmission that may cause much damage on seedlings (Carmona *et al.*, 2006; Schilder & Bergstrom, 1995). Epidemics of tan spot may be more severe during years with frequent rainfalls after stem elongation (DC31 (Zadoks *et al.*, 1974)) rather than before that development stage (Rees & Platz, 1983).

3.3.3 *Mycosphaerella graminicola*

Mycosphaerella graminicola (Fückel) J. Schroeter (anamorph *Septoria tritici* Berk. & M.A. Curtis) is the causal agent of septoria tritici blotch on wheat, also known as leaf blotch. The symptoms on the leaves are light brown to greyish long lesions following the leaf veins. The fungus enters the leaf through the stomata and grows intercellularly during the first days of infection. Then necrosis develops and black pycnidia are produced next to the stomata giving the typical appearance black dots in a row (Figure 4c) (Kema *et al.*, 1996). The conidia (Figure 3b) are long and thin and are dispersed in the same way as the conidia of *P. nodorum*. The life cycle of *M.*

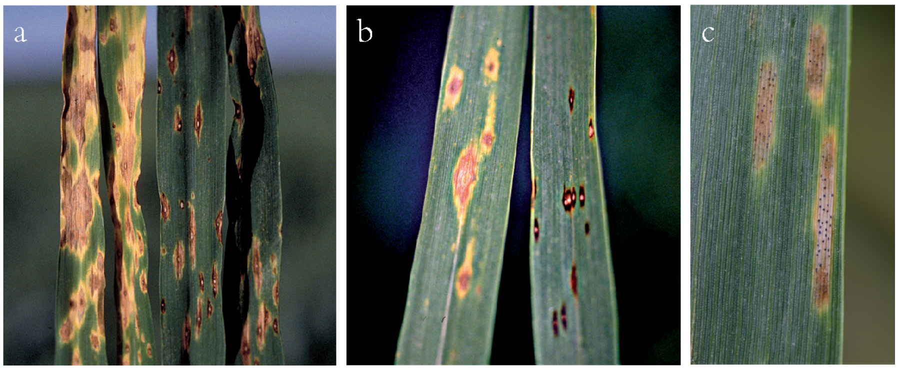

Figure 4. Wheat leaves with symptoms. a: tan spot symptoms at late to early stages of disease development, b: lesions of tan spot at an early stage (right) compared to an early stage of stagonospora nodorum blotch (left), c: symptoms of septoria tritici blotch (photo: Swedish Plant Protection Centres).

graminicola is very similar to the life cycle of *P. nodorum* (Eyal *et al.*, 1987; Shaner & Finney, 1976), but the incidence of sexual reproduction in Sweden is unknown.

3.3.4 Physiological leaf spots

Sometimes wheat leaves show necroses without any detected pathogen and this is referred to as physiological leaf spots. The symptoms are variable and often similar to various stages of tan spot and stagonospora nodorum blotch. The necrotic spots may appear either early in the season or on the upper leaves at the time of heading (Smiley *et al.*, 1993a). The cause of the spots is still unknown but there are hypotheses about abiotic stress such as chloride deficit, bright sun light or large fluctuations in temperature and weather conditions (Engel *et al.*, 2001; Smiley *et al.*, 1993b; Wu & von Tiedemann, 2004).

3.4 Genetic structure of pathogens

The reproductive system of a fungus influences not only the epidemiology due to dispersal methods, but also has also a strong impact on the genetic structure of the pathogen population. Asexual reproduction may lead to a fungal population consisting of few genotypes since many individuals have identical genome (Hartl & Clark, 1989; McDonald & Linde, 2002). The genetic structure of a population reproducing sexually may on the other hand be diverse with many unique genotypes due to recombination. Differentiation within and among populations would therefore be measured through gene diversity by analysing the number of alleles occurring for each gene (McDonald & Linde, 2002). Fungal species with mixed reproduction have an advantage since a strain with high fitness may increase in frequency through asexual reproduction and dispersal of the trait to other areas or populations through sexually produced windborne spores.

Other mechanisms of changes in the genetic structure within a population are mutations, genetic drift, migration and demographic bottlenecks (Agrios, 2005; Hartl & Clark, 1989; McDonald & Linde, 2002). Mutations occur randomly at a low frequency. If the mutation is neutral it can be either fixed in the population or deleted. If the mutation influences the fitness of the fungal strain the mutation can be selected for in the population. Another way of changing the genetic structure is through migration of individuals or windborne spores. Demographic bottlenecks occur when the population size is drastically reduced resulting in a population with low genetic diversity. Bottlenecks occur at different levels for example during introduction of a small population to a new area or after every fungicide application (Agrios, 2005; McDonald & Linde, 2002).

3.5 Aggressiveness of pathogen and susceptibility of host

Necrotrophic fungi, such as *P. nodorum*, produce host selective toxins or cell wall degrading enzymes to kill the host cells in order to retrieve the nutrients needed (Friesen *et al.*, 2008a). For infection to occur the host must produce the target receptor protein recognised by the toxin. Fifteen toxins have so far been identified in *P. nodorum* (Friesen *et al.*, 2008b). Four of them have been named SnToxA, SnTox1, SnTox2 and SnTox3, and they interact with the products of the host receptor genes *Tsn*1, *Snn1, Snn2* and *Snn3* respectively (Friesen *et al.*, 2008c; Liu *et al.*, 2006; Stukenbrock & McDonald, 2007).

One of the alleles encoding ToxA in *P. nodorum* is very similar to the single ToxA allele found in *P. tritici-repentis* and the recognition protein for ToxA is the same for both species (Friesen *et al.*, 2006). Those authors therefore assumed that the ToxA gene was horizontally transferred from *P. nodorum* to *P. tritici-repentis*, probably just prior to 1941. The symptoms observed in 1941 (Barrus, 1942; Johnson, 1942) have much resemblance to the necrotic lesions induced by ToxA in *P. tritici-repentis* (Friesen *et al.*, 2006).

Prior to the discovery of the toxins breeding for resistant cultivars concentrated on screening wheat breeding lines in search for less susceptible material (Ali *et al.*, 2008; Francki *et al.*, 2008; Tommasini *et al.*, 2007). Resistance was found to be polygenic resulting in a more complex breeding programme (Feng *et al.*, 2004). Breeding for resistant cultivars may be easier onwards after the discovery of the genes encoding toxin receptor in the wheat genome. If the parental lines lack any of the receptor genes they have good breeding value (Friesen *et al.*, 2007; Friesen *et al.*, 2008c). Crossbreeding with wild grass ancestors to wheat, which often are resistant to *P. nodorum* strains infectious to wheat, would be useful in further breeding programs (Oliver *et al.*, 2008). Even though the findings of toxins and receptors may revolutionise plant breeding, phenotypic analyses of the cultivars in virulence tests should continue to be an important part of the breeding program to accurately observe host-pathogen interactions (Goodwin, 2007).

The correlation of susceptibility between seedlings and adult plants has been an issue of discussion. The two development stages may have different susceptibility due to variations in active genes throughout the development of the plant. Winter wheat seedlings infected during autumn may not suffer from severe damage in terms of yield loss due to shoot elongation (Holmes & Colhoun, 1974). Inoculation of mature plants revealed that the upper leaves of adult plants were more susceptible than the seedlings. Contradicting results was found in a resistance test where older leaves were more susceptible than the flag leaf since the development of necrosis and pycnidia went faster on the two leaves beneath the flag leaf (Jönsson, 1989). Other studies on the susceptibility of seedlings and adult plants revealed good correlations of disease severity between the two development stages (Jönsson, 1985; Rufty *et al.*, 1981). But still, seedling experiments are a valuable tool to analyse the virulence of pathogens due to the advantages with experiments performed under controlled conditions at any time using multiple isolates and plant material (Arraiano *et al.*, 2001).

3.6 Prevention and treatment of fungal diseases

Good agricultural management is the most effective way of controlling plant diseases. Alternation of cereal crops with various types of non-host crops, such as oilseed rape, legume crops or even oats, decreases the sources of inoculum in the field. Infested seed may be one important inoculum source which affect the seedlings from the first stage of development (Bennett *et al.*, 2007). Seeds of cereal crops in conventional farming are often treated with fungicides to reduce the impact of the pathogens. Alternative treatments are aerated steam treatment (Forsberg *et al.*, 2005) or bio-control agents such as *Pseudomonas flourescens* (Saharan *et al.*, 2008) or *Bacillus pumilus* (Gonzalez & Trevathan, 2001).

Bio-control agents for prevention of attacks by leaf pathogens are a central subject of research with the intention to decrease the chemical impact on the environment. Yeasts and filamentous fungi have been reported to possess antagonistic capacities towards pathogenic fungi (Perelló *et al.*, 2003; Perelló *et al.*, 2006). *Ulocladium atrum* and *Clonostachys rosea* prevent sporulation of *Botrytis aclada* (Yohalem *et al.*, 2004). *P. nodorum* caused less disease when *Sporobolomyces roseus* and *Cryptococcus laurentii* var. *flavenscens* were present on the leaf surface or the spikes (Fokkema *et al.*, 1979). The fungi *Trichoderma harzianum* and *T. koningii* decrease the impact of *P. tritici-repentis* and *M. graminicola* when applied to seeds and leaves (Perelló *et al.*, 2006). The mechanism of antagonism is thought to be competition of nutrients (Fokkema *et al.*, 1979; Kessel *et al.*, 2001) or in the case of *Trichoderma* species, mycoparasitism such as hyphal penetration was observed (Perelló *et al.*, 2003).

Disease control in growing crops is widely performed by application of different kinds of fungicides. Approximately half of the winter wheat area in Sweden (*c.* 300,000 ha in total (SCB, 2008)) is treated with fungicides (Söderberg, 2008). In 2007 15.3% of the 10,600 tonnes of active substance (a.s.) of pesticides sold in Sweden was used in agriculture (Kemikalieinspektionen, 2008). Fungicides constituted 2% (206.5 tonnes a.s.) of the total purchase of pesticides, which was an increase of 19 tonnes due to the high disease incidence in 2007. The purchase of three common substances was as follows: azoxystrobin: 8.4 tonnes, cyprodinil: 15.6 tonnes and propiconazole 22.7 tones (of which a large amount was used in the wood industry) (Kemikalieinspektionen, 2008). The purchase of prothioconazole in 2007 was 21.3 tonnes and increased during 2008 (M. Andersson, Bayer CropScience, personal communication).

Antifungal substances are divided into different groups depending on their mode of action. They all inhibit different enzyme activities important to the fungus. Three of the most commonly used fungicide groups, strobilurins, triazoles and anilinopyrimidines, are presented below focusing on the function of the active substance.

3.6.1 Strobilurins

The first active substance within the chemical compound group strobilurins was developed from the natural fungicide produced by the fungus *Strobilurus tenacellus*. The substance inhibits the mitochondrial respiration by binding to the Q_o site (outer quinine oxidising pocket in complex III) at cytochrome *b*, which has the structure of a saddle (Esser *et al.*, 2004). Cytochrome *b* is a protein located in the inner mitochondrial membrane forming the core of the mitochondrial bc_1 complex (complex III). Strobilurins block the electron transfer process between cytochrome *b* and cytochrome c_1 which leads to inhibition of electron transport in the energy cycle. The consequences are disruption of the ATP production and thus the metabolism of the fungus will decrease (Esser *et al.*, 2004).

The uptake of active substance of strobilurin into the plant cells depends on formula, additives, mixtures with other products, application practices, weather conditions and the biological and physiological state of the crop to mention the most important ones (Bartlett *et al.*, 2002). Azoxystrobin is one of two strobilurins (picoxystrobin the other one) that move systemically in the xylem and protect younger, newly developed plant parts and provides good disease control all the way to the leaf tip.

Strobilurins have shown activity against ascomycetes, basidiomyctes, deuteromycetes and oomycetes. The active substance interact with the energy metabolism in order to prevent spore germination and infection, which are very energy demanding processes (Wong & Wilcox, 2001). Strobilurins may also have a curative effect due to the decrease in mycelial growth during the latency period (time between infection and the beginning of reproduction). Eradicant effects and antisporulant effects on both sexual and asexual stages have been reported in some fungal species (Bartlett *et al.*, 2002).

One of the reasons strobilurins have been used in such a large amount is that they have had a "greening effect" of the crop. The senescence of the leaves is delayed, even in absence of pathogenic fungi, resulting in increased grain weight and nitrogen content of the grains (Gooding *et al.*, 2000; Ruske *et al.*, 2003), but the increase in protein content in the grain was not significant or consistent.

3.6.2 Triazoles

Triazoles, such as propiconazole and prothioconazole, are used against many economically important pathogens on arable crops. Triazoles are characterised as demethylation inhibitors (DMI) since they inhibit the enzyme 14-α-sterol demethylase encoded by the *CYP51* gene, a member of the cytochrome P450 family (Siegel, 1981; Yoshida & Aoyama, 1987). This enzyme catalyses the production of ergosterol. Ergosterol is an important component of the fungal cell membrane and functions in the same way as cholesterol in animal cells (Bossche *et al.*, 1987; Mercer, 1984). Inhibition of 14-α-sterol demethylase leads to accumulation of eburicol, a precursor for ergosterol, to toxic levels and the growth of the fungus is halted or the hyphae become morphological deformed.

3.6.3 Anilinopyrimidines

Cyprodinil is a commonly used substance in the fungicide group anilino-pyrimidines (Knaufbeiter *et al.*, 1995). The biological mode of action includes inhibition of fungal methionine biosynthesis and secretion of hydrolytic enzymes. The chemical mode of action is still unknown but research has been performed on less sensitive strains of *Botrytis cinerea* in order to find the mechanisms behind the reduced sensitivity, and thereby elucidate the mode of action of the fungicide (Fritz *et al.*, 2003). Cyprodinil inhibits the elongation of hyphae in the host tissue or the formation of haustoruim, depending on fungal morphology, and may therefore work in a curative way (Knaufbeiter *et al.*, 1995). The necrotic area on the leaves does not increase and sporulation is inhibited. No cross-resistance with other fungicide groups has been reported for anilinopyrimidines (Knaufbeiter *et al.*, 1995).

3.7 Reduction in sensitivity to fungicides

During the past few years farmers in Denmark and in Skåne in Southern Sweden, but also in other parts of Europe, have noticed that the fungicide treatments have not been effective against septoria tritici blotch and tan spot (Almquist *et al.*, 2008; Jørgensen, 2007). Researchers have discovered that mutations have occurred in the mitochondrial genome of fungi, leading to substitutions in the amino acid sequence in cytochrome *b* (Grasso *et al.*, 2006; Sierotzki *et al.*, 2007) and the enzyme encoded by *CYP51* (Cools *et al.*, 2006; Leroux *et al.*, 2007). These substitutions have been associated with loss of sensitivity in pathogenic fungi against strobilurins and triazoles respectively.

In cytochrome *b* three amino acid substitutions have been identified at the amino acid position 129, 137 and 143. Alteration of the amino acid sequence leads to transformation of the binding site and the strobilurin can therefore not bind to the target site in cytochrome *b* (Esser *et al.*, 2004). The substitution at position 143, G143A (G= glycine, A= alanin, with the wild type amino acid first and the substituted amino acid as the last letter) is the most common one found among all species that have lost their sensitivity to strobilurins (Grasso *et al.*, 2006; Sierotzki *et al.*, 2007). A list of the pathogens and their substitutions are published on the home page of the Fungicide Resistance Action Committee (FRAC) (FRAC, 2007).

Insensitivity of triazoles has been reported in many important pathogenic fungi such as *M. graminicola*, *Blumeria graminis* f.sp. *tritici* (causing powdery mildew) and *Oculimacula acuformis* (causing eye spot, syn. *Tapesia acuformis*) (Cools *et al.*, 2006). The substitutions found in the genome of *M. graminicola* are located at position 137, 381 and 459-461, as well as the deletion of codons 459 and 460 (Fraaije, 2007; Leroux *et al.*, 2006; Leroux *et al.*, 2007; Zhan *et al.*, 2006). Deletion of amino acids at position 459 and 460 often co-occurs with the substitution I381V (I= isoleucine, V= valine) and the combination is associated with loss of sensitivity to triazoles (Brunner *et al.*, 2008; Lardinois *et al.*, 2006). The amino acid substitutions may have developed in the European population of *M. graminicola* and been dispersed by ascospores to other places in Europe (Brunner *et al.*, 2008). Danish and Swedish isolates of *P. tritici-repentis* showed decrease in sensitivity to triazoles during the growing seasons of 2006 and 2007 (Jørgensen, 2007). No amino acid substitutions associated with reduced sensitivity have been reported yet in *P. tritici-repentis*. Loss of sensitivity to triazoles has also been observed in *M. graminicola* as increased expression in five genes controlling the ABC transporters (ATP-binding cassette transporters) (Stergiopoulos *et al.*, 2003).

The sensitivity to fungicides can be analysed by sensitivity tests, either *in vitro* or in field trials (Brent & Hollomon, 2007). The sensitivity is measured as a dose-response curve from various concentrations of active substance estimating the EC_{50} value, which is the concentration of active substance needed for inhibition of 50% of mycelial growth (Brent & Hollomon, 2007).

4 Methodology and Results

4.1 Sampled fields for the studies

Leaves of winter wheat with visual symptoms of fungal infections similar to stagonospora nodorum blotch were collected during mid July in 2003, 2004 and 2005. The sampling locations were winter wheat fields with plots (appr. 20x20 m) that were not treated with fungicides. In total 13 fields were sampled in three counties of Sweden; Uppland, Västergötland and Östergötland (Table 1, Figure 5). The counties were referred to as regions in (I) and (II). One sampled plot in the county of Uppland was located in the outskirts of Uppsala (Ultuna), while the other two, Hagby and Husby, were located *c.* 20 km west of Uppsala *c.* 3 km apart. The two locations in Västergötland, Hästhalla and Skofteby, were located *c.* 10 km from each other. At Hästhalla the sampled plot of 2003 was located *c.* 600 m from those in 2004 and 2005, which were next to each other. Three locations were sampled in the county of Östergötland the first year; Lårstad, Yxstad and Mörby, within a distance of 30 km. The field sampled the second year at Yxstad was located *c.* 600 m from the first sampling location. The fields sampled during the three years at Mörby were located less than 200 m from each other. The crops were second year winter wheat (DC69-75 (Zadoks *et al.*, 1974)) and the fields were ploughed between the years, except for Ultuna 2003 which was organically cultivated with grass-clover ley as previous crop. The cultivars were either Kosack or Olivin.

Table 1. *Sampled winter wheat fields from three counties in Sweden. Diseased leaves were collected and used for isolations of* Phaeosphaeria nodorum *or for identification of fungal species on wheat leaves.*

County	2003	2004	2005
Uppland	Ultuna	Hagby[a]	Husby[a]
Västergötland	Hästhalla	Hästhalla, Skofteby[a,b]	Hästhalla
Östergötland	Lårstad, Yxstad[a], Mörby	Yxstad[a,b], Mörby	Mörby

[a] *Isolates from these fields were used in studies of genetic structure (II), isolate aggressiveness (III) and fungicide sensitivity (IV).*

[b] *Fields excluded from the study on fungal community on leaves (I).*

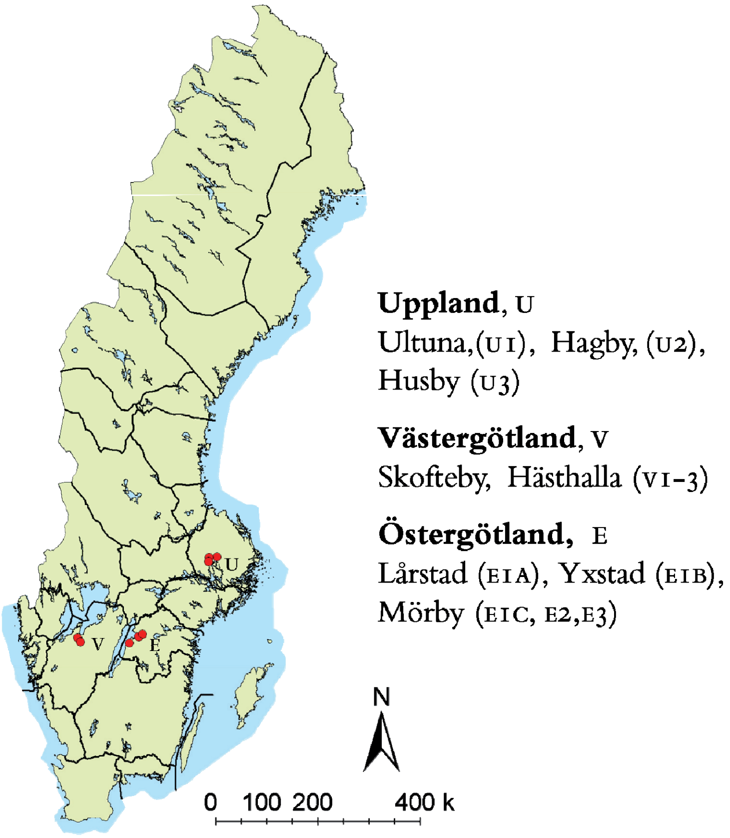

Figure 5. Sampling locations of 13 winter wheat fields where diseased leaves were collected during 2003-2005. Figures in parentheses are the field codes used in (I) (map: K. Perhans).

4.2 Spatiotemporal variation in the fungal community associated with wheat leaves showing necrotic leaf spots (I)

The fungal communities on necrotic wheat leaves were analysed using terminal restriction fragment length polymorphism (T-RFLP). The aim of the study was to investigate which fungal species are causing necrotic lesions on wheat leaves and to assess the presence of other fungi on the leaf.

In each of the eleven sampled fields (Table 1) twelve leaves, mostly flag leaves, were collected within an unsprayed plot (4 x 3 sites, approx. 6 m apart). The field coded EIC (Mörby 2003) was hierarchically sampled where ten leaves were sampled in the corners and at the two sampling sites in the centre of the plot, in total 66 leaves. The total number of leaves used in this study was 186.

Thirteen fungal species and one species complex were identified from the samples. Additional species were identified during cloning and sequencing of amplified fungal ITS-regions, which were not identified in the T-RFLP analysis. The fungal community consisted of three pathogens, seven species of yeast and three saprotrophs that were frequently occurring on the wheat leaves throughout the years. No variation was found within fields. The plots in the region of Uppland diverged in composition from the other two regions due to differences in the pathogenic fungal flora. The largest variation was found at the temporal level where the composition of the third year was different from the others, due to other dominating pathogenic fungi in the fields. *P. nodorum* was frequent during 2003 and 2004, *P. tritici-repentis* was present all years but at specific locations only, and *M. graminicola* dominated in two fields during 2005. One pathogen was often dominant within each field but *P. nodorum* and *P. tritici-repentis* often co-occurred on the same leaf during 2003 and 2004.

4.3 Mating type distribution and genetic structure are consistent with sexual recombination in the Swedish population of *Phaeosphaeria nodorum* (II)

P. nodorum were isolated from diseased leaves collected at 50 sampling sites within each of the five fields (marked with an 'a' in Table 1). After DNA extraction the genetic structure was analysed using the distribution of the two mating types and simple sequence repeats (SSR). The mating types within each field were randomly distributed in a 1:1 ratio when using clone corrected data. Four of the five loci studied were polymorphic while the fifth was monomorphic. In total 35 different alleles in five loci were found among the isolates from the five fields. The alleles and the mating type of each isolate were used to identify the genotype. In total 93 genotypes were found of which 69 were observed only once. Each field population had between 23 and 38 genotypes. The high frequencies of genotypes within each field together with the random distribution of the mating types are consistent with sexual recombination.

Ascospores are the ultimate evidence of mating and attempts to collect ascospores were made in the sampled fields during spring, summer and early autumn in 2004 and 2005 using a Burkard suction spore trap collecting spores directly on malt extract agar plates. Eight ascospore isolates were identified as *P. nodorum*. They were collected at Husby during May, July and September 2005.

The gene diversity of the *P. nodorum* isolates was low at some analysed loci, indication that the Swedish population has experienced at least one demographic bottle neck some time during the past. The time of the events could not be determined but the possible times are domestication of wheat or introduction of the crop to Sweden.

4.4 Variation in aggressiveness of *Phaeosphaeria nodorum* to six Swedish winter wheat cultivars (III)

The aggressiveness analysis was performed as a detached leaf test where six cultivars of winter wheat seedlings were inoculated with either 'high' ($1x10^6$ conidia ml^{-1}) or 'low' ($4.5x10^4$ conidia ml^{-1}) concentration of the inoculum suspension. The cultivars tested were Kosack, Aperitif, Olivin, Stava, SW Gnejs and SW Harnesk. Isolates of *P. nodorum* were tested for aggressiveness in three different experiments. Disease measurements included latency period and the ability to form pycnidia after inoculation. The leaf area covered by pycnidia was used for calculation of area under the disease progress curve, AUDPC. One isolate was used in the first experiment where the seedlings were inoculated with 'high' spore concentration in three different repeats. Significant differences were found in AUDPC between the cultivars but the variation between the inoculation times was too high for proper interpretation of these results, and AUDPC thus had limited usefulness in these studies.

In the second experiment five isolates were used for inoculation with 'low' inoculum concentration ($4.5x10^4$ conidia ml^{-1}). Significant differences in latency period were found between the isolates. This indicates that *P. nodorum* can vary in aggressiveness. There was no variation in susceptibility in the cultivars tested.

In the third experiment the latency period was significantly shorter when inoculated with $1x10^6$ conidia ml^{-1} compared to $4.5x10^4$ conidia ml^{-1}. The susceptibility of the cultivars did not vary. Ranking of the aggressiveness of the *P. nodorum* isolates was similar to the second experiment and consistent between 'high' and 'low' concentrations. Separation of aggressiveness was clearer with low inoculum concentrations. There was no interaction between isolates and cultivars.

4.5 Fungicide sensitivity of Swedish isolates of *Phaeosphaeria nodorum* (IV)

This is the first report of the sensitivity of *P. nodorum* to the antifungal substances azoxystrobin, cyprodinil and prothioconazole and the second for propiconazole (Peever *et al.*, 1994). Forty-two isolates collected during 2003-2005 and four reference isolates collected prior to the strobilurin release were tested *in vitro* for growth inhibition. The Swedish populations of *P. nodorum* are effectively controlled by propiconazole, prothioconazole and cyprodinil. The EC_{50} values of the two triazoles varied between 0.03 mg L^{-1} and 4.36 mg L^{-1}, while the EC_{50} values of cyprodinil varied between 0.02 mg L^{-1} and 0.43 mg L^{-1}. Fragments from the *CYP51* gene, known to carry a nucleotide substitution associated with loss of sensitivity to triazoles, was sequenced in 227 field isolates of *P. nodorum* along with the four reference isolates. Three nonsynonymous substitutions were found but they could not be related to the sensitivity of the isolates.

There was a large variation in sensitivity to azoxystrobin among the isolates. The EC_{50} values of the field isolates for azoxystrobin varied between 0.66 mg L^{-1} to estimations of far above 1000 mg L^{-1}. The EC_{50} values of the reference isolates ranged from 0.02 to 80.72 mg L^{-1}. A fragment of the gene encoding cytochrome *b* was sequenced for the 231 isolates mentioned above, in the search for the source of the loss of sensitivity. The substitution G143A, known to cause tolerance to strobilurins in other pathogenic fungi, was found in the majority of the isolates. The frequencies of isolates with the substitution were 79-98% in four of the fields while only 50% of the isolates collected at Hagby 2004 had the substitution. None of the reference isolates had any nucleotide substitutions in the sequenced region of either cytochrome *b* or *CYP51*.

5 Synthesis and Perspectives

5.1 Biology of *Phaeosphaeria nodorum*

Knowledge of the biology and epidemiology of a fungal disease contributes to the choice of management practices to prevent major disease outbreaks in a crop. One parameter to recognise is how the fungi are dispersed. In the case of the Swedish *P. nodorum* population the knowledge about the sexual part of the fungal life cycle has been confirmed (II). *P. nodorum* reproduces through both asexual and sexual reproduction. A strong indication of sexual reproduction is that the mating type distribution is in a 1:1 ratio, and thereby the prerequisites for mating are present. The genetic variation within fields was found to be diverse with many unique genotypes. The alleles of the loci were not linked to each other, which is consistent with frequent recombination occurring during sexual reproduction. On the other hand, it cannot be excluded that the population structure is influenced by asexual reproduction, since isolates with identical genotype were found. All of these may not be clones after all since identical genotypes could occur by chance.

The population size of *P. nodorum* has most likely fluctuated during the long period of domestication of wheat. The genetic study (II) revealed that the Swedish population of *P. nodorum* has most likely experienced at least one bottleneck, a drastic decrease in population size, some time in the past. It might have begun already in the Fertile Crescent where the ancestors of today's *T. aestivum* were first domesticated and the interaction between fungi and host began (Salamini *et al.*, 2002; Zohary & Hopf, 2000). When wheat cropping spread out of the Near East, the number of fungal strains that followed the seed to other areas or continents might have been limited. It is also possible that ascospores were dispersed by the wind to other continents.

Another suggestion is that the population structure changed when wheat was introduced to Sweden since the *P. nodorum* population is different from the Swiss and Danish populations studied earlier (Stukenbrock *et al.*, 2006). There is also a possibility that the harsher Swedish winters decrease the survival of the fungus on straw debris.

The presence of the sexual part of the life cycle of *P. nodorum* may have large consequences for agriculture. Ascospores are easily dispersed by wind, and favourable genetic and phenotypic characteristics can be spread effectively (Bathgate & Loughman, 2001). Various individuals of *P. nodorum* have different abilities to infect wheat and to produce progeny (III). If the ascospore is a product of mating between two aggressive isolates, the strain developed from the ascospore is most likely also aggressive. With increased aggressiveness the fungal strain produces more progeny (conidia or ascospores) which in their turn are dispersed further in the crop or to other fields. The impact of the aggressiveness of the fungal strain is also determined by the susceptibility of the wheat cultivar. Even though much effort has been made from the breeding companies to develop less susceptible cultivars, the wheat cultivars commonly grown in Sweden today are susceptible to *P. nodorum* both at the seedling stage (III) and as adults grown under field conditions (Larsson *et al.*, 2006).

It has been discussed whether seedlings are more susceptible than adult plants (*vide* 3.5). Plant breeding should involve screening of disease susceptibility on seedlings, plants or detached leaves, and adult plants in field trials. The technique with detached seedling leaves inoculated by spraying a spore suspension is a simple and time saving way of screening breeding material for less susceptible lines. Informative screenings could be performed with low inoculum concentration to simulate natural infection, even though the disease severity increased with increased inoculum concentration (III). The reproducibility of the technique should however be investigated further, since the variability of the isolates and cultivars was demonstrated during the repeated test of aggressiveness and host susceptibility. If the technique is standardised and adjusted for plant material, sporulation of isolates and inoculum concentration, the method would be efficient in determination of the susceptibility of cultivars.

5.2 Fungal community on necrotic wheat leaves

The composition of the fungal community on the leaves is influenced by management practises, cultivar susceptibility and dispersal of spores from various fungal species. Besides fungicides, environmental conditions such as humidity, temperature and nutrient supply are important parameters that determine the growth of the fungi on the leaves (Blakeman, 1985). The community consists of yeasts and saprotrophic fungi together with necrotrophic pathogens (I). The abundance of yeast and saprotrophs are similar within fields and across years and regions, but with some variation in species structure. Pathogenic fungi often co-occur even though one seems to dominate. During the third year of sampling (2005) the dominating pathogen in the field was another species than previous years, which may be correlated to the weather conditions with drier spring and more rainfall in June than in an average year (SJV, 2005a; SJV, 2005b).

Data obtained from a long term field inventory at Broadbalk, U.K., has shown a shift in occurrence of two of the most common pathogenic fungi, *P. nodorum* and *M. graminicola*, while *P. tritici-repentis* is not a problem in the U.K. (Shaw *et al.*, 2008). The pathogen abundance was found to be correlated to weather data in a short-term perspective where rain during spring increased the infections of both pathogens whereas *P. nodorum* was more abundant during warm summers. In a long term perspective the abundance of *P. nodorum* fluctuated from 1844 to 2002, with a peak during approximately 1960 to 1985. The peak coincides with the increased amount of sulphur emission from the use of fossil fuel, indicating that sulphur may be favourable for *P. nodorum*. *M. graminicola* dominated at Broadbalk during the last years of the twentieth century and the first years of the twenty-first century, while the frequency of the fungus was low during the rest of the twentieth century (Shaw *et al.*, 2008). Similar patterns of pathogen abundance have also been observed in Sweden, where we have seen a decline in *P. nodorum*, but not as drastic as the one in the U.K. (personal observation and communication with farmers and the Plant Protection Centres). *M. graminicola* and *P. tritici-repentis* have increased during the past years and can lead to severe yield reduction. However, the visual estimations of the abundance of *P. nodorum* and *P. tritici-repentis* may be delusive since the symptoms of tan spot and stagonospora nodorum blotch are hard to distinguish (*vide* 3.3). It is also difficult to see the *P. nodorum* pycnidia in field situations, even with a hand lens. The deposition of sulphur has decreased in Sweden during the last decades (Hultberg & Ferm, 2004) and it is possible that the change of fungal community was affected by the change in sulphur deposition in Sweden as well, but that is yet to be analysed.

5.3 Fungicide sensitivity of *P. nodorum*

Mutations of nucleotides that lead to reduced sensitivity to fungicides will be selected if the fungal population is treated with fungicides. One clear example is the substitution G143A in the gene encoding cytochrome *b*, abundant within the Swedish population of *P. nodorum* (IV). Since this is the first report on the sensitivity of *P. nodorum* to strobilurins, one can only speculate when and where that substitution occurred. It must have happened soon after the release of the strobilurins in the late 1990s since the substitution was frequent already in 2003 in samples from an untreated plot of the field. Strains with the substitutions have been selected by strobilurin treatments of the crop the previous year. The strains which had lost their sensitivity could therefore have overwintered in the stubble or originate from incoming ascospores carrying the substitution.

Based on the genetic variation in the mitochondrial DNA the G143A substitution occurring within the European populations of *M. graminicola* is thought to originate from four independent mutation occasions (Torriani *et al.*, in press). The substitution has then most likely migrated within Europe in a west-to-east direction through ascospore dispersal which founded new insensitive subpopulations. The dispersal pattern of the G143A substitution within the population of *P. nodorum* is still unknown but follows presumably the dispersal pattern of ascospores, conidia and harvest residues. Another speculation of the origin of the G143A substitution found in the *P. nodorum* population is that horizontal gene transfer occurred from another fungal species. *P. nodorum* may also have been the source of G143A substitutions found in other species. This theory is based on the suggestions that the gene encoding ToxA in *P. tritici-repentis* was transferred from *P. nodorum* due to the large resemblance to the toxin in *P. nodorum* (Friesen *et al.*, 2006; Knell, 2006). Friesen *et al*, (2008a) suggest that horizontal gene transfer is more common between species within the order Pleosporales, which contains both *P. nodorum* and *P. tritici-repentis*. The G143A substitution may therefore have been exchanged between them. The substitution in *P. tritici-repentis* was discovered prior to the one in *P. nodorum* but this has little bearing on which one was the donor and which one was the receipient.

The loss of sensitivity against strobilurins due to the G143A substitution within the population of *P. nodorum*, combined with the abundance of *P. nodorum* found within the fungal community, shows that this fungus is still an important issue for research and wheat growers. The fungus is present in the fields but due to the resemblance of stagonospora nodorum blotch to tan spot, we can underestimate the amount of either pathogen. This means that we have to consider the risk of reduced strobilurin efficacy in inhibition of all pathogens in all wheat growing areas in Sweden, not only the southern part of Sweden where reduced sensitivity of *M. graminicola* and *P. tritici-repentis* already has become an important problem (Almquist *et al.*, 2008; Jørgensen, 2007).

The results from the fungicide sensitivity test (IV) revealed that control of *P. nodorum* can still efficiently be achieved by using fungicides based on triazoles and anilinopyrimidine. Use of strobilurins is not advisable for crop protection due to the high frequency of the G143A substitution. Crop rotation and proper soil management are the most important ways of preventing fungal infection, by decreasing the amount of inoculum produced in the field. The crop cannot be protected from incoming ascospores of various species or airborne conidia that can start epidemics in the field.

The future of the fungicides is uncertain. The fungicides that are effective today may have lost their efficacy due to fungal evolution or they have been removed from the market in the future. Much depends on the new directives from the Commission of the European Union on the legalisation and usage of pesticides that are under consideration. The Swedish Board of Agriculture has written a report of the interpretations from the Swedish Chemical Agency (KemI) and the British Pesticides Safety Directorate (PSD) (Eriksson *et al.*, 2008). The interpretation made by PSD predicts a stronger effect on wheat production than the Swedish interpretation. According to PSD propiconazole may fall for the cut off criteria and cyprodinil will be substituted with another substance. Strobilurins may still be allowed for treatment against rust fungi. In practice, farmers are advised to use strobilurins in combination with triazoles against rusts (G. Berg, Plant Protection Centre, personal communication). Treatment of leaf spot diseases with strobilurins is not advisable due to the reduced efficacy against *M. graminicola* and *P. tritici-repentis*. Now we know that the Swedish populations of *P. nodorum* also have lost their sensitivity to strobilurins (IV) and farmers must therefore rely on the triazoles for controlling leaf spot diseases in wheat. The alternatives are limited since epoxiconazole, commonly used in fungicide testing, is not approved in Sweden and propiconazole has declined in usage during the last years due to use of other fungicides. This leads to increased reliance on those triazoles that exists on the market, of which prothioconazole dominated in 2008 (G. Berg, Plant Protection Centre, personal communication).

In an environmental perspective there are a few biological products for reduction of pathogens (*vide 3.6*) and research is ongoing for more and efficient products. Legislation, taxes, environmental policies and the market price of wheat and fuel are also aspects that influence the farmers' choice of plant disease management methods and strategies (Söderberg, 2008).

5.4 Final reflection

In summary, sexual reproduction and ascospores are of importance for the epidemiology of *P. nodorum* in wheat. This can lead to dire consequences for agricultural practises including the prevention of disease since epidemics can occur anywhere under favourable weather conditions if ascospores manage to start an epidemic. The fungal population founded by ascospores may carry the substitution that causes reduced sensitivity to strobilurins, which is another threat to modern agriculture. Breeding for resistant cultivars, with help of the knowledge of the toxin-receptor interaction would be an important first step. The use of them, together with good agricultural management practices, including a crop rotation that reduces the presence of inoculum, would be one way of reducing the incidence of stagonospora nodorum blotch.

This thesis began with the concept of "eat or be eaten". There are many species that take their nourishment from wheat, including us human beings. It is the human fight against pathogens for higher yield for bread baking, and this competition also takes place with saprotrophs and yeasts. The fungal species have their internal fight against each other. These interactions could be used in biological control in purpose to reduce the environmental impact caused by high treatments by chemical substances and increased fuel use due to more intensive soil management. Much can be learned from the ecology of the system and this can be used in plant pathology and elsewhere. Plant pathology will continue to be an important subject of research, since all species need nutrients for reproduction for their survival and it will always be a give and take, eat or be eaten.

6 Conclusions

I The wheat pathogen *Phaeosphaeria nodorum* is common in Sweden but with some variation between years. The disease may be hard to distinguish from tan spot due to the resemblance of the symptoms caused by *P. nodorum* and *Pyrenophora tritici-repentis*.

II Saprophytes and yeasts are common on wheat leaves together with leaf spot pathogens.

III The diverse genetic structure of the *P. nodorum* population indicates that the fungus mates in Sweden. The two mating types are evenly distributed within fields. The genetic structure is diverse with many unique genotypes and the studied alleles were randomly associated to each other which are consistent with frequent sexual reproduction.

IV Isolates of *P. nodorum* vary in aggressiveness to the cultivars commonly used in Swedish agriculture. Low inoculum concentration separated the isolates better than high inoculum concentrations. The cultivars have similar susceptibility to the fungus, at least at the seedling stage.

V The reproducibility of the detached leaf tests was not satisfactory if AUDPC was used as a disease measurement. Further adjustments and standardisations must be made before the method can be used reliably in susceptibility test against *P. nodorum* using AUDPC.

VI The Swedish population of *P. nodorum* is sensitive to propiconazole, prothioconazole and cyprodinil, even though there was variability among the isolates.

VII The majority of the *P. nodorum* isolates collected during 2002–2005 had the substitution G143A in cytochrome *b*, associated with loss of sensitivity to strobilurins. The result indicates that fungicides based on strobilurins would not be advisable in for disease control.

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Sammanfattning

Så länge det har funnits vete har det förmodligen funnits patogener som orsakar sjukdomar på veteplantorna. Flera av dem är svampar även om virus, bakterier, och nematoder också tar sin näring från plantorna. Forskare har teorier om att vissa svampsjukdomar uppstod i samband med att vetet för tolv tusen år sedan började odlas i "bördiga halvmånen" i Mellanöstern ("the Fertile Crescent"). När utbredningen av veteodlingen drogs norrut i Europa följde patogenerna med via utsädet elleer spreds via vindburna sporer. Under 1800-talet identifierades flera patogena svampar som angriper vete, mycket tack vare utvecklingen av mikroskopet. Förädlingen av vetesorter under 1900-talet ledde till plantor med längre ax, kortare strå och breda blad för att öka skördarna. Frodiga bestånd medförde att skördeförlusterna på grund av bladfläcksvampar ökade eftersom de gynnas av ett fuktigt mikroklimat. Ett kort avstånd mellan bladnivåerna förkortar dessutom spridningsavståndet för svamparna.

Svamppopulationen på veteblad med nekrotiska fläckar

Veteblad med nekrotiska fläckar kan härbärgera flera arter av svampar. Tretton arter och ett artkomplex identifierades från höstveteblad insamlade i elva fält fördelade i Östergötland, Västergötland och Uppland under tre år (Tabell 1, Figur 5). Tre arter var patogena, tre var saprofyter och resten var olika arter av jäst. Jästsvamparna förekom i alla fält vilket tyder på att de samexisterar med de patogena svamparna. Troligen har jästsvamparna inte så stor betydelse för grödan.

De patogena svamparna var *Mycosphaerella graminicola* (anamorph *Septoria tritici*) som orsakar svartpricksjuka, *Pyrenophora tritici-repentis* (anamorph *Drechslera tritici-repentis*) som orsakar vetets bladfläcksjuka samt *Phaeosphaeria nodorum* (anamorph *Stagonospora nodorum)* som orsakar brunfläcksjuka i vete.

P. nodorum dominerande i flera av fälten under 2003 och 2004 medan *M. graminicola* var vanligast i både Östergötland och Västergötland under 2005. I Uppland dominerade *P. tritici-repentis* under 2005 medan *P. nodorum* fanns i mycket liten mängd (I). Symptomen av brunfläcksjuka (Figur 1) och vetes bladfläcksjuka (Figur 4) är förväxlingsbara under senare sjukdomsstadier. Det bästa sättet att skilja svamparna åt är att studera de asexuella sporerna, konidierna (Figur 3).

Biologin hos *Phaeosphaeria nodorum*

I livscykeln för *P. nodorum* finns en asexuell och en sexuell fas (Figur 2). Den asexuella fasen innefattar bildandet av asexuella sporkapslar, pyknider, på blad, ax och och halmrester. Pykniderna innehåller konidier som tränger ut vid fuktig väderlek. De sprids via regnstänk mellan bladen i beståndet. Bildandet av pyknider och konidiespridning kan ske flera gånger under en växtsäsong. Om svampen når axet etablerar den sig på kärnorna. Vid skörd förs svampen vidare med utsädet till nästa odlingssäsong. *P. nodorum* kan också övervintra på växtrester i fältet. Cirkeln är sluten om konidier från pyknider som bildas på halmresterna kan infektera nya plantor året därpå. Smittat utsäde medför att veteplantans tillväxt hämmas eller att plantan dör.

Förekomsten av det sexuella stadiet hos *P. nodorum* i Sverige bekräftades vid en analys av svampens genetiska struktur (II). I den sexuella fasen av livscykeln möts två individer med parningstyperna MAT1-1 respektive MAT1-2. De båda parningstyperna förekommer lika frekvent i fälten och de är slumpmässigt fördelade, vilket ökar möjligheterna för sexuell reproduktion. Resultatet av den sexuella reproduktionen är en fruktkropp, pseudotecium, som innehåller ascosporer. Ascosporerna är vindburna vilket medför att de kan spridas till nya veteplantor såväl inom som mellan fält.

Olika individer av en svamp har oftast olika antal upprepningar och olika kombinationer av mikrosatelliter, vilka tillsammans med parningstypen utgör isolatets genotyp. Totalt identifierades 93 olika genotyper bland 227 isolat, varav 69 förekom endast en gång. Inom varje fält fanns det mellan 23 och 38 genotyper. Många unika genotyper tillsammans med slumpvis förekomst av parningstyperna i fält är en mycket stark indikation på att *P. nodorum* förökas på sexuell väg även i Sverige.

Olika isolat av *P. nodorum* har varierande nivå av aggressivitet mot vetesorter (III). Sex höstvetesorter som provades i ett aggressivitetstest hade ungefär samma mottaglighet för angrepp av brunfläcksjuka. Latensperioden för svampen förkortades då plantorna inokulerades med hög konidiekoncentration.

Bekämpning av växtpatogena svampar

Angreppen av *P. nodorum* kan förhindras eller bekämpas på flera sätt. En bra växtföljd där stråsäd alterneras med t.ex. oljeväxter och ärtväxter samt god jordbearbetning med framförallt plöjning, minskar halmmängden på markytan och därmed möjligheterna för inokulum att överleva och orsaka nya infektioner. Fungicider förhindrar myceltillväxt och sporgroning, genom att störa en viktig enzymaktivitet. Under 2000-talet har lantbrukare i Skåne och Danmark, men även i andra delar av Europa, märkt en försämrad effektivitet av fungicidbehandlingar mot *M. graminicola* och *P. tritici-repentis*. Orsaken är att någon av de aminosyror som bygger upp enzymet som substansen ska fästa vid har bytts ut, varvid fungiciden inte längre kan störa enzymet i fråga. Fyra substansers effektivitet mot *P. nodorum* undersöktes genom odling på agarplattor (IV). Cyprodinil som tillhör gruppen anilinopyrimidiner, samt propiconazole och prothioconazole, tillhörande gruppen triazoler var effektiva mot *P. nodorum*. Azoxystrobin, som är en strobilurin, hade delvis förlorat förmågan att hämma tillväxten av *P. nodorum*. Majoriteten av isolaten hade ett EC_{50}-värde (den effektiva koncentrationen som minskar tillväxten till 50 % av referensisolatens tillväxt) som var högre än referensisolatens genomsnittliga EC_{50}-värde. Vid sekvensering av den gen som kodar för cytokrom *b*, det protein som azoxystrobin ska fästa vid, hade majoriteten av isolaten aminosyran alanin istället för glycin vid position 143. Substitutionen som betecknas G143A, fanns hos 79 % av de 227 *P. nodorum*-isolat som sekvenserades. I ett fält var förekomsten av substitutionen endast 50 % bland de insamlade isolaten. Detta är den första rapporten någonsin om att *P. nodorum* har blivit okänslig mot strobiluriner.

Slutsats

Dessa resultat har betydelse för lantbruket i flera avseenden. I ett epidemiologiskt perspektiv är kunskapen om att *P. nodorum* kan spridas mellan fält via ascosporer och ge upphov till angrepp trots god växtföljd och jordbearbetning viktig för valet av odlingsstategier. En stor del av den svenska *P. nodorum*-populationen är okänslig mot strobiluriner and den andelen kan öka ytterligare genom selektion vid fungicidbehandling. Detta innebär att strobiluriner ej bör rekommenderas för behandling mot bladfläcksvampar i vete. Aktsamhet bör även iakttas vid användning av andra fungicider eftersom andra patogena svampar har förlorat sin känslighet mot exempelvis triazoler.

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Yours truly,
Eva

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