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Fungal communities in organically grown winter wheat affected by plant organ and development stage

Magdalena Grudzinska-Sterno · Jonathan Yuen · Jan Stenlid · Annika Djurle

Abstract The fungal community on the roots, stem bases, stems and grains of organically grown winter wheat was analysed using terminal restriction fragment length polymorphism (T-RFLP) combined with cloning and sequencing of the ITS region. The changes in the composition of fungi in different plant parts and over time as well as interactions between fungi were also investigated. Among 58 fungal taxa found the most common were *Davidiella macrospora*, *Cladosporium* spp., *Tetracladium maxilliforme*, *Didymella exitialis*, *Microdochium nivale* and an unidentified species within Ascomycetes. Several potential wheat pathogens were found: *Fusarium* spp. including *F. poae* and *G. avenacea* (*F. avenaceum*), *Microdochium nivale*, *Oculimacula yallundae*, *Parastagonospora nodorum* and *Zymoseptoria tritici* and most of them were present on all plant parts. Plant part affected the most the fungal colonization of wheat as was shown both by multivariate analysis of the whole fungal community as well as the analysis based on the identified species. The composition of fungal communities in different parts changed during the growing season but no pattern common for the whole crop could be observed. The most dynamic and significant changes were found among yeasts. Both positive and negative significant interactions between pairwise combinations of pathogens were observed. Positive significant associations were also found between pathogens and other fungi.

Keywords Fungal community · Wheat · T-RFLP · Fungal pathogens · Saprophytes · Growth stage · Plant part

Introduction

Plants are inhabited by fungi that interact with each other and ultimately affect the fitness of natural plant populations and the quality of agricultural crops. Saprophytic fungi living on a plant can protect the plant against pathogens (Liggitt et al. 1997; Perelló et al. 2002). The use of chemical control can, however, change the saprophytic mycoflora and thereby even facilitate the spread of pathogenic fungi (Müllenborn et al. 2008). Fungi colonizing the roots are also very important for plant health and development as they play a role in the suppression of diseases, stimulation of plant growth and in providing nutrients to the plant (Shivanna et al. 1996).

Several reports have described fungal populations on wheat and the most detailed studies including isolation of fungi from roots, culms, leaves, glumes and kernels were performed in Switzerland by Sieber et al. (1988) and recently in Canada by Vujanovic et al. (2012). In Argentina, Larran et al. (2007) isolated endophytic fungi colonizing the above ground parts of healthy wheat

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plants. Most reports focus however on the fungal microflora of particular parts of the plant such as roots (Bateman and Kwaśna 1999; Lemańczyk and Sadowski 2002), stem bases (Matusinsky et al. 2008), leaves (Blixt et al. 2010; Larran et al. 2002) or grains (Müllenborn et al. 2008; Nicolaisen et al. 2014).

Among the wheat fungal microflora pathogens such as *Gaeumannomyces graminis* and *Rhizoctonia solani* can be found on roots (Bockus et al. 2010). Pathogenic *Fusarium* species, *Microdochium nivale*, *Oculimacula yallundae*, *Parastagonospora nodorum*, *Pyrenophora tritici-repentis*, *Rhizoctonia cerealis* and *Zymoseptoria tritici* are often found on above-ground parts of plants (Bockus et al. 2010). The most serious wheat pathogens in Sweden are *Z. tritici*, *P. tritici-repentis*, *P. nodorum* causing leaf blotch diseases and fungi causing eyespot disease (*O. acuformis* and *O. yallundae*) (Wiik 2009). *Fusarium* species are also common on Swedish wheat (Lindblad et al. 2013) and the incidence of diseases caused by these pathogens is predicted to increase in the future (Roos et al. 2011). During recent years stripe rust (*Puccinia striiformis*) has also become an increasing problem in Sweden (Hovmøller et al. 2011).

Traditionally, cultivation-dependent methods have been used for the analysis of fungal communities in wheat (Bateman and Kwaśna 1999; Larran et al. 2007; Vujanovic et al. 2012). These methods are time consuming and laborious and unsuitable for analysis of a large number of samples. Moreover, the results may depend on the selected medium and the culture conditions, and therefore do not reflect the original community in the substrate (Pfender and Wootke 1988) since other species may be viable but are not culturable irrespective of medium.

Terminal restriction fragment length polymorphism (T-RFLP) is a high-throughput method used for studying fungal communities. It has been successfully used to analyse the composition of fungal populations in soil (Dickie et al. 2002; Lindahl et al. 2007), wood (Allmer et al. 2006) and wheat leaves (Blixt et al. 2010). T-RFLP allows the identification of individual fungal species in mixed samples by comparison of restriction fragments length from an unknown community (T-RFLP profiles) to a reference database of known T-RFLP patterns (Dickie et al. 2002). The reference database may be derived directly from the analyzed samples through cloning, sequencing and restriction cutting of amplicons (Lindahl et al. 2007).

The overall aim of this study was to investigate the dynamics of fungal populations in organically grown winter wheat by i) identification of fungi inhabiting below- and above-ground plant parts or organs, ii) investigation of changes in the composition of fungal communities during the growing season, and iii) investigation of relationships between pathogens and between pathogens and non-pathogenic fungi colonizing wheat.

This is, to our knowledge, the first published study where the fungal community of the winter wheat's roots, stem bases, stems and grains was investigated by culture-independent methods simultaneously.

Materials and methods

Sampling and sample processing

Winter wheat plants were collected in 2007 from three organically grown winter wheat fields located in Uppland and Västmanland regions in the eastern central part of Sweden. The previous crop in each field was red clover.

Plants were sampled at growth stages (GS) DC 30–32 (stem elongation, GS1), DC 65–69 (anthesis, GS2) and DC 85–90 (maturity, GS3) according to Zadoks et al. (1974). In each field, one row with 30 sampling sites at 10 m distance was established. On each sampling date, two or more plants were dug up from each site. Depending on the growth stage roots, stem bases, upper stems and grains were analyzed for the presence of fungi (Table 1).

The plants were soaked and washed in running water in order to remove soil. From each of two plants per sampling site (60 plants per field) the main stem was detached and the leaf sheaths removed. The roots were cut off from the stem (just below their points of attachment) and six to eight randomly chosen 3–5 cm root pieces containing root tips were put into a cassette and washed under running tap water over night. Next day the root pieces were washed for 5 min, 5 times, by shaking them in sterile distilled water and after drying put into a freezer (−20 °C).

Stem bases, 3–4 cm long, and upper stems consisting of the highest node and 1-cm segments on each side of it were put into cassettes and washed as described above. Grains were separated from the glumes, ground to a powder in a mill (Retsch, Ninolab AB) and stored in

Table 1 Sampling scheme of wheat plants from a single field during the growing season 2007

Growth stage GS	Plant part			
	Roots R	Stem base SB	Stem S	Grain G
GS1 Stem elongation (DC 30–32)[a]	30[b]	30	-	-
GS2 Anthesis (DC 65–69)	30	30	30	-
GS3 Maturity (DC 85–90)	30	30	30	30
total	90	90	60	30

[a] the growth stage is given according to Zadoks et al. (1974)

[b] number of sampling sites

dry conditions at room temperature until processing, which took place within one week.

DNA extraction

DNA was extracted according to the CTAB protocol (Gardes and Bruns 1993) with some modification. Roots, stem bases and upper stems were freeze dried and ground in 2 ml microcentrifuge tubes containing three autoclaved stainless steel nuts with 6.0 mm diameter in a FastPrep® preparation shaker (BIO 101® Systems). The roots were ground once at a speed of 4000 rpm for 40 s. For stem bases and upper stems three 20 s cycles at 4000 rpm were used. For extraction of DNA from grains 150 mg of ground powder were used.

To extract DNA, 1000 µl 3 % CTAB (3 % hexadecyltrimethylammonium bromide, 0.1 M Tris-HCl pH 8.0, 1.4 M NaCl, 0.02 M EDTA) was added to microcentrifuge tubes containing the homogenized plant material. The samples were vortexed and put in a heating-block at 65 °C for 2 h. After 10 min centrifugation at 7000 rpm the supernatant was transferred to a new 1.5 ml tube and 700 µl chloroform: isoamyl alcohol (24:1) was added. The samples were centrifuged for 8 min at 13,000 rpm, the supernatant transferred to a new 1.5 ml tube and precipitated with cold isopropanol. The pellet was washed with 70 % ethanol, re-suspended in 50 µl MiliQ water and stored in −20 °C.

Identification of fungi

For the identification of fungi in plant material, terminal restriction fragment length polymorphism (T-RFLP) was used and a reference database with the T-RFLP patterns of individual fungal species was developed. T-RFLP profiles in the reference database were obtained after cloning and sequencing of the fungal ITS region from 85 samples of roots, stem bases, stems and grains collected during this study. The database was complemented with the profiles of 42 fungal isolates collected from plants from one of the fields in this study, wheat roots from a field with pea as the previous crop, and grains collected from randomly chosen fields and stored in the collection of isolates in the Department of Forest Mycology and Plant Pathology.

In order to identify fungi in plant samples, the ITS region of nuclear rDNA was amplified with the fungal-species specific primer pair ITS1F (Gardes and Bruns 1993) and ITS4 (White et al. 1990) labelled with fluorescent dye D3 and D4 respectively (WellRED, Sigma-Proligo). In 25 µl PCR reaction, a final concentration of 0.2 µM of each primer, 0.2 mM dNTP, 2.75 mM $MgCl_2$, 0.3 U/µl ThermoRed DNA Polymerase (Saveen & Werner AB) and recommended reaction buffer Y was used. The PCR reaction contained 30 ng of total DNA. PCR amplification was performed in 2720 Thermal Cycler (Applied Biosystems) using the conditions: 94 °C for 5 min, 35 cycles of 30s at 94 °C, 30s at 55 °C and 30s at 72 °C, followed by final extension at 72 °C for 7 min. PCR products were separated on 1 % agarose gel stained with ethidium bromide and visualized under UV light (GelDoc, Bio-Rad Laboratories). PCR products were cleaned using JetQuick Kit (GENOMED) according to the manufacturer's protocol. Purified products (3 µl) were digested in separate reactions with *Alu*I (Roche), *Cfo*I (Promega) and *Taq*I (Fermentas). Digestions were in a total volume of 6 µl, included 2 U of enzyme, and were incubated for 4 h at 37 °C (*Alu*I and *Cfo*I) or 4 h at 65 °C (*Taq*I). Restriction digests were purified using Sephadex G-50 Medium (GE Healthcare Bio-science). The length of the terminal

fragments was analysed in a Beckman Coulter CEQ™ 8000 Genetic Analysis System using CEQ™ DNA Size Standard Kit-600.

T-RFLP patterns of samples were compared to the reference database using the software TRAMPR (TRFLP analysis and matching package for R) (FitzJohn and Dickie 2007). Fungal taxa were identified if minimum four out of six of the enzyme/primer combinations were present in the sample. The tolerance for the mismatches in peak location was set to 1.5 bases.

To set up the reference database the ITS region from 85 samples of different plant parts were amplified with nonfluorescent primers under the same conditions as described above. PCR products were pooled into seventeen cloning reactions, cloned into the PCR® 2.1-TOPO vector according to the protocol of TOPO TA Cloning Kit (Invitrogen Life Technologies) and transformed into One Shot TOP10 Chemically Competent *Escherichia coli*. All clones chosen for sequencing were tested for the presence of an insert by PCR with M13 forward and reverse primers. PCR amplicons from cloning were purified with the Agentcourt® AMPure® Protocol 00060IV024. In total, 386 positive clones were sequenced from both ends using ITS1F and ITS4 primers (sequencing by Macrogen, Seoul, S. Korea).

Sequences were manually edited and aligned using the SeqMan II software (DNA Star, Madison, WI, USA). Consensus ITS sequences were matched with DNA sequences from GenBank at NCBI using the BLAST search function (Altschul et al. 1997). The criterion for species identification was minimum 98 % similarity with a known ITS sequence. Sequences were submitted to GenBank under accession numbers: KC694147-KC694161, KC753399-KC753447, KC989057-KC989105.

In order to obtain T-RFLP profiles of the identified fungal species, the representative clones were subjected to T-RFLP analysis as described above for samples.

The isolates included in the reference database were identified by sequencing of the ITS region and subjected to T-RFLP analysis, as described above.

Statistical analysis

Analysis based on the identified species

In this analysis only samples in which the fungi were identified by matching of the restriction profile with the

patterns from the reference database were included ($N = 610$).

Species richness and the composition of fungal communities were analysed in relation to plant part and growth stage. Species richness was analysed in R by calculating species accumulation curves (SAC), showing the relationship between the number of observed taxa and the sampling intensity (Colwell and Coddington 1994). Fungal community composition was compared by calculating qualitative Sorensen similarity indices (C_S) (Magurran 1988).

The relationships between the composition of community and environmental variables of plant part, growth stage and field were analysed with the canonical correspondence analysis (CCA) in CANOCO 4.5 (Biometrics-Plant Research International, Wageningen, The Netherlands). The species matrix containing presence/absence data was used in the multivariate analysis. The percentage of the total inertia that was explained by the environmental variables was calculated by dividing the sum of all canonical eigenvalues by the sum of all eigenvalues. The statistical significance of the CCA was evaluated using a Monte Carlo permutation test with 499 permutations.

A series of general linear models were used to examine variation in the occurrence of the various fungal taxa in different plant parts and over time. Logistic regression analysis was used to determine whether the occurrence of the fungal taxa, expressed as the odds of finding a fungus, varied in the four different plant parts (roots, stem bases, stems, and grain). For these analyses, only fungi that occurred in more than 8 samples total were included. This was the minimum number of samples needed for the analysis. Logistic regression was also used for stratified analyses of three plant parts (roots, stem bases, and stems) to see if the occurrence of the different taxa, calculated as the odds, changed significantly during the growing season. The analysis was possible to perform only on the most common taxa, i.e. those that were identified in more than 5 % of all samples.

A log-linear model (Xu et al. 2005) was used to test the significance of co-occurrence of the most common fungi, including plant pathogenic fungi, and these analyses were also stratified by plant part. The general linear models analyses were carried out using the glm function in the R version 2.8.1. Upper and lower 95 % confidence intervals were calculated where relevant.

Analysis of the whole fungal community

For the analysis of the whole fungal community T-RFLP data from 790 samples were uploaded to the online TRFLP analysis software package T-REX (http://trex. biohpc.org/) (Culman et al. 2009). True peaks were identified for all samples of which the area exceeded the standard deviation (assuming zero mean) computed over all peaks and multiplied by one (Abdo et al. 2006). The peaks were then aligned using 1.5 bp clustering threshold in T-REX.

An Additive Main Effects Multiplicative Interaction (AMMI) model within T-REX was used to analyse differences in the fungal communities between plant parts, growth stages and fields (Culman et al. 2009). Binary data (presence/absence of peaks) rather than peak height or peak area were used in the analysis since this data type is the most informative and also recommended for multivariate analysis (Culman et al. 2008).

The AMMI model uses analysis of variance (ANOVA) to first divide the variation into main effects and interactions, and then applies PCA to the interactions to create interaction principal components axes (IPCAs) (Gauch 1992). The variation from the interactions between the main effects (here T-RFs vs. environments) reflect how microbial community profiles (T-RFs) differentially respond to the environments (Culman et al. 2008) and was therefore the most interesting for our study. The analysis was done using the combined data generated by TRFLP of all three different digestion enzymes. Scatterplots of the first two axes of AMMI were graphed with Minitab® Statistical Software v.16 (State Collage, PA).

Results

Reference database and identification of fungi in plant material

T-RFLP patterns of 67 fungal taxa occurring on wheat were collected in the reference database. Among them, 25 fungal taxa were identified by cloning and sequencing of the ITS region from plant samples and 21 were obtained by sequencing of fungi isolated from different parts of the plants. Twenty-one fungal taxa included in the database were identified both as clones and isolates.

A comparison of fungal sequences to reference sequences at NCBI resulted in 46 and 15 sequences identified to species and genus level respectively. Four sequences were identified to order and 2 sequences represented unidentified Ascomycetes.

For most of the fungal taxa a unique restriction pattern was obtained. For fungi from the genera *Fusarium* and *Cladosporium* the cutting of the ITS region with restriction enzymes *Alu* I, *Cfo* I, *Taq* I gave fragments very similar in length (Online Resource 1). This means that the species *Fusarium culmorum*, *F. graminearum* and *F. sporotrichioides* as well as *Cladosporium cladosporioides* and *C. tenuissimum* could not be distinguished during fragment analysis (TRAMPR). In the analysis of fungal communities these species are therefore presented as *Fusarium* spp. and *Cladosporium* spp.

Identification of fungi in the samples was performed by comparing the restriction patterns obtained from the samples to the fungal profiles collected in the reference database. Out of 810 samples collected, 790 samples were analysed by T-RFLP. Twenty samples were excluded from the fragment analysis due to lack of a high quality PCR product after amplification of the ITS region. Matching of T-RFLP profiles of samples with fungal profiles from the reference database identified fungi in 610 samples. These samples were used in analyses based on identified species. The identification of fungi failed for 180 samples.

Diversity of fungi

A total of 58 fungal taxa were identified using T-RFLP analysis (Fig. 1). Although none of the accumulation curves of fungal taxa reached an asymptotic value a decreased rate of accumulation of new taxa was observed with increasing sampling effort (Fig. 2).

Six fungal taxa from the reference library: *Gaeumannomyces graminis*, Mortierellales, *Mucor* sp., Pleosporales, *Rhizoctonia* sp. and *Sydowia polyspora* were not detected in the plant material.

The majority of fungi colonizing the winter wheat belonged to the phylum Ascomycota (75.8 %). The most common species were *Davidiella macrospora*, *Cladosporium* spp., *Tetracladium maxilliforme*, *Didymella exitialis*, *Microdochium nivale* and an unidentified ascomycete. These were all found in more than 20 % of the samples. One fifth of the identified fungal taxa belonged to Basidiomycota, and out of them the yeasts *Cryptococcus victoriae* and *Cryptococcus tephrensis* were the most frequent; 16.6 % (found in 101 samples) and 13.6 % (found in 83 samples)
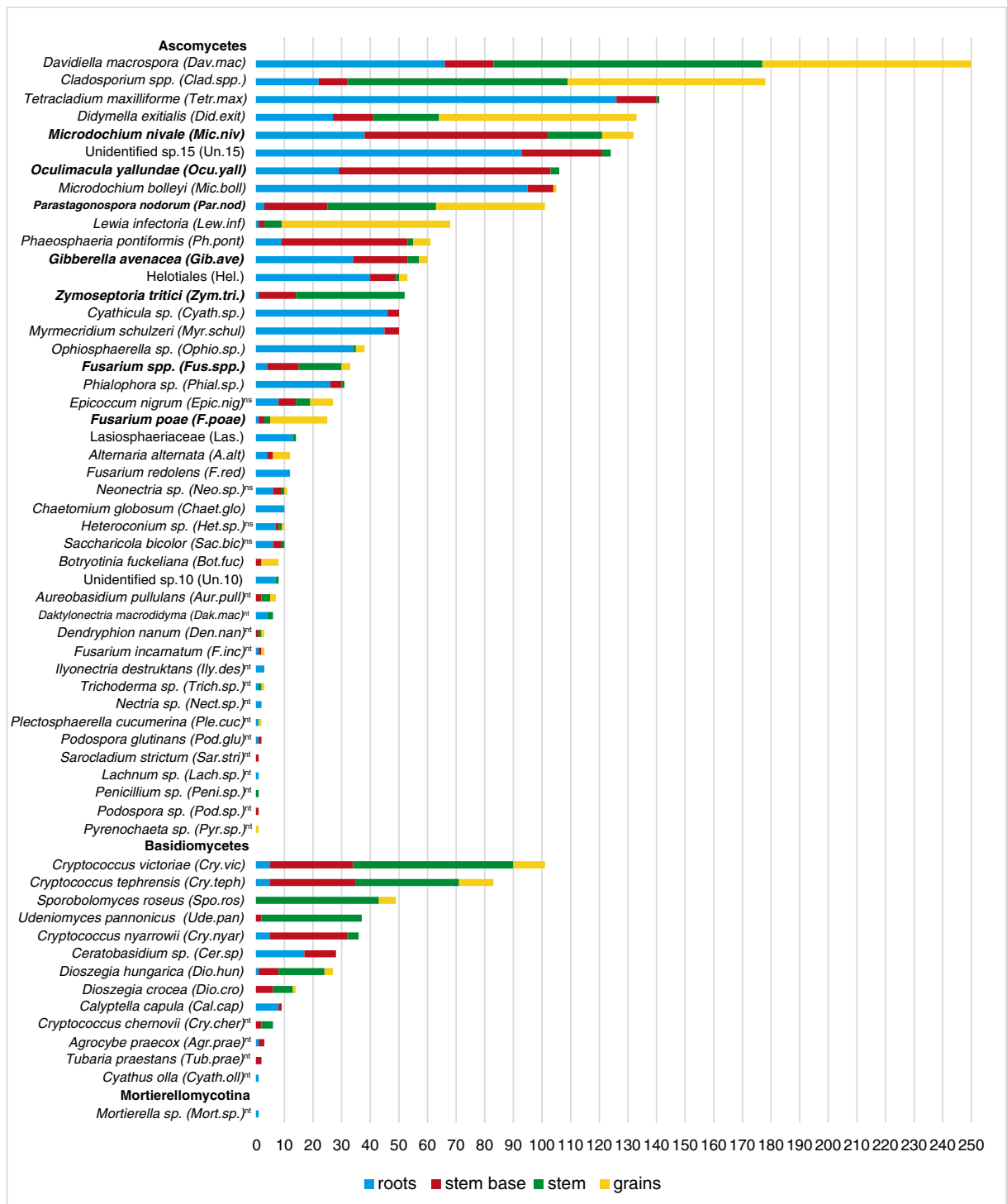
Fig. 1 Fungal taxa identified in different parts of winter wheat from three organic fields during one growing season using T-RFLP. The graph shows the number of samples in which the fungus was detected. Total number of samples $N = 610$, and for each plant part: roots $N = 194$, stem base $N = 194$, stem $N = 141$, grains $N = 81$. The taxon

code used in Fig. 3 is given in parenthesis. *Cladosporium* spp. represents *Cladosporium cladosporioides* and *Cladosporium tenuissimum*. *Fusarium* spp. represents *Fusarium culmorum*, *Fusarium graminearum* and *Fusarium sporotrichioides*. [ns] not significant in logistic regression analysis, [nt] not tested
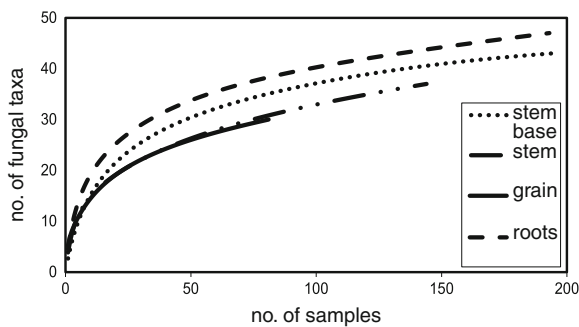
Fig. 2 Richness of fungal taxa detected in different parts of winter wheat, as expressed by the relationship between cumulative number of taxa found and sampling intensity

respectively. The subphylum Mortierellomycotina was represented by one genus, *Mortierella*, which was found only in roots. Since the ITS primers that were used do not work for identification of rust fungi and oomycetes the presence of species from those groups in the samples is not known. Among the fungi colonizing wheat, six potential wheat pathogens identified to species were found: *Fusarium poae* (found in 25 samples - 4.1 %), *Gibberella avenacea* (found in 60 samples - 9.8 %), *Microdochium nivale* (found in 132 samples - 21.6 %), *Oculimacula yallundae* (found in 106 samples - 17.4 %), *Parastagonospora nodorum* (found in 101 samples - 16.6 %) and *Zymoseptoria tritici* (found in 52 samples - 8.5 %). Other pathogens from the genus *Fusarium* were found but because of similar TRFLP profiles they could not be identified as separate species.

The highest number of taxa was found in roots (46 taxa), followed by stem bases (42), stems (36) and grain (29) (Fig 2). The average number of taxa per sample was 4.5 for roots (range 1–17), 2.6 for stem bases (range 1–16), 3.9 for stems (range 1–13) and 5.1 for grain samples (range 1–11).

Comparison of the fungal communities on different wheat parts using the Sorenson similarity indices (C$_s$) showed moderate to high similarities in community composition. The composition of the fungal population of stem bases had the highest similarity to the communities found on roots and stems (C$_s$ = 0.75), whereas the biggest differences were observed between the populations on grains and roots (C$_s$ = 0.62). Out of 46 fungal taxa identified on roots 23 were not detected on the grains. The most common among them were *Tetracladium maxilliforme*, an unidentified ascomycete, *Myrmecridium schulzeri* and *Cyathicula* sp., identified

in 64.9 %, 47.9 %, 23.2 % and 23.7 % of the root samples respectively (Fig. 1). Among fungi colonizing the grains, six taxa were found there but not on roots (Fig. 1). Most of the fungi colonizing grains also occurred on stem bases and stems. 12 taxa were specific only for one plant part. *Chaetomium globosum*, *Fusarium redolens*, *Ilyonectria destruktans*, *Lachnum* sp., *Nectria* sp., *Cyathus olla* and *Mortierella* sp. were unique for roots. *Podospora* sp., *Sarocladium strictum* and *Tubaria prestans* were found only in stem bases. *Penicilium* sp. was unique for stems and *Pyrenochaeta* sp. was unique for grains (Fig. 1). Seventeen taxa including four potential pathogenic species and the genus *Fusarium*, were found on all plant parts. The exceptions among the pathogens were *Z. tritici* and *O. yallundae*, which were not detected in grains.

Although pathogenic fungi were found in all plant parts differences between organs colonized were observed (Fig. 1). The most frequently detected pathogens in roots were *M. nivale*, *G. avenacea* and *O. yallundae* which were found in almost 20 %, 18 % and 15 % of the samples, respectively. These pathogens dominated also on stem bases where *O. yallundae* and *M. nivale* occurred in more than 30 % of the analyzed stem bases (P < 0.001). On stems the most frequently observed pathogen was *Z. tritici*, found in 27 % of the samples (P < 0.001), whereas on grains *P. nodorum* and *F. poae* occurred with the highest frequencies; found in 47 % and 25 % of the samples, respectively (P < 0.001).

Significant differences in the distribution of fungi between plant parts were observed for 35 fungal taxa (Fig. 1).

In the CCA model the relationship between identified fungal species and environmental variables was tested. All analysed variables, i.e. plant part, growth stage and field, were found to have small but significant effects on the composition of fungi in wheat (Table 2, Fig. 3). The most important source of variation was plant part, which explained 8.2 % of the total variance in fungal species. Growth stage and field explained 2.0 % and 1.5 % of the total variance in fungal community, respectively.

The AMMI analysis of the whole fungal population also showed that fungal community composition was the most affected by plant part as the samples grouped into four distinct clusters corresponding to roots, stem base, stem and grains (Fig. 4). Clusters of roots, stem base and stem were located close to each other.

Analysis of the source of variation showed that plant part had the greatest variation of interaction effects

Table 2 Variation in fungal community within fields, plant parts and growth stages analysed by canonical correspondence analysis (CCA)

Source of variation	Total inertia	Explained inertia	% explained
Field (F: F1, F2, F3)	12.218	0.187	1.5**[a]
Plant part (PP: R, SB, S, G)	12.218	1.004	8.2**
Growth stage (GS: 1, 2, 3)	12.218	0.247	2.0**
PP + GS + F	12.218	1.345	11.0**

[a] Monte Carlo permutation test, $P < 0.01$

(36.2 %), whereas the variation of interaction effects for growth stage and field was lower and stood at 10.19 % and 7.66 %, respectively (Table 3).

Seasonal variations

The fungal community colonizing roots and above ground parts of wheat changed during the growing season (Fig. 5, Online Resource 2). It is shown by the odds of finding fungi in plant parts obtained as a result of logistic regression analysis. The odds increased, decreased or fluctuated depending on fungus and plant part. As an example: the odds of finding the pathogen *Gibberella avenacea* in roots decreased from 0.25 in young plants to 0.06 in mature plants, whereas in the stem bases the odds increased from 0.02 to 0.11 during the growing season. The changes observed for this fungus in roots were statistically significant.

In roots of young plants *D. exitialis*, one unidentified ascomycete species and two pathogens, *G. avenacea* and *M. nivale*, were detected more often than in roots of mature wheat plants (confidence limits not overlapping) (Fig. 5, Online Resource 2). The opposite situation, but not statistically significant, was observed for *M. schulzeri* and the pathogenic fungus *O. yallundae*.

Three *Cryptococcus* spp. were detected with high frequency in stem bases early in the growing season (May) but they were absent in this plant part in the end of the season (July/August). In mature plants *C. victoriae* and *C. tephrensis* were frequently found on higher parts of the stem. A similar variation was observed for the pathogen *Z. tritici*, which was detected more often in stem bases of young plants than in older plants. The changes observed for this fungus in stem bases were statistically significant. In mature plants this fungus was not found in the stem bases but on higher parts of the stem.

Between anthesis and maturity of the wheat there was a significant decrease in the detection of yeasts, *Sporobolomyces roseus* and *Udeniomyces pannonicus*, in stems while the detection of pathogens, *M. nivale* and *P. nodorum*, in stems increased significantly during this period.

Fig. 3 Seasonal and plant part dependent variation of fungal community analysed by canonical correspondence analysis. R: roots, SB: stem base, S: stem, G: grain, GS1: growth stage1 (stem elongation); GS2: growth stage 2 (anthesis), GS3: growth stage 3 (maturity); taxon code is explained in Fig. 1
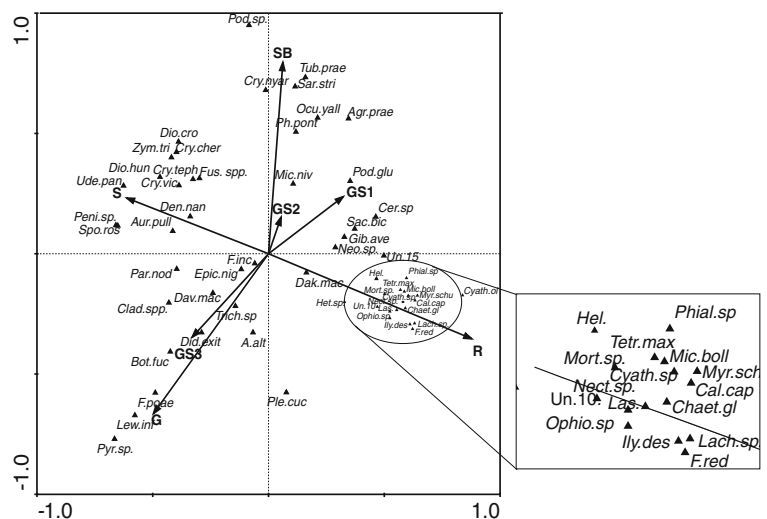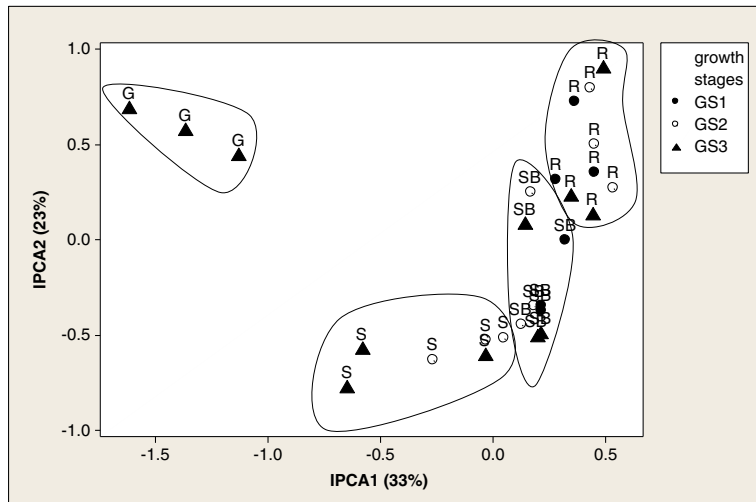
Fig. 4 AMMI analysis of fungal
T-RFLP data from roots (R), stem
bases (SB), stems (S) and grains
(G) of winter wheat during one
growing season

Although the fungal community colonizing the roots changed over time, the Sorenson similarity index was stable ($C_s = 0.78$–0.81). For stem bases it fluctuated over time, showing the highest turnover between fungi inhabiting young and mature plants ($C_s = 0.52$).

Relationships among fungi

Among 63 pair-wise combinations of common wheat pathogens detected in roots, stem bases and stems only 10 showed significant interactions (Table 4). Most of them were positive, which means that the presence of one pathogen favoured the presence of the other. The most significant interactions were observed in the upper part of the stem whereas in the roots only one interaction out of 21 tested was significant.

Fusarium spp. showed the most interactions with other pathogens. In the stem there was a positive interaction between *Fusarium* spp. and *F. poae* and *G. avenacea*. At the same time *Fusarium* spp. showed negative interaction with *P. nodorum* and *Z. tritici*. In

the stem base positive interaction was observed between *Fusarium* spp. and *Z. tritici* while there was a negative interaction between *M. nivale* and *O. yallundae*.

The strongest positive interactions ($P = 0.001$) were observed in three combinations of pathogens: *G. avenacea* – *M. nivale* in roots, *P. nodorum* – *G. avenacea* in stem bases and *P. nodorum* – *Z. tritici* in the stem.

Almost all pathogens, except for *F. poae*, showed significant interactions with the most common saprophytic fungi and/or yeasts (Table 5). Most of the interactions were positive. The only negative interaction was observed between *M. nivale* and *D. macrospora* in grains.

The pathogen *P. nodorum* was the fungus which showed the most positive interactions with the common non-pathogenic fungi. Positive interactions were detected with yeasts in the stem and with the saprophytic fungi *Cladosporium* spp. and *D. macrospora* in all plant parts except for the roots. In the stem *Z. tritici* also showed positive interactions with these fungi. In roots positive

Table 3 Percent of variation in T-RFLP fingerprints from each source

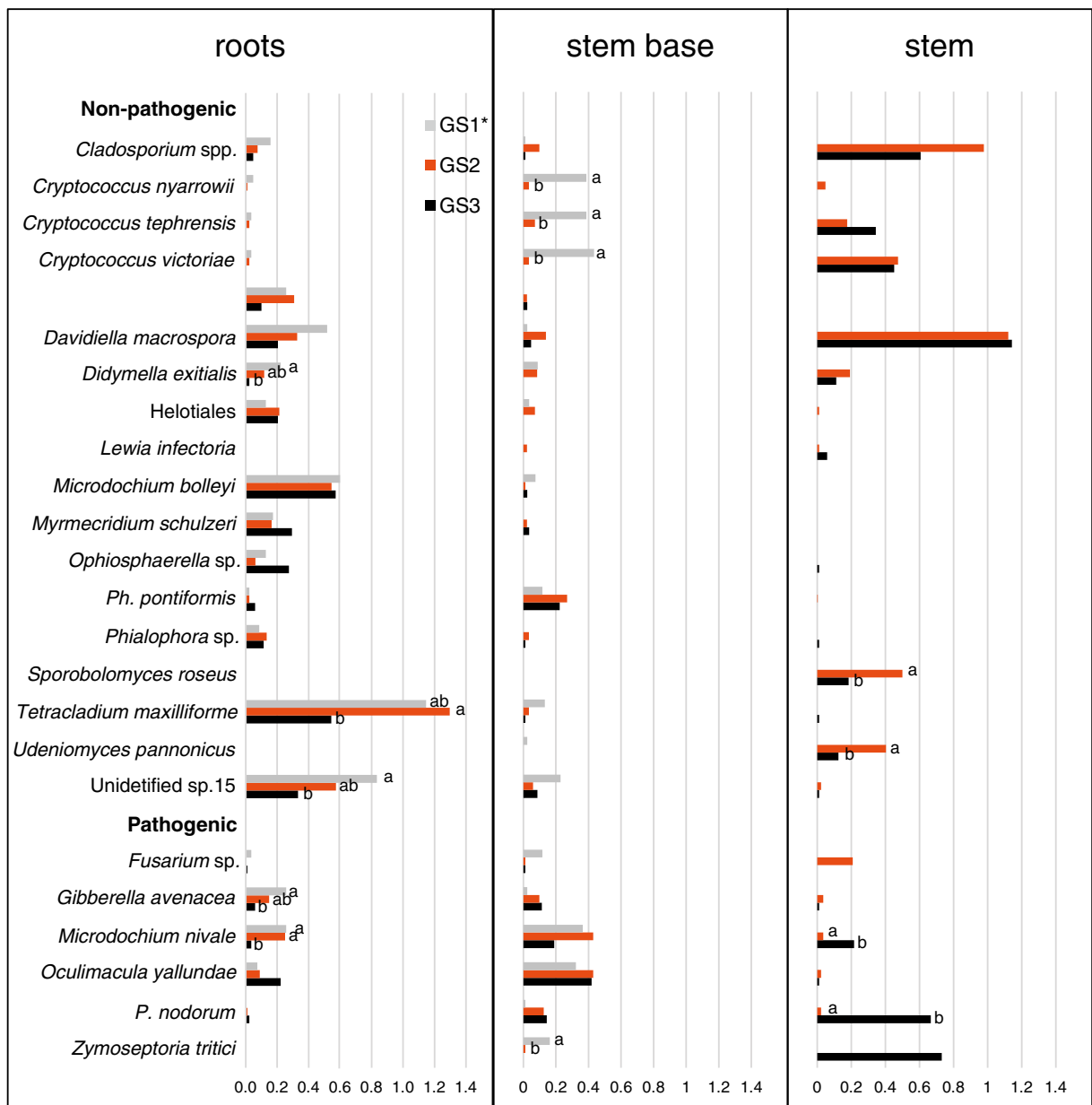|		Plant part (PP)	Growth stage (GS)	Field (F)	PP + GS + F
Main effect		63.75	89.75	92.33	51.7
	T-RF	62.20	89.50	91.86	49.5
	Environment	1.55	0.25	0.47	2.2
Interaction		36.23	10.19	7.66	48.1
	Pattern	35.47	9.30	6.41	40.9
	Noise	0.76	0.89	1.25	7.2

Fig. 5 The occurrence of the most common fungal taxa on winter wheat during one growing season analysed by logistic regression and shown as the odds of finding fungi in plant parts. Statistically significant changes are marked with different letters. [*]growth stage 1, 2, 3: GS1 (stem elongation), DC30–32; GS2 (anthesis), DC 65–69; GS3 (maturity), DC 85–90 according to Zadoks et al. (1974)

interactions were observed between *G. avenacea* and all non-pathogens tested except for *Cladosporium* spp. *M. nivale* also showed positive interactions with all saprophytic fungi in the roots.

A formal disease assessment was not carried out in any of the three fields, but a general overview showed no remarkable occurrence of any specific disease on any part of the plants. Disease surveys in 2007 from the

same area as where the fields were located report weak attacks of eyespot (*Oculimacula yallundae*) and tan spot (*Pyrenophora tritici-repentis*), and few cases of take-all (*Gaeumannomyces graminis*). Septoria tritici blotch (*Zymoseptoria tritici*) was the most common leaf spot disease but it did not exceed 20 % incidence until after the end of anthesis to early milk stage (DC 69–73) (Waern and Sandström 2007).

Table 4 Results of log-linear analysis of two-pathogen interaction for common pathogens of wheat

Plant part	Fungus	Z. tritici	F. poae	Fusarium spp.	G. avenacea	M. nivale	O. yallundae
Roots	*Parastagonospora nodorum*	ns[a]	nt[b]	ns	ns	ns	ns
	Zymoseptoria tritici		nt	nt	nt	nt	nt
	Fusarium poae			nt	nt	nt	nt
	Fusarium spp.				ns	ns	ns
	Gibberella avenacea					P***	ns
	Microdochium nivale						ns[c]
Stem base	*Parastagonospora nodorum*	ns	ns	ns	P***	ns	ns
	Zymoseptoria tritici		ns	P*[d]	ns	ns	ns
	Fusarium poae			ns	ns	ns	ns
	Fusarium spp.				ns	ns	ns
	Gibberella avenacea					ns	ns
	Microdochium nivale						N**
Stem	*Parastagonospora nodorum*	P***	ns	N**[e]	ns	ns	ns
	Zymoseptoria tritici		ns	N**	ns	ns	ns
	Fusarium poae			P**	P*	ns	ns
	Fusarium spp.				P*	ns	ns
	Gibberella avenacea					ns[c]	ns[c]
	Microdochium nivale						ns

[a] ns, *, ** and *** - not significant and significant at $P = 0.05$, $P = 0.01$ and $P = 0.001$, respectively

[b] not tested because of too low number of samples

[c] not significant according to Fisher's exact test but significant in log-linear model

[d] positive interaction between pathogens

[e] negative interaction between pathogens

Discussion

The fungal community colonizing organically growing wheat represents fungi of different ecology. They can be classified according to their functions as wheat pathogens (incl. minor pathogens), grass pathogens, saprophytic filamentous fungi often present on wheat, yeasts, and fungi with undetermined function in this system.

The most important factor affecting the colonization of wheat by fungi was plant part as was shown both by the multivariate analysis based on individual species as well as on the whole fungal population. Both methods showed also that differences in fungal communities between plant parts were greater than between growth stages or fields. Organ specificity has been reported in several studies (Larran et al. 2007; Sieber et al. 1988; Vujanovic et al. 2012) indicating that fungi could adapt to the particular microecological and physiological conditions present in a given organ. Species richness and diversity was the highest in roots. It could be attributed to the special conditions in this plant part. Roots create a relatively stable and nutrient rich environment, compared to above ground organs, which favours the survival of many fungal species (Vujanovic et al. 2012). Although the fungal community of roots was the most diverse it was closely related to the community on stem base and stem, as was shown by the multivariate analysis.

Most fungi were able to colonize more than one plant part. Four dominating species *D. macrospora*, *Cladosporium* spp., *D. exitialis* and *M. nivale* were found both on roots and above parts of wheat which indicates that these fungi were the best adapted to colonize wheat.

Seasonal variation in the composition of fungal communities in different parts of wheat was observed during this study. In roots the contribution of non-pathogenic fungi decreased during the growing season, while the contribution of pathogens varied depending on the species. Some pathogens, such as *M. nivale* and

Table 5 Results of log-linear analysis of the interaction between common pathogens of wheat and the most common saprophytic fungi and yeasts

Plant part	Fungus	Cladosporium spp.	D. macrospora	Unidentified sp. 15	T. maxiliforme	C. tephrensis	C. victoriae
Roots	P. nodorum	ns[a]	ns	ns	ns[c]	ns	ns
	Z. tritici	nt[b]	nt	nt	nt	nt	nt
	F. poae	nt	nt	nt	nt	nt	nt
	Fusarium spp.	ns	ns	ns	ns	ns	ns
	G. avenacea	ns	P**	P**	P**	P*	P***
	M. nivale	P**[d]	P*	P*	P**	ns	ns
	O. yallundae	ns	ns	ns	P**	ns	ns
Stem base	P. nodorum	P*	P***	ns	P*	ns	ns
	Z. tritici	ns	ns	P*	ns	P***	P***
	F. poae	ns	ns	ns	ns	ns	ns
	Fusarium spp.	ns	ns	ns	ns	P***	P***
	G. avenacea	ns	P*	ns	ns	ns	ns
	M. nivale	ns	P*	ns	P*	ns	ns
	O. yallundae	ns	ns	ns	ns	ns	ns
Stem	P. nodorum	P***	P***	ns	ns	P**	P***
	Z. tritici	P*	P***	ns	ns	P***	P***
	F. poae	ns	ns	ns	ns	ns	ns
	Fusarium spp.	P*	P*	ns	ns	ns	ns
	G. avenacea	ns	ns	ns[c]	ns	ns	ns
	M. nivale	ns	ns[c]	ns	ns	ns	ns
	O. yallundae	ns	ns[c]	P***	P*	ns	P**
Grain	P. nodorum	P***	P**	-	-	ns	ns
	F. poae	ns	ns	-	-	ns	ns
	Fusarium spp.	ns	ns	-	-	ns	ns
	G. avenacea	ns	ns	-	-	ns	ns
	M. nivale	ns	N*[e]	-	-	ns	ns

[a] ns, *, ** and *** - not significant and significant at $P = 0.05$, $P = 0.01$ and $P = 0.001$, respectively

[b] not tested because of too low number of samples

[c] not significant according to Fisher's exact test but significant in log-linear model

[d] positive interaction between pathogens

[e] negative interaction between pathogens

G. avenacea, causing pink snow mold and Fusarium Head Blight, were more common in young roots, whereas *O. yallundae*, causing eyespot, was more common in old roots. Similar patterns of colonization of wheat roots by saprophytic fungi have been reported by Lemańczyk and Sadowski (2002). They also observed that the pathogens became dominant with the development of the plant.

In stem bases *M. nivale* and *O. yallundae*, but not *G. avenacea*, were more common in young plants. The colonization of different parts of wheat by the pathogens depends on their aggressiveness as well as on the tissue specificity or systemic spread from the point of infection. During the growing season more aggressive fungi, like *O. yallundae*, could infect the plant and replace the weaker ones. Matusinsky et al. (2008) observed that *Oculimacula* spp. and *R. cerealis* infections increased with the development of the crop but these pathogens infected only plants previously colonized by *M. nivale*. Decrease of non-pathogenic fungi may also result from rapid colonization of tissue by more aggressive fungi in which case the non-pathogen would not be able to function as an effective antagonist against the pathogen.

On the above ground parts of wheat the most dynamic and significant changes were observed among yeasts. Yeasts are commonly found both on healthy wheat leaves and stems (Larran et al. 2002) as well as on leaves infected by pathogens (Blixt et al. 2010; Perelló et al. 2002). Our results show that yeasts also colonize roots, stem bases and grains. A clear decrease of white-pigmented yeasts from the genus *Cryptococcus* observed in stem bases late in the season can be due to the strong competition for nutrients with pathogens and saprophytes colonizing stem bases during plant development. Environmental conditions could also influence the growth of yeast in stem bases. On the upper parts of the stem the pattern of colonization was different. Two species of *Cryptococcus* were abundant in the uppermost node of the mature plants and even during anthesis, while *Udeniomyces pannonicus* and *Sporobolomyces roseus* occurred in high abundance in the stem during anthesis and then decreased significantly.

Some pathogens were found on plant parts where they normally do not occur. Examples are the leaf pathogens *Zymoseptoria tritici*, causing Septoria tritici blotch, and *Parastagonospora nodorum*, causing Stagonospora nodorum blotch, which were found on stem bases and sporadically on roots, and the stem base pathogen *Oculimacula yallundae* found on the stem

around the uppermost node. These infestations could come from surviving inoculum on old crop debris or perennial grass weeds or from airborne ascospores. In organically grown wheat seedborne *P. nodorum* could be another inoculum source and symptoms would usually develop on the coleoptile (Shah and Bergstrom 1999). Our findings of *P. nodorum* on the roots could indicate that seedborne inoculum also is able to infect roots. Seedborne inoculum of *Z. tritici* is not considered as important, although in Argentina proof of natural seed infestation has been presented (Consolo et al. 2009). In the case of *O. yallundae* it has been shown that the ability of this fungus to colonize the plant depends on the pathogenicity of isolates. More pathogenic isolates of *O. yallundae* are able to penetrate the leaf sheaths causing stem infection whereas the less pathogenic are unable to spread beyond the leaf sheaths (Lucas et al. 2000).

F. poae, involved in the Fusarium Head Blight (FHB) complex, was detected in grains most frequently during this study. It is a relatively weak pathogen compared to *F. culmorum* and *F. graminearum*, which are also in the FHB complex, but it produces several mycotoxins, including trichothecenes, beauvericin and enniatins (Stenglein 2009). An increasing occurrence of *F. poae* has been observed in many countries in Europe (Xu et al. 2005). Dominance of *F. poae* among FHB pathogens increases the risk of grain contamination with a broad spectrum of mycotoxins produced by this fungus and indicates that this species can become more important in the future.

Microdochium bolleyi and *Didymella exitalis*, known as minor pathogens of wheat, were frequently detected. *M. bolleyi*, common on wheat and barley roots, can infect the plant causing discoloration on crown and roots, and some isolates can also reduce plant emergence and shoot dry weight (Gonzalez and Trevathan 2000). *D. exitalis* causes leaf scorch of cereals. There is no information about infection of grains by this fungus but our results demonstrate that *Didymella exitalis* was very common in the grains. The source of infection was probably air-borne ascospores produced by the fungus late in the season on senesced leaves (Cromey et al. 2004).

The grass pathogen *Ophiosphaerella* sp. causing spring dead spot disease in bermudagrass (Smiley et al. 2005) was found among the fungi colonizing wheat roots and grain. This is the first report of *Ophiosphaerella* sp. from wheat in Sweden. The role

of *Ophiosphaerella* sp. in wheat is unknown but it has been shown that damage can occur in wheat after oats and in wheat monoculture (Kwaśna et al. 2010) suggesting that this fungus also can be a potential wheat pathogen.

Significant interactions between some pathogens were detected, particularly in the upper part of the stem. Most of the combinations of two pathogens showed positive relationships in which the presence of one pathogen could favour the occurrence of the other. *Z. tritici* and *P. nodorum* have similar life cycles and environmental requirements (Bockus et al. 2010) and it can explain the positive interaction observed between them in the stem samples. Negative interactions between these species and *Fusarium* spp. in the upper part of the stem could indicate that they compete in the colonization of the stem tissue. The opposite situation was observed in stem bases (Table 4). *Z. tritici* and *P. nodorum* are better adapted to infect leaf tissue than the stem base but it seems that in the presence of other pathogens these fungi are also able to colonize stem bases.

Positive interactions were also found in combinations of *Fusarium* spp. with *F. poae* and *G. avenacea* (anamorph *F. avenaceum*) which confirms that pathogenic *Fusarium* species often occur as species assemblages (Pettitt et al. 2003; Xu et al. 2005). The occurrence of and interactions between *Fusarium* species cannot be further resolved with the method used in our study.

In the stem bases a negative association was observed between *M. nivale* and *O. yallundae*, suggesting that these pathogens are antagonists or that conditions favouring one pathogen do not favour the other. The results are in contrast to our knowledge about these fungi. *M. nivale*, causing brown foot rot and snow mold, and *O. yallundae*, causing eyespot, have previously often been found together in the stem bases (Bateman et al. 2000; Matusinsky et al. 2008). In mixed infections it is not clear which of them is the primary or secondary colonizer (Matusinsky et al. 2008). In two fields from our study either *M. nivale* or *O. yallundae* dominated and they were found together only sporadically. In the third field *M. nivale* was the most frequent but mixed infections were observed more often compared to the other fields. These differences between fields influenced the log-linear analysis resulting in a negative interaction between *M. nivale* and *O. yallundae* when all fields were analyzed together.

Most of the wheat pathogens showed a significant positive interaction with saprophytic fungi meaning that the presence of non-pathogens favoured the colonization by pathogen, and/or vice versa. We found no indications that any of the saprophytic fungi could be regarded as potential antagonist preventing infections by pathogens. Antagonistic effect of saprophytic fungi inhabiting wheat against wheat pathogens has been observed frequently but only under experimental conditions (Liggitt et al. 1997; Müllenborn et al. 2008; Perelló et al. 2002). In the field the interactions between fungi colonizing the same tissue are more complex and influenced by many external factors, which sometimes can facilitate the growth of both pathogenic and non-pathogenic fungi.

We developed a reference database containing T-RFLP patterns of 67 fungal taxa, which were obtained after T-RFLP analysis of sequenced clones and isolates of fungi colonizing different parts of wheat. Although most of the fungal taxa collected in the reference library were found in the analyzed plant samples, unknown fungal profiles which did not match with patterns from the library were observed. These profiles can represent fungi rarely occurring on wheat and thereby unidentified by cloning or isolation and not included in the reference database.

The unknown fungal profiles can also represent isolates of fungal species already collected in the library but differing in sequences within the ITS region. The fungal ITS rDNA gene region contains both variable and highly conserved sequences (Thies 2007). Sequence variations in the ITS region within the same fungal species have been reported (Avis et al. 2006; Vujanovic et al. 2009). If the variation occurs in the restriction enzyme recognition site fragments of different length within the same species are obtained after cutting with restriction enzymes. This would result in different T-RFLP profiles for different individuals of the same species. The risk that some patterns will not be identified can be decreased by including as many T-RFLP profiles of one species as possible in the database (Dickie and FitzJohn 2007).

We could not identify fungi in 180 samples. The reason could be poor quality of restriction profiles obtained after T-RFLP. One of the criteria for the identification of fungal taxa in TRAMPR was the presence of peaks for minimum four out of six of the enzyme/primer combinations in a sample. Samples which did not fulfil this requirement could not be analysed by the program. Another reason for the unsuccessful identification of taxa could be that the obtained profile did not match with any of the fungal pattern from the library.

The internal transcribed spacer (ITS) region of the rDNA genes is commonly used for identification of fungal species and analysis of fungal communities (Allmer et al. 2006; Blixt et al. 2010; Dickie et al. 2002; Kwaśna et al. 2008; Lindahl et al. 2007). In the present study, however, the species *Fusarium culmorum*, *F. graminearum* and *F. sporotrichioides* as well as *Cladosporium cladosporioides* and *C. tenuissimum* could not be distinguished by analysis of the ITS region, with the restriction enzymes that were used, because of its low polymorphic nucleotide sequences. Very low variation in the sequence of the ITS region in the genus *Fusarium* has already been reported (Edel et al. 1997; Yli-Mattila et al. 2004). Recent phylogenetic studies of *Fusarium* have shown that the translation elongation factor 1α (TEF-1α) gene is a useful region for identification and distinguishing between species (Edel et al. 1997; Edel-Hermann et al. 2015; Kristensen et al. 2005). *C. cladosporioides* and *C. tenuissimum* are very closely related species (Bensch et al. 2010). Our results indicate high similarity between sequences of ITS regions resulting in difficulties in separation of those species by analysis of restriction profiles. Schubert et al. (2007) reported that within five genes (ITS, actin, calmodulin, translation elongation factor 1-α and histone H3) used for identification of species in the *C. herbarum* complex only ITS data did not provide any resolution.

The fungal population on organically grown winter wheat was very diverse. It consisted of fungi, which specifically colonized below- and above-ground parts of the plants. The composition of fungi changed during crop development but a pattern common for the whole crop could not be observed. However, for the particular plant part of wheat some patterns in the dynamics of fungal populations were found.

Among the fungi colonizing wheat some wheat pathogens were very common. They changed over time and also slightly between fields together with the other fungi found on the wheat. Most of the pathogens were specialized on plant part, a few of them were also found in unexpected places indicating the high adaptive ability of these fungi.

Significant interactions between pathogens and saprophytic fungi were observed but no evidence that any of the saprophytes could be considered as potential antagonists against pathogens was found.

Compliance with ethical standards

Conflict of Interest The authors declare that they have no conflict of interest.

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