Eur J Plant Pathol (2010) 126:373–386
DOI 10.1007/s10658-009-9542-z

Spatiotemporal variation in the fungal community associated with wheat leaves showing symptoms similar to stagonospora nodorum blotch

Eva Blixt · Åke Olson · Björn Lindahl · Annika Djurle · Jonathan Yuen

Received: 27 February 2009 / Accepted: 1 October 2009 / Published online: 23 October 2009

Abstract The fungal communities on wheat leaves showing symptoms similar to stagonospora nodorum blotch were analysed using terminal restriction fragment length polymorphism (T-RFLP). Collection of diseased leaves was carried out in eleven winter wheat fields located in three regions of Sweden during mid-July in 2003–2005. Fourteen different fungal species were found on the leaves out of which thirteen were identified to the species level and one to the genus level. The majority of the samples had between one and four species present of which at least one was a pathogen. Among the analysed leaves three major leaf pathogens were found: *Phaeosphaeria nodorum* was common during 2003 and 2004, *Mycosphaerella graminicola* dominated during 2005. *Pyrenophora tritici-repentis* was present in all fields, but sometimes in just a few samples. *Phaeosphaeria nodorum* and *P. tritici-repentis* often co-occurred on the same leaf. In addition, seven species of yeast and three saprophytes frequently occurred on the leaves every year. The variation in fungal community was largest between the different years while the region of Uppland diverged from the other two regions in species composition. No significant differences in fungal communities were found within a single field, indicating a uniform community at the lowest spatial level.

Keywords Fungal community · Fungal pathogen · Necrotic lesion · Saprophyte · stagonospora nodorum blotch · Wheat · Yeast

E. Blixt (✉) · Å. Olson · B. Lindahl · A. Djurle · J. Yuen
Department of Forest Mycology and Pathology,
Swedish University of Agricultural Sciences,
P.O. Box 7026, SE 750 07 Uppsala, Sweden
e-mail: Eva.Blixt@mykopat.slu.se

Introduction

Wheat, *Triticum aestivum,* is one of the largest staple food crops grown globally today. Grain yield can be significantly reduced by pathogens that degrade leaf or other host tissues leading to decrease of nutrient assimilation. Several fungal pathogens cause necrotic lesions on wheat leaves and the most common ones are *Phaeosphaeria nodorum* (anamorph *Stagonospora nodorum* causing stagonospora nodorum blotch, *Pyrenophora tritici-repentis* (anamorph *Dreschlera tritici-repentis*) causing tan spot and *Mycosphaerella graminicola* (anamorph *Septoria tritici*) causing septoria tritici blotch. Winter wheat seedlings can also be damaged by *Monographella nivalis* (anamorph *Microdochium nivale*) causing pink snow mold, *Ascochyta tritici* (causing ascochyta leaf spot) and *Cochliobolus sativus* (anamorph *Bipolaris sorokiniana*) causing spot blotch. The stem base and spike can be affected by various *Fusarium* species. These pathogenic fungi are found in many parts of the world where wheat is grown and their importance varies between regions and climatic conditions (Wiese 1987).

Necrotic leaf spot pathogens can often be distinguished by the symptoms and the morphology of the spores produced. The early symptoms of stagonospora nodorum blotch are dark brown lesions, often surrounded by chlorosis, that later expand to irregular lesions (Eyal et al. 1987). Pycnidia of *P. nodorum* are tan or brown and scattered in the lesion. They can be difficult to discover since they are partly embedded in the leaf tissue and their colour is very close to that of the lesion. The cirrhi containing pycnidiospores have an orange to pink colour. *Phaeosphaeria avenaria* f.sp. *triticea* causes symptoms similar to stagonospora nodorum blotch, but the pycnidiospores are larger than those of *P. nodorum* and the cirrhi are white.

The early symptoms of tan spot are small lens- or diamond-shaped tan to brown lesions, often densely scattered over the leaf surface. The lesions are surrounded by a yellow border and often a dark spot can be seen in the centre. In later stages the margins of the tan-coloured spot becomes darker and the centre of the lesion turns into a lighter colour. Lesion shape becomes more irregular as lesions coalesce. The typical lesions of tan spot will still have a grey spot in the centre (Dreschler 1923). The symptoms can vary depending on wheat cultivar and race of the pathogen. The presence of a greyish spot in the centre of the lesion is often used to distinguish tan spot from stagonospora nodorum blotch. A quick method to distinguish between stagonospora nodorum blotch and tan spot is to examine the early symptoms along with the formation and morphology of the conidia and pycnidiospores. *Pyrenophora tritici-repentis* produces free conidia attached to conidiophores on the leaf surface during the asexual stage of the life-cycle, while *P. nodorum* produces pycnidia, containing pycnidiospores.

The symptoms of septoria tritici blotch are long light brown to greyish lesions following the leaf veins. Black pycnidia are produced in the substomatal chambers giving the typical appearance of black dots in a row (Eyal et al. 1987; Shaner and Finney 1976). These pycnidia are smaller than those of *P. nodorum* but easier to see since they protrude more from the leaf surface and have a darker colour. The cirrhi from *M. graminicola* pycnidia are white.

The incidence of *M. nivalis*, *A. tritici* and *C. sativus*, is often low under Swedish conditions and their symptoms can be distinguished from those of other leaf-spotting fungi, due to their colour and time of appearance. Other types of fungi, such as saprotrophs and yeasts, can also be found on wheat leaves; the amount and species diversity is dependent on environmental factors (Kinkel 1997).

Identification of fungal species in complex communities can be efficiently performed by surveys and assessment of plant samples using molecular methods. The most frequently used genomic region for species identification of fungi is the internal transcribed spacer of the ribosomal genes (ITS-region) (White et al. 1990). The two non-coding ITS-regions flanking the rDNA subunit 5.8s, ITS1 and ITS2, are species-specific in nucleotide sequence composition. A fungal-specific primer has been developed, ITS1f, which together with primer ITS4 is used to amplify a 400–800 bp region (Gardes and Bruns 1993; Kitts 2001).

Terminal restriction fragment length polymorphism (T-RFLP) is a method that identifies species based on variations in fragment lengths after digestion of the PCR amplified fragment by restriction enzymes (Dickie et al. 2002; Kitts 2001). The method relies on a reference database of restriction fragment lengths from identified taxa. Such a database is derived directly from the samples to be analysed, through cloning and sequencing of amplicons. By restriction cutting of the cloned amplicons, a unique combination of fragment lengths are recorded for each identified species. T-RFLP patterns derived from mixed communities are then compared with the fragments derived from sequenced clones, in order to identify species within the communities (Lindahl et al. 2007).

Natural fluctuations in population dynamic processes such as immigration, density and resource limitations, can be found among fungal species occurring on the phylloplane, *i.e.* leaf and stem surfaces on plants (Kinkel 1997; Woody et al. 2007). The density and diversity of the fungal community on wheat leaves has been shown to increase as the plant develops during the season (Blakeman 1985; Remlein-Starosta 2004), but also varies between seasons (Legard et al. 1994). Yeasts colonise leaves early in the season and are often dominant during mid-season, whereas filamentous fungi increase later (Behrendt et al. 1997; Blakeman 1985). The population dynamics of yeasts may fluctuate even on a daily basis as the microclimate changes between day and night (Nix et al. 2008). Environmental conditions are important for fungal

development on the phylloplane. Humidity, temperature and nutrient supply are parameters that, besides fungicides, determine the growth of the fungi (Blakeman 1985). Sulphur dioxide emissions have been associated with the increase of white-pigmented yeasts and *P. nodorum* on wheat leaves (Remlein-Starosta 2004; Shaw et al. 2008). Pink-pigmented yeasts are on the other hand inhibited by sulphur emission (Dowding and Richardson 1990).

The spatial distribution of fungi on leaves varies due to leaf habitat conditions and irregular deposition of inoculum (Andrews and Harris 2000). The majority of epiphytic fungi are found on the abaxial (lower) surface of the leaf, which may be due to a higher nutrient availability and a more suitable microclimate in terms of moisture and temperature. Studies on spatial distribution of fungi on a larger scale have often been conducted to find a means for prediction and prevention of epidemics (Almquist et al. 2008; Engle et al. 2006). Variation in abundance and even presence of pathogens at larger spatial scales may fluctuate with agricultural management, location and weather conditions. The latter is often important in determining the distribution and incidence of pathogens within and among fields as well as their temporal variation.

The aim of this study was to investigate fungal communities on diseased winter wheat leaves showing symptoms similar to stagonospora nodorum blotch and the variation in species composition over time and different spatial levels. Leaf samples were collected from three consecutive years across different spatial scales; within single fields, within regions (200 m–30 km) and across regions (100–300 km).

Materials and methods

Sampling

Collection of diseased wheat leaves showing symptoms similar to stagonospora nodorum blotch was made during mid-July in 2003, 2004 and 2005, with years subsequently referred to as year 1, 2 and 3. The sampling locations were eleven commercial winter wheat fields with plots (appr. 20×20 m) which were not treated with fungicides (used for disease warning services by the Swedish Board of Agriculture). The fields were located in three different regions of Sweden: Uppland, (U), Västergötland (V) and Östergötland (E) (Fig. 1, Table 1). The regions are about 100–300 km apart. In each region, samples were collected during three subsequent years (U1, U2, U3, *etc*.) and from different fields each year. In Västergötland and Östergötland the fields were located within a 600 m distance, whereas in Uppland they were within 20 km from each other. In the region of Östergötland three

Fig. 1 Sampling locations of winter wheat fields within three regions of Sweden. U: Uppland (U1, U2, U3); V: Västergötland (V1, V2, V3); E: Östergötland (E1a, E1b, E1c, E2, E3) (map: K. Perhans, Dept. of Ecology, SLU, Uppsala)

Table 1 Sampling strategy for the survey of the fungal community on wheat leaves exhibiting symptoms similar to stagonospora nodorum blotch

County of Sweden	Year of sampling	Field name	Cultivar	Precrop	No. of samples collected	No. of samples used in analyses
Uppland (U)	2003	U1	Olivin	Ley (organic)	12	10
	2004	U2	Olivin	Winter wheat	12	12
	2005	U3	Kosack	Winter wheat	12	12
Västergötland (V)	2003	V1	Olivin	Winter wheat	12	12
	2004	V2	Olivin	Winter wheat	12	12
	2005	V3	Olivin	Winter wheat	12	9
Östergötland (E)	2003	E1a	Kosack	Winter wheat	12	12
		E1b	Olivin	Winter wheat	12	12
		E1c	Olivin	Winter wheat	66[a]	12[b], 59[c]
	2004	E2	Olivin	Winter wheat	12	11
	2005	E3	Olivin	Winter wheat	12	9
Total no. of samples					186	123[b], 172[d]

[a] Nine additional leaves were collected from six of the sampling sites (corners and middle of the second and third row)

[b] One random sample from each of the twelve sampling sites

[c] Only the leaves from the sites with multiple collection was used for test of within-field variation

[d] All samples were used in some analyses

field plots (E1a, E1b and E1c) within 30 km distance were sampled in year 1 (Table 1).

The cultivar grown in the sampled fields was Olivin except in the fields E1a and U3 where the cv. Kosack was grown. All crops were second year winter wheat and the fields were ploughed between the two wheat crops, except for the field sampled in Uppland 2003 (U1), which was organically cultivated with a clover-grass ley (ploughed under) as the previous crop.

Approximately ten diseased leaves, mostly flag leaves, with well defined necrotic lesions were collected at early grain-fill, DC 69–75 (Zadoks et al. 1974), at each of twelve sampling sites within each sampling plot, referred to as fields. Along each of four rows in a plot samples were taken at three sites which were approx. 6 m apart. The original aim of the leaf collection was to study the population of *P. nodorum* (Blixt et al. 2008), so the leaves were selected based on symptoms caused by this fungus. No systematic disease measurements were taken. One single leaf per sampling site within a field (12 per field for U1-3, V1-3, E1a, E1b, E2, and E3) was randomly selected for this study, *i.e.* a total of 120 leaves. For further analysis of the variation within fields, the field named E1c was hierarchically sampled. In addition to a single leaf from each of the 12 sampling sites, nine supplementary leaves were sampled at six of the sampling sites, *i.e.* each corner of the plot and from the middle site of the second and third row. In total 66 leaves were sampled from this field. The total number of leaf samples used in the study was 186 (Table 1).

DNA-extraction

The leaves were freeze-dried and ground in a 2 ml microcentrifuge tube containing two M5 stainless steel nuts at a speed of 4.5 units for 30 s in a FastPrep® preparation shaker (Qbiogene). DNA was extracted using the Qiagen DNeasy Plant Mini kit protocol with minor modifications: 400 µl of buffer AP1 was added to a 2 ml microcentrifuge tube containing 20 mg of leaf powder and five glass beads. The tubes were shaken at 5,000 rpm for 2×30 s in a Precellys® preparation shaker (Bertin Technologies); 21 U of RNase A was added and the samples were incubated at 65°C for 60 min, and 130 µl of buffer A2 was then added and the samples centrifuged for 5 min at 13,000 rpm (16,200×*g*). The protocol was then followed and the DNA was re-suspended in 50 µl dilution buffer (DNeasy Plant Mini Kit, Qiagen).

Identification of fungal species

Terminal restriction fragment length polymorphism (T-RFLP) was used to identify the fungal species. The ITS region was amplified with the primers ITS1f (Gardes and Bruns 1993) and ITS4 (White et al. 1990). The primers were labelled with fluorescent dye D3 and D4 respectively (Well red, Sigma-Proligo). The PCR solution of 20 µl contained 0.5 ng total DNA μl^{-1}, 2.75 mM $MgCl_2$ (final concentration), 0.2 mM dNTP, 0.2 µM of each primer, 0.03 U μl^{-1} ThermoRed DNA Polymerase (Saveen & Werner AB) and corresponding reaction buffer Y (10X). The PCR conditions were 94°C for 2 min, 30 cycles of 30 s at 94°C, 30 s at 55°C and 30 s at 72°C, followed by a 7 min extension (2720 Thermal Cycler, Applied Biosystems). Negative controls were included in every PCR. The PCR products were separated on 1.0% agarose gel (Agarose D-1, Conda), half-strength TBE, at 3.3 V cm^{-1} for 90 min. The gels were stained with ethidium bromide and visually analysed under UV light (GelDoc, Bio-Rad Laboratories).

The PCR products were digested with restriction enzymes *Alu*I (Roche), *Cfo*I (Promega) and *Taq*I (Fermentas) according to the manufacturers' manual. The digested PCR products were purified by first adding 400 µl autoclaved Sephadex G-50 Medium suspension (GE Healthcare Bio-science) to each well of the 96-well plate supplied by the manufacturer, which was then centrifuged for 2 min at 1,500 rpm to remove additional water. Ten micro-litres of the digested PCR products were then added to the Sephadex G-50 medium and centrifuged again at 300 rpm for 2 min. The purified PCR products were collected from the lowermost 96-well plate. The length of the terminal fragments were analysed in a Beckman Coulter CEQ™ 8000 Genetic Analysis System using CEQ™ DNA Size standard Kit-600 (Beckman Coulter) as an internal standard.

Assembly of clone library

To set up a reference library of fungal species, PCR products of the ITS region, using non-fluorescent primers, were cloned using the TOPA TA Cloning Kit with the PCR® 2.1-TOPO vector and One Shot TOP10 chemical component *Escherichia coli* (Invitrogen). Based on the outcome of a preliminary multivariate analysis of T-RFLP patterns (see "Statistical analysis"), PCR products from the eleven fields were pooled into seven samples that were cloned individually. In total, 152 bacterial clone colonies were selected and each was suspended in 100 µl sterile H_2O, 25 µl of which were used directly for PCR. The PCR solution of 50 µl had the same concentrations and conditions as above but with different primers: M13 Forward and M13 Reverse. The PCR products were purified with the Agencourt ® AMPure® Protocol 000601v024. Sequencing of the clones was performed by Macrogen (Seoul, S Korea) from both ends using the ITS1f and ITS4 primers.

The clone sequences between primer sites ITS1f and ITS4 were assembled to 99% accuracy using SeqMan Genome Assembler (DNAstar Inc.) and the species were identified by comparison with reference sequences at the National Centre for Biotechnology Information (NCBI, www.ncbi.nlm.nih.gov) using the BLASTN algorithm (Altschul et al. 1990). Positive identification was made if the entire fragment between ITS1f and ITS4 was aligned to a species. In order to obtain T-RFLP patterns of the identified species, selected clones were once again re-amplified, using fluorescent primers, PCR products were cut with restriction enzymes and fragment lengths analysed as described above. Supplementary species patterns were kindly provided by Dr Magdalena Grudzinska-Sterno at the Department of Forest Mycology and Pathology, SLU, Uppsala, Sweden.

Identification of species within samples

The T-RFLP patterns of the identified species in the clone library were used for identification of species within each sample. Positive identification was made if both terminal restriction fragments from each restriction enzyme were present in the PCR product (six in total) or the entire ITS-amplicon in cases where restriction sites were missing, which sometimes was the case for *Alu*I. For each sample and primer/restriction enzyme combination, the amplitude of the fragment peaks was normalised as a proportion of the total fluorescence. The relative abundance of each species within each sample was calculated as the average normalised fluorescence of the unique fragments of the species using the following formula:

$$\text{Abundance} = \frac{\sum_{i=1}^{n} \left(\frac{F_{\text{fragm.}}}{F_{\text{Tot.}}} \right)_i}{n}$$

n : number of unique fragments

Statistical analysis

The pooling of PCR products for cloning was based on a preliminary clustering of samples by the multivariate technique detrended correspondence analysis (DCA) using canoco version 4.5 for Windows (Microcomputer Power) (Hill and Gauch 1980). The data used during the DCA procedure was the normalised amplitude of the terminal restriction fragments (all enzymes separately) from the ITS1f primer end.

To depict variation in fungal community composition within and between fields a DCA was performed using relative species abundances based on species-unique fragments from all six primer/enzyme combinations. Relationships between geographical location, sampling year and community composition within and among fields were tested for statistical significance using canonical correspondence analysis, CCA (ter Braak 1986). For statistical analyses of differences between fields, regions and years the 12 samples per field were used (Table 1). Within-field variation was tested using ten leaves from six sample sites where multiple collections were made in field E1c. The percentage of the total inertia that was explained by the environmental parameters was calculated by dividing the sum of all canonical eigenvalues by the sum of all eigenvalues. The significance of the CCA was tested using 1000 Monte Carlo permutations.

Results

Total DNA from 186 leaves was extracted and the fungal ITS region amplified with either ordinary or fluorescent primers for cloning or T-RFLP analysis, respectively. PCR products from the eleven fields were pooled and cloned in seven individual samples.

Clone library

One hundred and fifty-two clones were sequenced and 96 of them clustered into 16 groups corresponding to unique sequence types (Table 2). These sequences showed 98–100% identity to reference sequences at

Table 2 Fungal species identified on wheat leaves showing symptoms similar to stagonospora nodorum blotch based on cloned fragments of the ITS-regions

	Species	GenBank No	% identity.	No. of clones	%[a]	Species code
Pathogenic fungi	*Blumeria graminis*	AB273566	99	1	1.0	–
	Mycosphaerella graminicola	EU019297	100	9	9.4	M. gram
	Phaeosphaeria avenaria f.sp. *triticea*	AY196988	99	2	2.1	(grouped with P. nod)
	Phaeosphaeria nodorum	AF181710	100	30	31.3	P. nod
	Pyrenophora tritici-repentis	AM887510	100	18	18.8	PTR
Saprotrophic fungi	*Davidiella macrospora*	EU167591	100	5	5.2	Dav. mac
	Lewia infectoria [b]	AY154692	99	1	1.0	Lew. in
	Udeniomyces pannonicus	AB072232	100	6	6.3	Uden. pa
Yeasts	*Bullera globospora*	AF444407	99	1	1.0	Bul. glo
	Cryptococcus victoriae	AJ581048	99	1	1.0	Cry. vic
	Cryptococcus wieringae	AF444383	99	3	3.1	Cry. we
	Cryptococcus sp.[c]	AF444354[c]	98	4	4.2	Crypt. sp.
	Dioszegia crocea [d]	AJ581068	99	3	3.1	Dio. cr
	Dioszegia fristingensis	EU070927	98	2	2.1	Dio. fri
	Dioszegia hungarica	AB049614	99	8	8.3	Dio. hu
	Sporobolomyces roseus	AY015438	99	2	2.1	Spo. ros
Sum of clones and percentage				96	100	

[a] Percentage of the 96 clones successfully sequenced and identified to species or genus level

[b] anamorph *Alternaria infectoria*

[c] Short fragment aligned to *C. chernovii* (98% identity)

[d] syn. *Bullera crocea*

NCBI and corresponding taxa were identified for fifteen of them. One sequence group could only be determined at the genus level. Thirty-one sequenced clones were identified as PCR-chimeras of sequences from different taxa. The sequencing procedure failed for 25 clones. The most frequent clones were identified as *P. nodorum* (31.3% of total identified clones, Table 2). Two clones were identified as the pathogen *P. avenaria* f.sp. *triticea*, but were pooled together with *P. nodorum* in the subsequent T-RFLP analysis, since the restriction fragments from the separate clones could not be distinguished during fragment analysis. Four species, *M. graminicola, Davidiella macrospora, Bullera globospora* and *Cryptococcus victoriae* did not have any restriction sites for *Alu*I within the ITS-region, and thus the PCR product appeared uncut in the fragment analysis. Separate analyses of the sequences of ITS1 and ITS2 from the chimeras identified the following species: *M. nivalis, Ascochyta* sp., *Cladosporium* sp., *Epicoccum* sp., *Cryptococcus nyarrowii* and *Dioszegia aurantiaca.* These species were not used in further analyses due to lack of complete restriction fragment profiles of the species.

Fungal community within fields

The fungal communities from 186 wheat leaves from three regions, three years and 11 fields were analysed with T-RFLP. Fourteen samples were removed from the study due to unsuccessful PCR amplification. The restriction fragments from the samples were matched with the fragment patterns of the clones. All cloned species, except for *Blumeria graminis*, were recovered as T-RFLP patterns among the samples (Table 3). The majority of the samples contained 1–4 identified species, of which at least one was a recognised pathogenic fungus (Fig. 2). The maximum observed species richness was ten species in a sample, recovered from three different samples.

All major detected fragment peaks could be assigned to an identified species, except for two large peaks cut by *Taq*1 in ITS2, one small peak cut by *Taq*I in ITS1 and three small peaks cut by *Cfo*I. Four small peaks were not identified for either ITS1 or ITS2 when the restriction enzyme *Alu*I was used. The percentage of total fluorescence assigned to identified clones per field was between 55–96%, with the lowest percentage in field U1 and the highest in field V1. The average across all fields was 72%.

Three recognised pathogenic fungi were identified among the samples and they were the most abundant species. *P. nodorum* and *P. tritici-repentis* were found in all eleven fields. These two species corresponded to >50% of fluorescence associated with the identified fungal species in some of the samples (Table 3, Fig. 3). The pathogen *M. graminicola* was found in eight of the fields and dominated in field V3 and E3. Basidiomycetous yeasts, especially *Dioszegia crocea* (syn. *Bullera crocea*) and *Dioszegia hungarica*, as well as the saprotroph *Davidiella macrospora* were abundant in all fields, except for U3 (Fig. 3).

There was no significant (P=0.758) difference in community composition within field E1c (n=59) (Table 4). The analysis of the adjacent fields in region E showed differences in community composition between fields that could explain 26% of the inertia (P=0.001). The fields E1b and E1c had similar fungal communities, but the samples from E1a diverged (Fig. 4). E1a had a different cultivar but the effect of the cultivars was small (explained 9% of total inertia) when analysing all fields.

The composition of fungal communities between fields varied across all regions and years (Fig. 5). Across all regions, there was an overall separation between years, (20% of inertia, P=0.001), driven by a higher incidence of basidiomycetous yeasts during year 1, highest abundance of *P. nodorum* during year 2 and higher abundance of *M. graminicola* (E & V) and *P. tritici-repentis* (U) during year 3 (Figs. 3 & 6). Across all years, an overall separation between regions was observed (12% of inertia, P=0.001). Samples from region U diverged from regions E and V (Fig. 5), particularly during year 1 (high incidence of basidiomycetous yeasts) and year 3 (high incidence of *P. tritici-repentis* (Figs. 3 & 6).

Discussion

The community structure present on wheat leaves exhibiting symptoms similar to stagonospora nodorum blotch were of low complexity. In most cases, the fungal communities consisted of less than five species although a few samples from 2003 and 2004 had up to ten species identified. At least one of the three main pathogens, *P. nodorum, M. graminicola* or *P. tritici-repentis*, was found on almost every sampled leaf and often dominated the fungal flora on the leaf. This

Table 3 The frequency of fungal species identified using T-RFLP on wheat leaves showing symptoms similar to stagonospora nodorum blotch from eleven winter wheat fields in the Uppland, Västergötland, and Östergötland regions of Sweden, 2003–2005 (n=123)

Species	U1,[a] 2003	V1, 2003	E1a, 2003	E1b, 2003	E1c[b], 2003	U2, 2004	V2, 2004	E2, 2004	U3, 2005	V3, 2005	E3, 2005	Sum of sp. in samples
Mycosphaerella graminicola	0	0	1 (1:0)[c]	1 (1:0)	0	5 (4:0)	1 (1:0)	8 (8:0)	0	10 (0:1)	8 (0:3)	34
Phaeosphaeria nodorum	3 (3:0)	12 (1:2)	12 (2:2)	12 (0:10)	11 (1:5)	12 (1:7)	12 (11:1)	10 (0:2)	4 (4:0)	1 (1:0)	10 (0:0)	99
Pyrenophora tritici-repentis	2 (2:0)	10 (3:0)	10 (3:0)	2 (2:0)	2 (2:0)	9 (5:1)	3 (3:0)	2 (2:0)	12 (0:11)	1 (1:0)	1 (0:1)	54
Davidiella macrospora	5 (5)	1 (1)	3 (3)	0	2 (2)	2 (2)	1 (0)	10 (8)	2 (2)	5 (4)	6 (4)	37
Lewia infectoria	0	0	6 (6)	2 (2)	0	0	2 (2)	0	0	0	0	10
Udeniomyces pannonicus	1 (1)	2 (2)	9 (4)	0	2 (1)	4 (3)	1 (1)	10 (7)	0	2 (2)	0	31
Bullera globospora	3 (3)	1 (1)	1 (1)	0	1 (1)	0	1 (1)	0	0	0	0	7
Cryptococcus victoriae	6 (6)	0	2 (2)	0	1 (1)	0	1 (1)	0	0	5 (5)	2 (2)	17
Cryptococcus wieringae	0	0	3 (3)	0	0	0	1 (1)	0	0	0	0	4
Cryptococcus sp.	9 (0)	0	0	0	0	0	0	0	0	0	1 (1)	10
Dioszegia crocea	8 (6)	12 (11)	8 (8)	2 (2)	5 (5)	6 (6)	3 (3)	9 (9)	0	1 (1)	1 (1)	55
Dioszegia fristingensis	2 (1)	7 (6)	8 (3)	1 (0)	5 (1)	3 (2)	3 (1)	4 (4)	0	2 (0)	1 (1)	36
Dioszegia hungarica	6 (5)	12 (0)	9 (7)	11 (3)	11 (1)	6 (6)	3 (2)	8 (7)	1 (1)	9 (8)	1 (1)	69
Sporobolomyces roseus	6 (5)	7 (6)	6 (4)	0	1 (1)	2 (2)	0	8 (7)	1 (1)	4 (3)	3 (3)	38
No. of samples	10	12	12	12	12	12	12	11	12	9	9	123

[a] *U* Uppland, *V* Västergötland, *E* Östergötland

[b] One random sample from each of the twelve sampling sites

[c] The first (or only) number in the parentheses shows the number of samples where the fungus represents <10% of the total fluorescence, and the number after the colon sign shows the number of samples where the respective fungal pathogen represents >50%

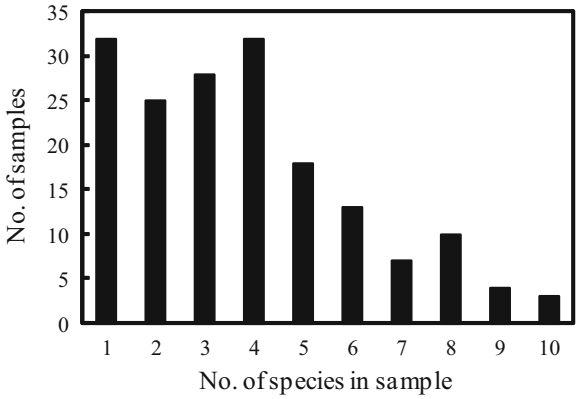

Fig. 2 Frequencies of samples with a specific number of species identified in each of the wheat leaves originating from eleven fields and from three regions in Sweden. All samples from field E1c are included, $n=172$ leaves

indicates that leaves exhibiting symptoms similar to stagonospora nodorum blotch are strongly associated with the presence of pathogenic fungi during the development stages when the samples were collected. In general, the dominant pathogen co-occurred with epiphytic species of yeasts, saprophytes or one of the other two main pathogens. In rare occasions all three pathogens were present in the same sample, but in these cases one of them still dominated. *Phaeosphaeria nodorum* and *P. tritici-repentis* often co-occurred on the leaf and in a few samples they contributed equally to the community.

We observed that different pathogens dominated in different years, with *P. nodorum* dominating the first and second year and *M. graminicola* or *P. tritici-repentis* during the third year of sampling. Fluctuation in occurrence of pathogenic fungi between years is common and can to a large extent be explained by weather factors (Djurle 1996). Conclusions about consistent shifts in pathogen frequencies cannot be drawn from this study. Shifting frequencies of *P. nodorum* and *M. graminicola* over long periods of time were analysed from the long-term samplings at Broadbalk, U.K, and a decrease in sulphur deposition was one of the suggested causes of the decreased occurrence of *P. nodorum* (Shaw et al. 2008). The observation that *P. nodorum* incidence has decreased in Sweden since the 1990s while the incidence of *M. graminicola* and *P. tritici-repentis* respectively has increased remains to be explained.

The variation in fungal communities between the fields was high as shown by the inertia explained by the variation within region and the scattered samples in the figure of DCA for regional level (Fig. 4). The samples from all regions and years were also scattered (Fig. 5). The samples from each field were close together in the two figures indicating that the variation was larger between fields than within fields, which was confirmed with the CCA analysis. The low variation within fields can be explained by adjacent migration of fungi within fields or that the microclimate in the crop might favour certain species resulting in low variation in species composition within a field.

Our result of higher abundance of *P. nodorum* during years 1 and 2 differed from the reports from the Plant Protection Centres, which instead reported high abundance of *P. tritici-repentis* (SJV 2003, 2004). This was probably due to the sampling strategy in the current study, where leaves with symptoms similar to stagonospora nodorum blotch were selected for collection. Dominance of *M. graminicola* within fields in regions E and V was also observed by farmers and extension workers in 2005 (SJV 2005). The dominance of *M. graminicola* was seen already during sampling when it was difficult to find leaves with symptoms similar to stagonospora nodorum blotch. The weather conditions may have had an impact since the spring of 2005 was drier and the early summer had more rain than in an average year (SJV 2005). There were no distinct differences in temperature during spring and early summer between

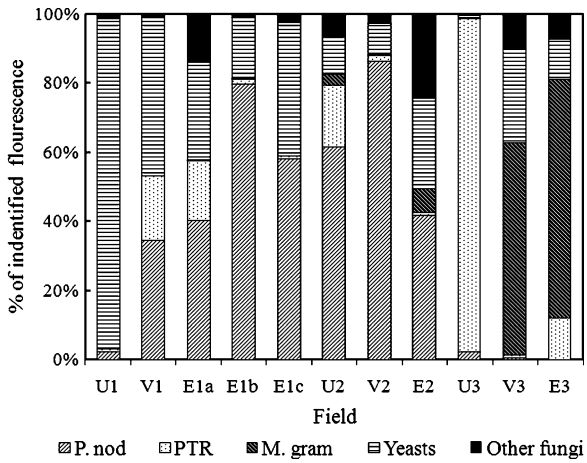

Fig. 3 Quantification of fluorescence associated with identified species as percentage of the total fluorescence from species identified within each field. P. nod: *P. nodorum*, PTR: *P. tritici-repentis*, M. gram: *M. graminicola*. U: Uppland, V: Västergötland, E: Östergötland

Table 4 Variation in fungal community among sites, fields, regions and years as determined by canonical correspondence analyses (CCA)

Source of variation	Sample set	Total inertia	Explained inertia	% explained
Within field	E1c[a]	2.303	0.175	8, ns
Fields within Region	E1a, E1b, E1c[b]	1.195	0.305	26, ***[c]
Region	U, V, E	3.274	0.382	12, ***
Year	1,2,3	3.274	0.652	20, ***
Region + Year	All fields	3.274	1.108	34, ***

[a] Ten samples were collected at six sampling sites in field E1c (corners and middle of the second and third row)

[b] One random sample from each of the twelve sampling sites

[c] Stars indicate Monte Carlo permutations $P=0.001$

2005 and a normal year. Rain during spring increases the incidence of both *M. graminicola* and *P. nodorum*, and *P. nodorum* is more abundant during warm summers (Shaw et al. 2008).

The composition of the fungal community in the region of Uppland differed from the other two regions, mainly in the low frequency of pathogens during year 1 and the almost complete dominance of *P. tritici-repentis* during year 3. The cultivar in 2005 was Kosack but it has similar resistance levels to tan spot and stagonospora nodorum blotch as cv. Olivin (Larsson et al. 2006). The agricultural management in field U1 (organic) could explain the divergence of the fungal community compared to the other fields (conventional). The previous crop in this field (U1) was not wheat, which could mean that there was less initial inoculum of the pathogens detected in this study. The crop canopy was not dense and plants were short, potentially leading to a drier microclimate, which would be less favourable for disease development. A short straw would on the other hand facilitate splash dispersed pathogens reaching the upper leaves. Moreover, even under the favourable, moist, weather conditions present during the spring and the middle of the summer in Uppland in 2003, a shorter and lighter crop canopy would likely to have dried out more rapidly, potentially leading to less favourable conditions for disease (SJV 2003). *Pyrenophora tritici-repentis* dominated in the wheat fields of Uppland in 2003, whilst *M. graminicola* and *P. nodorum* rarely occurred (SJV 2003). In the absence of epidemics of pathogenic fungi in field U1, shown by small and few lesions on the leaves, the epiphytic fungi, especially *Cryptococcus* sp., dominated in the DNA pool.

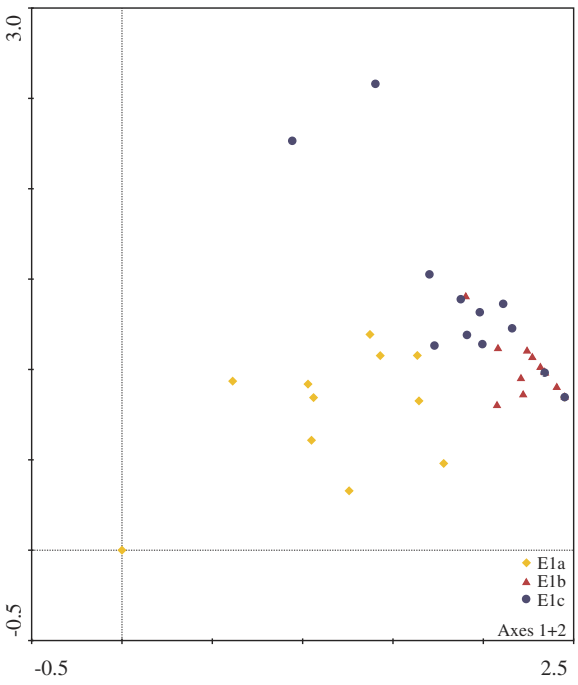

Fig. 4 Similarity of fungal communities on diseased leaves collected in three fields from the region of Östergötland (E) during year 1, based on detrended correspondence analysis (DCA)

Some saprotrophic fungi were common in a few fields. However, species of *Lewia* (anamorph *Alternaria*) and *Davidiella* (anamorph *Cladosporium*) were scarce or could not be detected through cloning. This may be due to the date of sampling, since the senescence of the plants had not progressed far, or that the cloning was not successful in detecting species from these genera. The species of yeast found on the wheat leaves are similar to those from earlier studies. Species within the genera *Sporobolomyces*

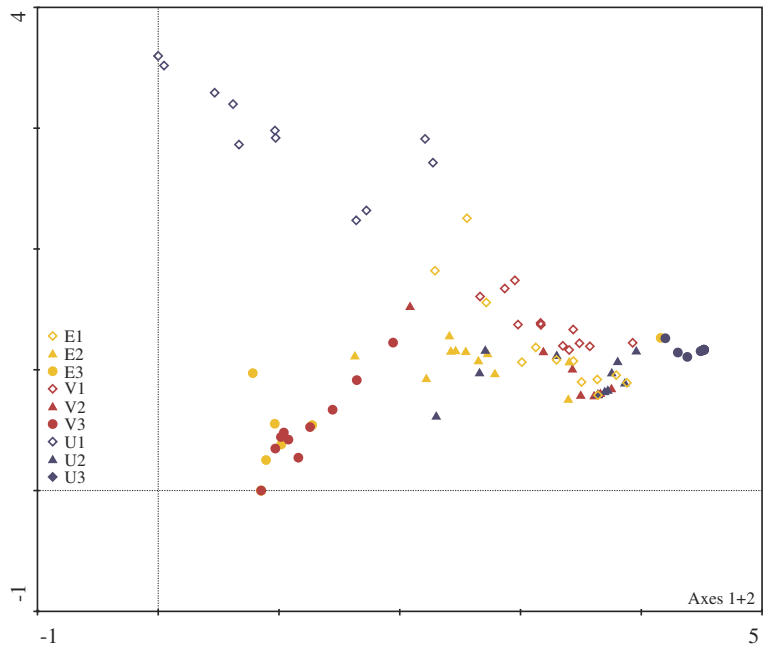

Fig. 5 Similarity of the fungal communities in nine fields and three regions (U, V & E) during 2003–2005, based on detrended correspondence analysis (DCA). The first year of region E is represented by twelve random samples from the three fields

(pink-pigmented yeasts) and *Cryptococcus* (white-pigmented yeasts) have been reported as most abundant during heading, but also genera such as *Candida, Geotrichum* and *Trichosporon* (Remlein-Starosta 2004), as well as other sets of fungal species have been found on healthy wheat leaves (Larran et al. 2007). Epiphytic yeasts found on two wild-grass species, creeping bentgrass (*Agrostis palustris*) and tall fescue (*Festuca arundinacea*), were associated with the pathogenic fungi *Sclerotinia homoeocarpa* and *Thanatephorus cucumeris* (anamorph *Rhizoctonia solani*) (Allen et al. 2004). Higher numbers of yeast colony-forming units were observed on leaves affected by either of the pathogens, suggesting that yeasts metabolise nutrients from the leaking dying cells. The yeast community was found not to vary across space or time during two previous trials (Allen et al. 2004). In the present study, yeasts were common in the fields

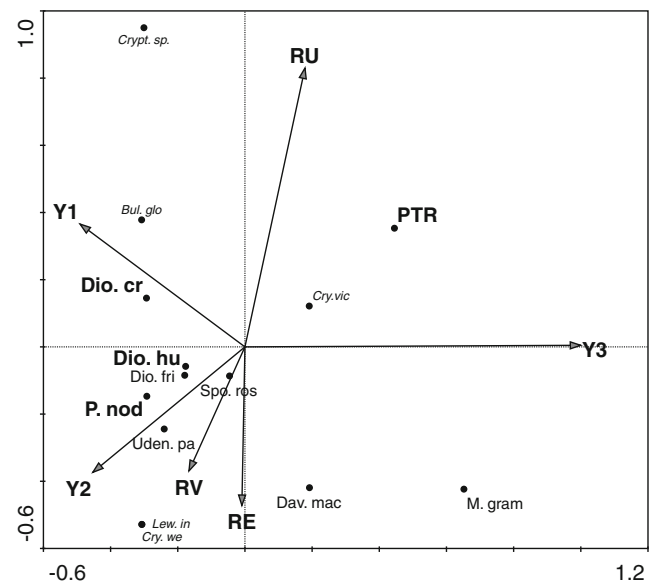

Fig. 6 Spatial and temporal variation of fungal communities in eleven fields and three regions 2003–2005 analysed with canonical correspondence analysis (CCA). RU: Uppland, RV: Västergötland, RE: Östergötland, Y1: 2003, Y2: 2004, Y3: 2005. For explanation of species names see Table 1. Font sizes are used to indicate how common the different species are (large font = most common)

with pathogens and there was a tendency for a variation in the abundance of yeasts, but neither was statistically significant. However, yeasts were dominating where the pathogens were low in frequency (U1), which may be due to less competition for nutrients.

The complexity of the fungal community as well as the ecological functions of the fungal species on the phylloplane have not been thoroughly investigated (Andrews and Harris 2000). Yeasts have antagonistic capacities towards pathogenic fungi. Less disease caused by *P. nodorum* occurred when *Sporobolomyces roseus* and *Cryptococcus laurentii* were present on the leaf surface or the spikes (Fokkema et al. 1979). Other yeasts, such as *Rhodotorula glutinis* and *Cryptococcus* sp., and the filamentous fungus *Trichoderma harzianum* inhibit growth and prevent germination of *Botrytis cinerea* (Buck 2002; Dik et al. 1999). The fungi *T. harzianum* and *T. koningii* decrease the impact of *P. tritici-repentis* and *M. graminicola* when applied to seeds and leaves (Perelló et al. 2006). *Ulocladium atrum* and *Clonostachys rosea* prevent sporulation of *Botrytis aclada* (Yohalem et al. 2004). The mechanism of antagonism is thought to be competition for the nutrients on the leaf surface (Fokkema et al., 1979). In the case of *Trichoderma* species, mycoparasitism involving hyphal penetration has been observed (Perelló et al. 2003). Fatty acids produced by the yeast *Pseudozyma flocculosa* have been shown to have antagonistic effects on the growth of *B. graminis* (Avis et al. 2000).

Identification of fungal species using cloning of the ITS region and subsequent matching of restriction fragments from the clones and the samples enables community analysis in large sample sets (Lindahl et al. 2007). Only a few fragments obtained from the leaf samples did not match fragments obtained from the sequenced clones, indicating that the cloning approach was successful in isolating the majority of the dominant species within the sampled fields. One drawback was that T-RFLP was unable to distinguish between *P. nodorum* and *P. avenaria* f.sp. *triticea*, since their ITS sequences are so similar. When comparing the clones in this study with sequences published in GenBank we found matches to *P. nodorum* both with and without two extra nucleotides (CT).

The total amount of fungal DNA could vary depending on the fungal occurrence in the sample, implying that the method used here only provides relative information on species abundances. However, at sample level, the percentage of fluorescence associated with identified species was in general high, which further supports the efficiency of this method.

Since the sampling plan was designed to include leaves with symptoms of stagonospora nodorum blotch, and that these are similar to those of tan spot, it was not surprising to find that both fungi occurred on the leaves during the first two years of collection. The dominance of *P. tritici-repentis* in U3 was not expected since *P. nodorum* had been isolated from >30 sampling sites in this field prior to the experiment (Blixt et al. 2008). The isolates of *P. nodorum* collected previously in U3 originated from leaves incubated under damp conditions with continuous near-UV light, and since the focus was only on *P. nodorum* numerous lesions caused by *P. tritici-repentis* could have been ignored. The environmental conditions during the incubation were also beneficial for *P. nodorum* (Eyal et al. 1987), which might have favoured isolation of that fungus in the previous study, and hence the deviation between other Swedish studies and the present one. The results from the current study are yet another example of the difficulty in distinguishing symptoms of tan spot from stagonospora nodorum blotch just at a glance, as reported earlier (Almquist et al. 2008; Engle et al. 2006). In the inventory of pathogenic fungi on wheat made by Almquist et al. (2008), *P. nodorum* was much more frequent after Q-PCR identification than when observed visually in the field. The symptoms of *M. graminicola* are so distinct that they may have been subject to negative selection during the collections.

We could not identify any other pathogenic fungi in this study. The sampling dates were too early in the season to detect rust fungi such as *Puccinia striiformis* var. *striiformis* or *P. triticina*. It was probably too late for *B. graminis*. Absence of these pathogens in the samples could indicate that the cultivars in the study are less susceptible but the severities of rust and powdery mildew were in general low during the years in question according to reports from the Swedish Board of Agriculture, subdivision Plant Protection Centre (SJV 2003, 2004, 2005).

The variation in composition of fungal communities within fields varied across both time and space. Differences between years had the largest effects on the fungal communities, due to a shift in dominating pathogens in 2005 compared to the two previous

years. In addition, the fungal communities in Uppland differed from those in Västergötland and Östergötland, which were more similar to each other. Low variation in fungal community composition was found within a single field (E1c). This was also supported by the dense scattering of the samples from individual fields in the DCA (Figs. 4 & 5). The cvs Olivin and Kosack have similar sensitivity to tan spot and stagonospora nodorum blotch, but Olivin is slightly more sensitive to septoria tritici blotch (Larsson et al. 2006) and the cultivars did not have a large impact on the fungal community composition, shown by CCA.

The total fungal community on the wheat leaves was found to consist of 14 species of which one only could be identified to genus level (*Cryptococcus* sp.). The fungi detected were epiphytes, saprotrophs and pathogens. Both yeasts and filamentous fungi were represented. Three species of pathogenic fungi were the most abundant on the wheat leaves together with three species of basidiomycetous yeasts. Necrotic lesions on wheat leaves were associated with one of the three major pathogens, *P. nodorum, P. tritici-repentis* and *M. graminicola*. A single pathogen dominated within each field, but *P. nodorum* and *P. tritici-repentis* often co-occurred during 2003 and 2004. In 2005 the occurrence of *P. nodorum* was lower than for the two previous years while *M. graminicola* dominated in two regions and *P. tritici-repentis* in the third.

Acknowledgements The authors wish to thank Magdalena Grudzinska-Sterno for help with translations and contributions to the clone library, and Katarina Ihrmark and Ylva Persson for technical support. We would also like to thank the Swedish Board of Agriculture (SJV), subdivision Plant Protection Centres for providing information about the sampling locations. The project was funded by the Swedish University of Agricultural Sciences, (SLU) and the postgraduate school IMOP (Interactions between Micro-Organisms and Plants) at SLU.

References

Allen, T. W., Quayyum, H. A., Burpee, L. L., & Buck, J. W. (2004). Effect of foliar disease on the epiphytic yeast communities of creeping bentgrass and tall fescue. *Canadian Journal of Microbiology*, *50*(10), 853–860.

Almquist, C., Lerenius, C., Filipsson, C. & Jonsson, A. (2008). *Bestämning av förekomst av patogena svampar i vete med PCR-teknik. Report 18. Dept. Soil Sciences, Division of precision agriculture, Swedish University of Agricultural Sciences* [online]. Available from: http://po-mv.slu.se.

Altschul, S. F., Gish, W., Miller, W., Myers, E. W., & Lipman, D. J. (1990). Basic local alignment search tool. *Journal of Molecular Biology*, *215*(3), 403–410.

Andrews, J. H., & Harris, R. F. (2000). The ecology and biogeography of microorganisms of plant surfaces. *Annual Review of Phytopathology*, *38*, 145–180.

Avis, T. J., Boulanger, R. R., & Belanger, R. R. (2000). Synthesis and biological characterization of (Z)-9-heptadecenoic and (Z)-6-methyl-9-heptadecenoic acids: Fatty acids with antibiotic activity produced by *Pseudozyma flocculosa*. *Journal of Chemical Ecology*, *26*(4), 987–1000.

Behrendt, U., Muller, T., & Seyfarth, W. (1997). The influence of extensification in grassland management on the populations of micro-organisms in the phyllosphere of grasses. *Microbiological Research*, *152*(1), 75–85.

Blakeman, J. P. (1985). Ecological succession of leaf surface microorganisms in relation to biological control. In C. E. Windels, et al. (Eds.), *Biological control on the phylloplane* (pp. 6–30). St. Paul: American Phytopathological Society.

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*(4), 634–641.

Buck, J. W. (2002). In vitro antagonism of *Botrytis cinerea* by phylloplane yeasts. *Canadian Journal of Botany-Revue Canadienne De Botanique*, *80*(8), 885–891.

Dickie, I. A., Xu, B., & Koide, R. T. (2002). Vertical niche differentiation of ectomycorrhizal hyphae in soil as shown by T-RFLP analysis. *New Phytologist*, *156*(3), 527–535.

Dik, A. J., Koning, G., & Kohl, J. (1999). Evaluation of microbial antagonists for biological control of *Botrytis cinerea* stem infection in cucumber and tomato. *European Journal of Plant Pathology*, *105*(2), 115–122.

Djurle, A. (1996). *Glume blotch, wheat, weather and field conditions.* Uppsala: Diss. Swedish University of Agricultural Sciences.

Dowding, P., & Richardson, D. H. S. (1990). Leaf yeasts as indicators of air-quality in Europe. *Environmental Pollution*, *66*(3), 223–235.

Dreschler, C. (1923). Some graminicolous species of *Helminthosporium*. *Journal of Agricultural Research*, *24*, 641–739.

Engle, J. S., Madden, L. V., & Lipps, P. E. (2006). Distribution and pathogenic characterization of *Pyrenophora tritici-repentis* and *Stagonospora nodorum* in Ohio. *Phytopathology*, *96*(12), 1355–1362.

Eyal, Z., Scharen, A. L., Prescott, J. M., & van Ginkel, M. (1987). *The Septoria diseases of wheat: Concepts and methods of disease management*. Mexico: CIMMYT.

Fokkema, N. J., Denhouter, J. G., Kosterman, Y. J. C., & Nelis, A. L. (1979). Manipulation of yeasts on field-grown wheat leaves and their antagonistic effect on *Cochliobolus sativus* and *Septoria nodorum*. *Transactions of the British Mycological Society*, *72*, 19–29.

Gardes, M., & Bruns, T. D. (1993). ITS primers with enhanced specificity for Basidiomycetes—application to the identification of mycorrhizae and rusts. *Molecular Ecology*, *2*(2), 113–118.

Hill, M. O., & Gauch, H. G. (1980). Detrended correspondance-analysis—an improved ordination technique. *Vegetatio*, *42* (1–3), 47–58.

Kinkel, L. L. (1997). Microbial population dynamics on leaves. *Annual Review of Phytopathology*, *35*(1), 327–347.

Kitts, C. L. (2001). Terminal restriction fragment patterns: a tool for comparing microbial communities and assessing community dynamics. *Current Issues in Intestinal Microbiology*, *2*(1), 17–25.

Larran, S., Perello, A., Simon, M. R., & Moreno, V. (2007). The endophytic fungi from wheat (*Triticum aestivum* L.). *World Journal of Microbiology & Biotechnology*, *23*(4), 565–572.

Larsson, S., Hagman, J., & Ericson, L. (2006). *Stråsäd, Trindsäd, Oljeväxter, Potatis. Sortval 2006*. Uppsala: SLU.

Legard, D. E., McQuilken, M. P., Whipps, J. M., Fenlon, J. S., Fermor, T. R., Thompson, I. P., et al. (1994). Studies of seasonal changes in the microbial populations on the phyllosphere of spring wheat as a prelude to the release of a genetically modified microorganism. *Agriculture, Ecosystems & Environment*, *50*(2), 87–101.

Lindahl, B., Ihrmark, K., Boberg, J., Trumbore, S., Högberg, P., Stenlid, J., et al. (2007). Spatial separation of litter decomposition and mycorrhizal nitrogen uptake in a boreal forest. *New Phytologist*, *173*(3), 611–620.

Nix, S. S., Burpee, L. L., Jackson, K. L., & Buck, J. W. (2008). Short-term temporal dynamics of yeast abundance on the tall fescue phylloplane. *Canadian Journal of Microbiology*, *54*(4), 299–304.

Perelló, A., Mónaco, C., Simón, M. R., Sisterna, M., & Bello, G. D. (2003). Biocontrol efficacy of *Trichoderma* isolates for tan spot of wheat in Argentina. *Crop Protection*, *22*(9), 1099–1106.

Perelló, A. E., Mónaco, C. I., Moreno, M. V., Cordo, C. A., & Simón, M. R. (2006). The effect of *Trichoderma harzianum* and *T. koningii* on the control of tan spot (*Pyrenophora tritici-repentis*) and leaf blotch (*Mycosphaerella graminicola*) of wheat under field conditions in Argentina. *Biocontrol Science and Technology*, *16*(8), 803–813.

Remlein-Starosta, D. (2004). The occurrence of yeast-like fungi on winter wheat leaves. *Progress in Plant Protection*, *44* (2), 1043–1046.

Shaner, G., & Finney, R. E. (1976). Weather and epidemics of septoria leaf blotch of wheat. *Phytopathology*, *65*(7), 761–766.

Shaw, M. W., Bearchell, S. J., Fitt, B. D. L., & Fraaije, B. A. (2008). Long-term relationships between environment and abundance in wheat of *Phaeosphaeria nodorum* and *Mycosphaerella graminicola*. *New Phytologist*, *177*(1), 229–238.

SJV. (2003). *Växtskyddsåret 2003, divided by county* [online]. Available from: http://www.sjv.se/amnesomraden/vaxtmiljovatten/vaxtskydd/radgivningochinformation/informationsmaterial/vaxtskyddsaret/tidigarear.4.111089b102c4e186cc80003343.html.

SJV. (2004). *Växtskyddsåret 2004, divided by county* [online]. Available from: http://www.sjv.se/amnesomraden/vaxtmiljovatten/vaxtskydd/radgivningochinformation/informationsmaterial/vaxtskyddsaret/tidigarear.4.111089b102c4e186cc80003343.html.

SJV. (2005). *Växtskyddsåret 2005, divided by county* [online]. Available from: http://www.sjv.se/amnesomraden/vaxtmiljovatten/vaxtskydd/radgivningochinformation/informationsmaterial/vaxtskyddsaret/tidigarear.4.111089b102c4e186cc80003343.html.

ter Braak, C. J. F. (1986). Canonical correspondence analysis: a new eigenvector technique for multivariate direct gradient analysis. *Ecology*, *67*(5), 1167–1179.

White, T. J., Bruns, T. D., Lee, S. B., & Taylor, J. W. (1990). Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In M. A. Innis, et al. (Eds.), *PCR protocols—A guide to methods and applications* (pp. 315–322). San Diego: Academic.

Wiese, M. V. (1987). *Compendium of wheat diseases* (2nd ed.). St. Paul: American Phytopathological Society.

Woody, S. T., Ives, A. R., Nordheim, E. V., & Andrews, J. H. (2007). Dispersal, density dependence, and population dynamics of a fungal microbe on leaf surfaces. *Ecology*, *88*(6), 1513–1524.

Yohalem, D. S., Nielsen, K., Green, H., & Jensen, D. F. (2004). Biocontrol agents efficiently inhibit sporulation of *Botrytis aclada* on necrotic leaf tips but spread to adjacent living tissue is not prevented. *Fems Microbiology Ecology*, *47*(3), 297–303.

Zadoks, J. C., Chang, T. T., & Konzak, C. F. (1974). A decimal code for growth stages of cereals. *Weed Research*, *14*(6), 415–421.