Succession of the fungal endophytic microbiome of wheat is dependent on tissue-specific interactions between host genotype and environment

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Succession of the fungal endophytic microbiome of wheat is dependent on tissue-specific interactions between host genotype and environment

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HIGHLIGHTS
- Host genotype and environment are drivers of the wheat endophytic fungal microbiome.
- The effect of host genotype on the microbiome is independent of environment.
- Abiotic factors influence phyllosphere, but not rhizosphere fungal communities.
- Airborne fungi are the main inoculum source for the microbiome in leaf and seed.
- Pedigree/Relatedness of wheat cultivars is not reflected in the microbiome.

GRAPHICAL ABSTRACT

ABSTRACT
Fungi living inside plants affect many aspects of plant health, but little is known about how plant genotype influences the fungal endophytic microbiome. However, a deeper understanding of interactions between plant genotype and biotic and abiotic environment in shaping the plant microbiome is of significance for modern agriculture, with implications for disease management, breeding and the development of biocontrol agents.

For this purpose, we analysed the fungal wheat microbiome from seed to plant to seeds and studied how different potential sources of inoculum contributed to shaping of the microbiome. We conducted a large-scale pot experiment with related wheat cultivars over one growth-season in two environments (indoors and outdoors) to disentangle the effects of host genotype, abiotic environment (temperature, humidity, precipitation) and fungi present in the seed stock, air and soil on the succession of the endophytic fungal communities in roots, flag leaves and seeds at harvest. The communities were studied with ITS1 metabarcoding and environmental climate factors were monitored during the experimental period.

Host genotype, tissue type and abiotic factors influenced fungal communities significantly. The effect of host genotype was mostly limited to leaves and roots, and was location-independent. While there was a clear effect of plant genotype, the relatedness between cultivars was not reflected in the microbiome. For the phyllosphere microbiome, location-dependent weather conditions factors largely explained differences in abundance, diversity, and presence of genera containing pathogens, whereas the root communities were less affected by abiotic...
factors. Our findings suggest that airborne fungi are the primary inoculum source for fungal communities in aerial plant parts whereas vertical transmission is likely to be insignificant. In summary, our study demonstrates that host genotype, environment and presence of fungi in the environment shape the endophytic fungal community in wheat over a growing season.

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1. Introduction

Plant-associated fungi can have important functions in growth and health of their host plant, by playing roles in disease resistance, nutrient acquisition, and abiotic stress tolerance (Hardoim et al., 2015; Schlaeppi and Bulgarelli, 2015; Wani et al., 2015). Recently, the implications of plant-microbe interactions in modern agriculture have received much attention, potentially contributing to plant breeding strategies (Kavamura et al., 2020; Pieterse et al., 2016), development of microbe-based biocontrol (Backman and Sikora, 2008; Orozo-Mosqueda et al., 2018; Syed Ab Rahman et al., 2018), and general understanding of the effect of plant domestication on the microbiome (Kavamura et al., 2020; Pérez-Jaramillo et al., 2018, 2016). Internally colonising endophytic fungi are thought to have evolved with their host (Bidartondo et al., 2011; Redecker et al., 2000), and due to their lifestyle are potentially more subjected to host genetic control than externally colonising microorganisms (Ahlholm et al., 2002; Saikkonen et al., 2010, 2004). Despite the clear evidence of the economic significance of fungi in the plant microbiome, both as pathogens (Savary et al., 2019) and promoters of plant health (Saikkonen et al., 2004; Van Wees et al., 2008; Zamioudis and Pieterse, 2011), their roles in the microbial community, especially those displaying an endophytic life-style, are still poorly investigated compared to bacteria. Considering the position of wheat as a staple food and main source of nutrition for a large proportion of the world population (Curtis and Halford, 2014; Lobell et al., 2009), studying the factors influencing the associated fungal community that also comprises many devastating pathogens (Krattinger et al., 2009; Savary et al., 2019), is of particular relevance. In this study, the term endophytic encompasses all microorganisms internally colonising the plant, despite their pathogenic or mutualistic lifestyle on the host plant (Jørgensen et al., 2020).

Plant genetic control of the internal microbial community is of considerable interest for crop plant breeding and towards exploring the possibility to design a ‘healthy’ microbiome (Morella et al., 2020). In laboratory-based studies, individual host plant mutations were found to affect composition of synthetic bacterial communities in the phyllosphere (Bodenhausen et al., 2014; Chen et al., 2020; Horton et al., 2014) and in the rhizosphere (Lundberg et al., 2012). In an agricultural setting of networks of interacting biotic and abiotic factors, the magnitude of the effect of genetic traits might vary depending on environmental conditions and microbial communities in the environment. Thus, the role of genetic traits established under controlled conditions might be overestimated and context-dependent traits overlooked (Cooper et al., 2009; Yan et al., 1999). A genotype effect on the fungal or bacterial microbiome has been found for closely related species of cereals (Sapkota et al., 2015), accessions/cultivars of wheat (Sapkota et al., 2017; Simonin et al., 2020) and wheat cultivars with genetically-determined height differences (Kavamura et al., 2020) and drought resistance (Azarbad et al., 2020). However, the lack of knowledge on the degree of genetic relatedness limits the conclusions that can be drawn from these results. Furthermore these studies did not attempt to separate the microbiome into epiphytes and endophytes.

Interactions between the host genotype and the growth location has a major effect on shaping the plant microbiome (Azarbad et al., 2020; Fitzpatrick et al., 2018; Rojas et al., 2020b). Previous studies investigated the effect of field location on the fungal wheat microbiome of wheat leaves (Sapkota et al., 2017, 2015; Schlatter et al., 2019), but since field sites can differ in both biotic and abiotic factors, conclusions on which factors account for the variation are difficult to make. Environmental conditions, different microbial communities in the environment and soil properties can vary substantially between field sites. Location-dependent differences between the environmental microbiome as source of microbial inoculum have an influence on the plant microbiome (Rojas et al., 2020b; Simonin et al., 2020). We designed the current pot experiment to dig deeper into implications of these interactions by using seed stock of cultivars harvested in the same field, and growing the plants indoors and outdoors in the same batch of soil and at the same location. By inclusion of air samples from both environments, we could explore further the origin and contribution of the airborne fungal inoculum to the microbiome in different tissues. To our knowledge, the importance of the air microbiome has not previously been in included in plant microbiome studies although there is a body of evidence to support its role (Maigien et al., 2014).

While soil (Zarraonaindia et al., 2015), air (Maigien et al., 2014), and seeds (Hodgson et al., 2014) have been investigated individually as sources of microbial inoculum, this is the first comprehensive study exploring the complex interaction between all three inoculum sources, environmental factors and plant genotype. Furthermore, we analyse microbiomes from roots, flag leaves and harvested seed. This experimental design allows us to address the following questions: Firstly, how do environmental differences between locations shape the wheat microbiome? To which extent does plant genotype (cultivar) contribute to fungal community variation, and is genetic relatedness and resistance level to Septoria tritici blotch (STB) reflected in the fungal community? Are tissue-specific communities equally influenced by the investigated factors? Finally, what are the inoculum sources for endophytic communities of wheat in different tissues and is there evidence for vertical seed-transmission to below- or above ground tissues?

We show that seeds, leaves and roots harbour distinct fungal communities that are differentially affected by the environment; that the effect of host genotype is specific to leaves and roots and not dependent on environment; that genetic relatedness is not reflected in the microbiome; and that the microbiome in the phyllosphere is shaped by airborne fungi and the rhizosphere by soil fungi. These observations can have practical implications in an agricultural context, since knowledge of the factors naturally shaping the microbiome can serve as a guide to improve the mode of application and thereby also potentially efficiency of microbial biocontrol agents.

2. Materials and methods

2.1. Experimental design

The seed material used in this project comprises 12 British winter wheat cultivars: Cinnabar, Durin, Hedgehog, Hobbit, Hornet, Longbow, Mandate, Maris Beacon, Mithras, Norman, Rapier and Sportsman that were grown and harvested from the same field at the John Innes Centre, UK. The cultivars were selected based on relatedness and differences in resistance levels to STB based on quantitative resistance and presence/absence of the qualitative STB resistance gene Stb15. The experiment was carried out at the field station ‘Højbakkegaard’ at the University of Copenhagen in Taastrup, DK. Ten pots per cultivar (eight + two reserve pots) were filled with 10 L well-mixed sandy loam soil from an organic
field close to the experimental location, where wheat had been grown the previous year. Five wheat seeds were sown in each pot and covered with a 4 cm layer of 'Perlite' (sowing date: 27.4.2017). Four samples of 50 g soil were collected for microbiome analysis and stored at −20 °C until further processing. After a germination period of one week in the greenhouse, the wheat seedlings were vernalised for 8 weeks at 5 °C. Subsequently, five pots of each cultivar were placed outside (Fig. 1a) into the field environment in close vicinity to the greenhouse (29.06.2017). The remaining five pots were transferred to a greenhouse chamber, where no other plants were present. Plants were irrigated throughout the growth period with water supplemented with fertiliser (final solution: EC 2.0, using Pioner NPK Basis 14-2-23 +Mg + mikro brun', Horticoop Scandinavia A/S, Tilst, Denmark). One treatment with the fungicide ‘Prosaro’ (active ingredients prothioconazole and tebuconazole, Bayer AG, Leverkusen, Germany) for powdery mildew control was applied to all plants (6.7.2017), 6 weeks before the first plant sampling time point, when individual colonies of powdery mildew were observed on single plants. The wheat cultivars varied greatly in resistance to powdery mildew so it was important to prevent an epidemic.

2.2. Air sampling

Starting at the time point when the plants were transferred to the field or controlled greenhouse environment, airborne microorganisms were sampled in both locations using OSP samplers (Gesamtstaubprobenahme, CIS by BGI, INC Waltham, MA, USA) connected to ‘Apex’ air sampling pumps (Casella CEL, Buffalo, NY, USA). This sampling method was previously used successfully for sampling of airborne fungi and bacteria (Frankel et al., 2012). The two air samplers were mounted with polycarbonate filters (37 mm, pore size 1.0 µm, GVS Life Sciences, Zola Predosa, Italy) and airborne microorganisms sampled for approx. 14 h at a flow rate of 3.5 L min⁻¹ on a fixed day every week, by placing the air samplers in close proximity to the plants. The filters were collected directly after sampling and stored at −80 °C until further processing. Air sampling
was carried out for 12 weeks, until the seeds were mature and harvested, and a total of 24 filters were collected from both sampling locations.

2.3. Plant sampling and sample processing

After 8 weeks in the greenhouse or field, leaves and roots were sampled at anthesis (BBCH 65) within two consecutive days. From four pots (four biological replicates) one plant was sampled from each, collecting the whole roots and four flag leaves from individual tillers. Roots were washed in tapwater until soil was removed, and further processed the same day together with leaf samples. When the plants were matured (BBCH 92), four ears were collected from one plant, growing in the same pot from which leaves and roots were sampled at anthesis, and seeds separated from the husk. For plants grown in the field environment, the seeds took two weeks longer to mature and were taken into a greenhouse chamber to ripen before sampling, because of wet weather conditions outside.

2.3.1. Surface sterilisation

Leaf, root and seed samples were processed on the day of sampling under sterile conditions (laminar flow bench). The samples were surface steriltised by an initial rinse in 70% ethanol, incubated for 2 min in NaOCl followed by three washing steps with sterile MilliQ water. The concentration of NaOCl for the surface sterilisation was adjusted depending on the plant tissue. Thus, 0.5% was used for leaves, 3% for roots and 7% for seeds. Subsequently, samples were frozen at −20 °C until further processing. All further processing and handling steps were carried out under sterile conditions in a laminar flow bench.

2.3.2. Sample processing

The samples were transferred to 20 mL vials (Polyvials V, HDPE, Zinsser Analytic, Frankfurt am Main, Germany) and lyophilised. The samples were ground using zirconium oxide grinding balls on a shaker (Fluid Management Inc., Wheeling, IL, USA) until pulverised. The balls were previously surface sterilised with 0.5 M HCl for 3 min under shaking. Two 10 mm balls were added to each seed sample and four 5 mm balls to each leaf and root sample. Between processing steps, the samples were stored at −20°C.

2.4. Sequencing of fungal communities

2.4.1. DNA extraction

All steps were carried out under sterile conditions in a laminar flow bench. DNA was stored at −80 °C, and the number of thaw-freeze cycles minimised.

Soil

DNA was extracted from 400 mg soil using the ‘NucleoSpin Soil’ kit (Macherey-Nagel, Düren, Germany), following the manufacturer’s instructions and adding an additional lysis step of 5 min vortexing with glass beads. Clean-up of eluted DNA was performed with ‘E.Z.N.A. MicroElute DNA Clean-up’ kit (Omega bio-tek, Georgia, USA), following the manufacturer’s protocol.

Plant samples

DNA was extracted from leaf, root and seed samples using the ‘E.Z.N.A. SP Plant DNA’ kit (Omega bio-tek, Georgia, USA), following the manufacturer’s instructions, with an extension of the lysis step by vortexing for 10 min in SP1 buffer with 1 g 0.7 mm sterile ‘Garnet Beads’ (Omni International Inc., Georgia, USA).

Air samples

Air sample filters were cut into several smaller pieces and subsequently, DNA was extracted as described above for plant samples.

2.4.2. Generation and processing of ITS1 metabarcoding data

The ITS1 (nuclear ribosomal internal transcribes spacer 1) region was used as target for metabarcoding in this study. It was suggested as a universal DNA barcode marker for fungi and has been widely used in fungal community studies (Schoch et al., 2012). To optimise unbiased coverage of the fungal endophyte community in the plants and to minimise amplification of plant ITS1, different PCR primers for metabarcoding studies (Bokulich and Mills, 2013; Toju et al., 2012) were compared. The selection was made based on highest number of mismatches to the wheat ITS region of ten wheat cultivars (downloaded from NCBI) and highest similarity to ten abundant wheat associated ascomycete and basidiomycete species, using the Muscle alignment tool of CLC Workbench. The primer pair ITS1-F (Gardes and Bruns, 1993) and 58A2R (Martin and Rygiewicz, 2005) was selected, since it has also been successfully used in recent fungal wheat microbiome studies (Sapkota et al., 2017, 2015). Primers with Illumina adapters (58A2R-NGS (51 bases) GTC TTC CGG AGA TGT GTA TAA GAG ACA GCT GCC TTC TTT ATC GAT & ITS1F-NGS (55 bases) TCG GCC TCA AGT CAT GTG TAT AAC AGA CAG CTT GGT CAT TAA GAA GTA A) were used for amplification of the fungal ITS1 region. Three PCR reactions each with 20 ng DNA were carried out for each sample, using the ‘DreamTag PCR Master Mix’ (Thermo Scientific, Massachusetts, USA) in a final volume of 25 μL. All PCR reactions were carried out in a 2720 thermal cycler (Applied Biosystems, Massachusetts, USA) with an initial DNA denaturation step of 94 °C for 5 min, followed by 30 cycles at 94 °C for 30 s, 48 °C for 30 s, 72 °C for 40 s, and a final elongation step at 72 °C for 10 min. Subsequently, the PCR products were cleaned using the ‘Wizard SV Gel and PCR Clean-Up’ kit (Promega, Madison, USA), following the manufacturer’s protocol, and the DNA concentration adjusted. Indexing and sequencing was performed on a total of 364 samples in one lane of Illumina HiSeq 2 × 250 by ‘Macrogen’, Seoul, Republic of Korea. Sequencing resulted in 55,212,889 reads, with 92.2 ± 1.0% of the bases reaching a Phred quality score of >Q30.

Processing of the sequencing data was performed in QIIME2 (2018.4) (Bolyen et al., 2019). Sequence quality control, including denoising, trimming and chimera removal was achieved with DADA2 (Callahan et al., 2016), which outputs amplicon sequence variants (ASVs). Sequences with >90% identity to the wheat ITS1 region (NCBI FJ609737.1) were removed for downstream analyses. On average, 20.45% of the reads in plant samples resulted from amplification of plant DNA, with generally higher percentages in sample types with low abundance of fungal DNA. From 153,369 ± 57,870 (mean ± SD) observations per sample, 94,499 ± 55,212 remained after quality filtering and removal of plant-derived sequences. The q2-feature classifier (Bokulich et al., 2018) was used for taxonomic classification of ASVs, using a dynamic classifier that assigned a taxonomy based on 97% or greater sequence identity to the UNITE database (Nilsson et al., 2019) (version released 1.12.2017). ASVs that were not assigned to kingdom level were removed from the dataset. The sequencing data were deposited at the European Nucleotide Archive (EBI-ENA) under the accession number ERP124281.

2.5. Statistical analyses

Data analyses and preparation of figures were performed in R version 3.5.2 (Team, 2018), using the packages ‘limfe’ (Bates et al., 2015), ‘vegan’ (Oksanen et al., 2015) and ‘ggplot2’ (Valero-Mora, 2010).

We made non-metric multidimensional scaling (NMDS) plots to visualise the grouping of the observations based on dissimilarities. NMDS was performed based on Jaccard dissimilarity using the ‘vegdist’ function from the ‘vegan’ package in R (Oksanen et al., 2015), as a measure of the dissimilarity of all observation based on their ASV composition. We also tested Bray-Curtis dissimilarity, but did not achieve convergence. The NMDS plots were made with three dimensions, on the data with omitted observations of less than 1000 ASV reads (10 observations).
Furthermore, we only examined the ASVs, which had a total count over all observations of more than 0.1% of the total ASV count.

Diversity was measured by the Shannon index using the ‘vegan’ package in R (Oksanen et al., 2015). Shannon and NMDS scores were analysed with a linear mixed model with fixed effects: location + cultivar + tissue, and interactions: (location × cultivar × tissue or location × cultivar + tissue × cultivar and tissue × location) and random effect of pot. All linear mixed models were fitted with the lme4 package (Bates et al., 2015).

The ‘adonis’ function from the ‘vegan’ R package (Oksanen et al., 2015) was used to obtain an impression of how much of the variance in the data set was described by each covariate. We plotted the $R^2$ value, which was calculated by normalising the sequential sum of squares. These were found by adding one covariate at a time then calculating the sequential sum of squares each time. The linear model with Shannon index as response and covariates cultivar, location, and tissue was fitted.

The abundance of the most abundant genera was displayed using two kinds of abundance plots: relative and absolute. The relative abundance plots show bars indicating the relative abundance of each genus in the specific observation. The absolute abundance plots show the absolute count of each of the most abundant ASV’s averaged over the four observations for each cultivar and location.

Venn diagrams were made to compare the ASV composition within different environments. Each intersection of a number of areas represent the number of ASVs, which are present in all the intersecting areas.

**Fig. 2.** Tissue, environment and cultivar influence the α- and β-diversity of endophytic fungal communities in wheat. Sample sizes are n = 96 for leaf, roots and seed, respectively, n = 24 for seed stock, n = 24 for air, and n = 4 for soil. (a) Mean Shannon diversity index was higher at experimental site outside (and differed for tissues in the additive linear model). Error bars represent the standard error of the mean. (b) Wheat cultivars (x-axis) had a minor, but significant effect on Shannon diversity. Each point represents the mean of four replicates, hence the standard error of the mean (shaded area) should not be considered too strictly. (c) The factors ‘tissue’, ‘location’ and ‘cultivar’ had a significant effect on the non-metric multidimensional scaling 1 (NMDS1) score ($\text{ANOVA}, F_{22,206} = 2.52, P < 0.00037$), even though the cultivar effect was not detectable in the NMDS. NMDS including all data, coloured by ‘tissue’ (top), ‘location’ (middle), and ‘genotype/cultivar’ (bottom). Distance between observations is based on Jaccard dissimilarity, and included ASVs, which reflected >0.1% of the total number of observations. Ten observations with <1000 occurrences were omitted. (d) The effect size of experimental site was larger in above- than below-ground plant tissue. NMDS of ‘seed’ (top), ‘leaf’ (middle), and ‘root’ (bottom) coloured by environmental site IN (greenhouse) and OUT (field).
3. Results

We cultivated twelve closely related winter wheat cultivars (Arraiano and Brown, 2016) with different levels of resistance to Septoria tritici blotch (STB) (Arraiano et al., 2009) and varying in the presence or absence of the Stb15 gene (Arraiano and Brown, 2006) in two different locations over one generation (Fig. 1a, b, c). The two locations had the same environmental microbiome in soil, air and seed stock, but different abiotic environmental conditions (Fig. 1d–g). Replicated experimental blocks within the locations, sampling of different plant tissues (Fig. 1c), and monitoring of all sources of fungal inoculum allowed us to assess multiple scales of host plant and environmental factors that influenced composition and abundance of the fungal microbiome (Fig. 1h).

We collected 4 bulk soil samples, 24 air samples, and 312 plant samples. Plant and soil samples were taken in four biological replicates. The 24 seed stock samples sequenced contained one biological replicate only (~10 seeds) and were therefore sequenced with two technical replicates. The remaining 288 plant samples represented 192 individual plants (two per pot, one for leaf/root and one for seeds). There were 8 pots for each of the 12 cultivars, so there were 96 leaf, 96 root and 96 seed samples in total. Each pot represented one biological replicate of the cultivar (Fig. S1). The 24 air samples contained only one biological replicate for the 12 dates and 2 location and were therefore sequenced with two technical replicates. The remaining 288 plant samples represented 192 individual plants (~10 seeds) and were therefore sequenced with two technical replicates each. The 4 soil samples were taken from the same batch of soil used as growth medium for all plants, and treated as biological replicates.

We identified 5099 amplicon sequence variants (ASVs) that were assigned to fungi at >97% identity. The phyla represented in the samples were 89.3% Ascomycota, 6.1% Basidiomycota, 0.4% Mortierellomycota, 0.03% Glomeromycota and to a minor extent other phyla. Rarefaction curves indicated that sequencing depth covered the fungal diversity in the samples sufficiently (Fig. S2).

3.1. Plant tissue, location and genotype influence fungal communities

All three tested experimental factors ‘cultivar’, ‘location’, and ‘tissue’ significantly affected α-diversity of fungal communities measured by the Shannon index (Table 1). While the two-way interaction between ‘tissue’ and ‘cultivar’ or ‘location’ were also significant, the ‘cultivar × location’ interaction did not significantly influence community diversity. The sample and plant tissue type displayed the largest effect on Shannon diversity of fungal communities (Fig. 2a, NMDS of all data in three dimensions in Fig. S4). The soil microbiome was most diverse and diversity in plant samples was highest in roots, lower in seeds, whereas leaves harboured the least diverse microbiome. We investigated the impact of the factors ‘tissue’, ‘location’ and ‘cultivar’ on the fungal community with non-metric multidimensional scaling (NMDS). ‘ Cultivar’, ‘tissue’ and ‘location’ all had significant effects on the NMDS1 score, even though the ‘cultivar’ effect was not obvious from the NMDS plot (Fig. 2c). The effect of ‘tissue’ and ‘location’ were clearly visible in the NMDS plot (Fig. 2c, d). Even though the effect of ‘cultivar’ was significant (P = 0.008, Table 1), the relatedness between cultivars, disease resistance to STB and presence of an STB disease resistance gene did not correlate with differences in the fungal microbiome (Fig. S3).

3.2. Seeds, leaves and roots of wheat harbour distinct fungal communities

Using the adonis test, ‘tissue’ (seed, leaf, root) was estimated to account for 30.76% of the total variation in the dataset (Fig. 3). Therefore, from the factors investigated in this study, ‘tissue’ had the largest effect on endophytic fungal communities of wheat. The effect was reflected in both community richness (Fig. 4) and the significant differences in diversity in a linear mixed model (P = 0.0001, Table 1), of Shannon diversity (Fig. 2a). Root samples displayed the highest diversity, with the number of unique ASVs being three times higher than in seed and leaf (Fig. 5). Meanwhile, seed samples from the outside field location contained the largest number of ASVs, as a measure of richness (Fig. 4). The seed stock samples harboured similar diversity, but reduced

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**Table 1** Effect of experimental factors on α-diversity of fungal communities associated with wheat.

<table>
<thead>
<tr>
<th>Factor</th>
<th>Shannon</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cultivar</td>
<td>$\text{Chi}^2_1 = 30.393$</td>
</tr>
<tr>
<td></td>
<td>$P = 0.008$</td>
</tr>
<tr>
<td>Location</td>
<td>$\text{Chi}^2_1 = 58.534$</td>
</tr>
<tr>
<td></td>
<td>$P &lt; 0.0001$</td>
</tr>
<tr>
<td>Tissue</td>
<td>$\text{Chi}^2_1 = 286.138$</td>
</tr>
<tr>
<td></td>
<td>$P &lt; 0.0001$</td>
</tr>
<tr>
<td>Cultivar × location</td>
<td>$\text{Chi}^2_1 = 8.395$</td>
</tr>
<tr>
<td></td>
<td>$P = 1$</td>
</tr>
<tr>
<td>Cultivar × tissue</td>
<td>$\text{Chi}^2_1 = 66.272$</td>
</tr>
<tr>
<td></td>
<td>$P &lt; 0.0001$</td>
</tr>
<tr>
<td>Location × tissue</td>
<td>$\text{Chi}^2_1 = 29.422$</td>
</tr>
<tr>
<td></td>
<td>$P &lt; 0.0001$</td>
</tr>
</tbody>
</table>

Statistics describe linear mixed models of Shannon diversity (Shannon – cultivar + location + tissue + (1 | pot), $P = \Pr(\text{Chi})$ with single term addition or deletions. $P$-values are Bonferroni corrected by multiplication with 6.
richness compared to seed outside samples (Fig. 2a, Fig. 5), potentially due to the storage of the seed stocks for approx. five years before the start of this study. Generally, abundance and diversity of fungal communities was lowest in leaf samples.

A large fraction of the ASVs observed in plant samples was unique to each tissue (Fig. 5d), and only few genera constituted shares of >1% in different tissues (Fig. 4). These included *Fusarium*, *Alternaria*, *Neoscytalon* and *Acremonium* spp.

3.3. Environment has a stronger effect on above-ground than below-ground plant parts

After tissue, environment (‘location’) had the second strongest effect on fungal communities in wheat in NMDS and adonis tests. Mean Shannon diversity index was significantly higher at the experimental site outside in the linear mixed model (P < 0.0001, Table 1). In addition, the number of common ASVs between tissues was larger outside than

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**Fig. 4.** Several genera across cultivars contain cereal pathogens, are tissue-specific and affected by location. Mean abundance of dominant (>1% of total reads) fungal genera in different tissues of 12 wheat cultivars (names abbreviated) at the two locations IN and OUT. Genera with lower abundance (<1% of total reads) are represented in “other”. Air samples over the time period from one location are summarised.

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**Fig. 5.** Different wheat growth locations harbour distinct microbial communities. Venn diagrams with intersections displaying common ASVs in the shared areas. (a–c) Common ASVs of plant tissues (a) seed, (b) leaf and (c) root with the surrounding environment (seed stock, air, and soil). (d) ASVs shared between the tissues. The diagrams sum the data between locations. Diagrams separated by location are displayed in Fig. S5. Presence of fungi in the immediate environment is the major driver of communities in different plant parts. Bar chart of relative number of ASVs that overlap with the environmental communities in air, seed stock and soil. Eighty percent of the ASVs in all tissues were not present in the environment, and not taken into consideration for the bar chart.

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inside in Venn diagrams separated by ‘location’ (Fig. S5), indicating that the conditions outside were more favourable for colonisation by generalist fungi that are not plant tissue specific.

Temperatures outside were generally lower than inside the greenhouse and showed considerably higher fluctuations (Fig. 1d). Whereas both temperature and humidity in the greenhouse were relatively stable and did not fluctuate to a major extent (Fig. 1d, e), plants outside experienced on average 5 °C lower temperatures and considerably larger fluctuations between days. Furthermore, the plants outside were subjected to varying precipitation and wind intensity (Fig. 1f, g) throughout the period of the experiment. Fungi in the air did not differ significantly between the inside and outside location (Figs. 2a, 4).

All plant tissues were affected by the environmental differences between locations, but the magnitude differed between plant tissues. Whereas seed samples displayed a clear separation by location in the NMDS (Fig. 2d), leaf samples from the two locations clustered more closely and the even more so the root samples. The observations from NMDS were also reflected in the Shannon diversity of the interaction between ‘tissue’ and ‘location’ (Fig. 2a), indicating that the differences between locations mainly affected the phyllosphere microbiome, and to a smaller extent the root microbiome. The adonis test for the individual tissues (Fig. 3) confirmed that location was, with an effect size of 47.67%, the major driver of the seed microbiome, whereas the effect on leaf and seed was 12.72% and 2.71%, respectively. The largest share (47.67%), the major driver of the seed microbiome, whereas the effect size on seeds was only 12.67% and the major variation of 48% was explained by ‘location’. Interestingly, the cultivar effect in the adonis test between locations (Fig. 3), was larger inside (12.67%) than outside (5.55%), even though the interaction was found not to be significantly associated with diversity (Table 1). Microbiome abundance and diversity measured by the Shannon index did not correlate with relatedness between cultivars (data not shown), and neither the different levels of resistance to STB in the cultivars (Fig. S3). Presence of the major qualitative resistance gene Stb15 did not have a significant effect either (data not shown).

3.4. Fungi in the immediate environment are the major driver of communities in different plant parts

When comparing the soil and air microbiome with plant tissue samples by NMDS (Fig. 2c), NMDS scores for soil samples were located in the root cluster, while seed and leaf communities clustered closest to the air and the seed stock cluster. This could indicate that airborne inoculum is the major shaping factor of the leaf and seed microbiome, while soilborne fungi provide the inoculum for the root microbiome. Fungal diversity was significantly higher in roots than in seeds and leaves, likely reflecting the extensive microbial diversity in the immediate environment of the soil (Fig. 2a). Only 5% of the ASVs in air were also present in soil, indicating that the two are largely independent sources of microorganisms (Fig. 5).

Eighty percent of the ASVs in leaf, seed, and root, did not overlap with the microbiome of the local environment (soil, air, and seed stock) (Fig. 5a–c). While fungi can proliferate within the host plant tissue and reach the detection limit, individual spores in environmental samples might escape detection. After subtracting the unique observations for tissues, almost 80% of the remaining ASVs of root were shared with soil, but only 15–20% with the seed stock and air (Fig. 5e). On the other hand, 60% of ASVs in leaves and seeds were shared with the air. A large share (50%) of the phyllosphere ASVs overlapped with the seed stock, and this overlap was location-dependent, indicating that the seed stock was not the origin of these ASVs. A fraction of 25% of ASVs in the seed stocks were also present in air or soil, representing fungi that are generally present in the environment and not specific to seeds.

3.5. Host genotype influences leaf and root communities, but has a weaker effect on seed fungal communities

Although the effect of cultivar on the fungal microbiome was not apparent in the visualization of NMDS, ‘cultivar’ had a significant effect on Shannon diversity (P = 0.008, Table 1). According to the adonis test (Fig. 3), cultivar explained 5.92% of the variation in the total dataset, but differed for individual tissues. Whereas cultivar explained approx. 25% of the variation observed in leaves and roots, the effect size on seeds was only 12.67% and the major variation of 48% was explained by ‘location’. Interestingly, the cultivar effect in the adonis test between locations (Fig. 3), was larger inside (12.67%) than outside (5.55%), even though the interaction was found not to be significantly associated with diversity (Table 1). Microbiome abundance and diversity measured by the Shannon index did not correlate with relatedness between cultivars (data not shown), and neither the different levels of resistance to STB in the cultivars (Fig. S3). Presence of the major qualitative resistance gene Stb15 did not have a significant effect either (data not shown).

3.6. Abundance of pathogenic fungi differs between environments

Fungal genera containing pathogens of wheat dominated the fungal microbiome in all plant tissues and both locations, even though only few disease symptoms visibly developed on the plants during the experimental period. We observed symptoms of powdery mildew (Blumeria graminis) and individual pustules of leaf rust (Puccinia recondita) on the foliage, and symptoms of Fusarium infection (Fusarium spp.) on the heads of the plants grown outside. Powdery mildew symptoms were treated with a single application of fungicide at six weeks before sampling, since a heavy infestation would be predicted to modify the fungal communities considerably. Nevertheless, Blumeria was the predominant genus in the leaf microbiome (Fig. 4). Observation of individual spots of leaf rust on leaves outside were consistent with the elevated abundance of Puccinia spp. in those samples (Fig. 4), and chlorotic spots on the lower leaves of the outside plants. Even though the genus Zymoseptoria was highly represented in outside leaves, we did not observe the typical symptoms of STB (Zymoseptoria tritici). However, differences in Zymoseptoria abundance in leaves between locations were reflected in the air (Wilcoxon rank sum test, \( P = 0.040 \)), even though the diversity of total airborne fungal communities between locations did not differ significantly. Similar to leaves, seed microbiomes were dominated by genera containing pathogens or opportunistic pathogens. We observed pinkish fungal growth on the wheat heads from outside (but not inside) as signs of Fusarium infection and Fusarium head blight, and indeed Fusarium was the most abundant genus in those samples. The genera Fusarium, Monographella, Alternaria and Cladosporium were significantly overrepresented outside compared to inside, indicating that the conditions outside were more favourable for these fungi. Meanwhile, endophytic Penicillium and Acremonium spp. were significantly more abundant in seeds inside.

The fungal community of roots was generally more diverse. However, apart from pathogens, genera with reported mutualistic interaction potential were among the most abundant. The main pathogenic genera Fusarium (Fusarium root rot), Gaeumannomyces (take-all), and Microdochium were also responsive to location. The mutualistic endophytic Clonostachys spp. were present primarily in samples from outside, and endophytic Acremonium spp. in samples from inside; this was also observed in seed samples. In general, fungal genera in roots were affected by location to a lesser extent than seed- and leaf-associated ones.

4. Discussion

We used metabarcoding targeting ITS1 to test which factors and interactions contribute to shaping of the fungal microbiome of wheat roots, leaves and harvested grains under partially controlled conditions.
Thus, we analysed the microbiome in different tissues from seeds to plant to seeds and studied how different potential sources of inoculum contributed to shaping of the microbiome. To our knowledge, this is the first microbiome study to consider the effects of airborne microorganisms and climate factors under field conditions as well as the relatedness (pedigree) and presence of disease resistance genes of the cultivars on the microbiome. The British winter wheat cultivars used in this study were originally released in the 1960s to the 1990s, and previously investigated for genetic traits associated with susceptibility and resistance to STB (Arraiano and Brown, 2016, 2006). From these studies, we selected cultivars in pairs of parent and offspring that possessed different levels of STB resistance (Fig. 1b). Genetically, the resistance of the selected cultivars was mainly based on quantitative resistance genes, which provide low-to-moderate resistance, apart from the qualitative resistance gene Stb15 that was present in some cultivars.

The host genotype contributed significantly to shaping the leaf and root communities of fungal endophytes, which has not previously been demonstrated in wheat. However, cultivar effect on microbiome (including epiphytes) has been shown in studies of wheat leaves (Sapkota et al., 2017, 2015) and to some extent in the wheat rhizosphere (Simonin et al., 2020). In leaves, the effect of cultivar has been found to increase with leaf age, whereas the effect of the environment decreased (Sapkota et al., 2017). On the other hand, Comby et al. (2016) found no clear cultivar effect by investigating endophytic communities by isolation (Comby et al., 2016). However, the method was likely the limiting factor, since lower diversity is obtained generally from isolation studies as compared to the metabarcodeing approach and furthermore, only two wheat cultivars were compared. Nevertheless, differences in microbial communities were observed according to host maturity and host organs in this isolation approach (Comby et al., 2016), reflecting previous metabarcoding-based findings (Gdanetz and Trail, 2017; Sapkota et al., 2017) and the different fungal communities we observed between leaves, roots and seeds in this study.

Even though the genotype significantly influenced the endophytic fungal diversity in wheat, the genetic basis of the variation is unclear. Fungal diversity did not correlate with relatedness between genotypes (data not shown), which would be an indicator of certain genetic traits that affect the microbial community. Furthermore, neither the variation in quantitative resistance to STB in the cultivars (Fig. S3), nor the presence of the major qualitative resistance gene Stb15 had a significant effect (data not shown). Potentially, differences in physiological and anatomical traits between the cultivars are important determinants of the endophytic microbial communities. Even though the cultivars in this study were closely related, they displayed large variation in height, leaf size, ear shape, amount of epicuticular wax, and other traits. Physiological leaf attributes have previously been shown to differentiate microbial communities between spruce species, e.g., water content, transpiration rate, stomatal conductance, and nitrogen/phosphorous/potassium content (Li et al., 2018). Furthermore, a number of individual host mutational alterations in Arabidopsis that introduced alterations in surface structure, cell wall and defence signalling and PAMP-triggered immunity, an integral part of the plant’s immune system, were shown to affect composition and abundance of the bacterial community (Bodenhausen et al., 2014; Chen et al., 2020). Interestingly, the mutation affecting pattern-triggered immunity affected only the endophytic community, but not the epiphytic community in leaves of Arabidopsis (Chen et al., 2020), also emphasising differences between fungi and bacteria in interactions with the host plant. This indicates that mapping out physiological differences between cultivars might highlight characteristics for genotypedependent differences in microbial communities. For this purpose, a more extensive set of well-described wheat cultivars is required.

In experiments investigating the influence of the environment on the plant microbiome in form of different physical locations, the environment/location is mostly considered as one individual factor, and not separated into the effects of the abiotic environment/weather (temperature, wind, rain, UV radiation) and the biotic environment (fungi present in the seed, soil and air). However, depending on the genotype, wheat cultivars are likely to respond differently to both abiotic and biotic variables, because of presence of, e.g., resistance genes and physiological properties that influences, e.g., adaptation to climate. With the setup used in this study, we were partly able to separate the effects of the abiotic from biotic environment, since the same biotic factors (fungi present in seed stock and soil) were used indoors and outside and we determined the microbiome contribution of the air. Location-dependant effects on the wheat microbiome have previously been observed (Nicolaisen et al., 2014; Rojas et al., 2020b; Sakpota et al., 2017, 2015), but in these studies, the location effect was a combination of biotic and abiotic factors. As third factor investigated in this study, we found a significant effect of the location difference between field and greenhouse on the fungal communities in wheat. However, this effect was most likely not related to the composition of the fungal communities in the two environment, since the diversity of fungal communities present in the soil and air did not differ significantly between inside and outside (Fig. 4). It was previously found that the fungi present in air indoors are primarily determined by dispersal from the air outdoors (Adams et al., 2013), supporting our observation. The location-dependant differences we observed in plant microbiome composition and abundance therefore likely resulted from differences in the environmental conditions. Higher temperatures, less temperature fluctuations and absence of mechanical stress by wind and rain provided optimal growth conditions for wheat grown in the greenhouse. In the endophytic community, these factors were reflected by lower abundance and diversity of fungi. Furthermore, outdoors, there would be longer periods with high humidity, which encourages the establishment and development of diseases as well as increased interactions with airborne microorganisms. These factors also affect the general defence level of the plants.

Around 60% ASVs in leaves and seeds (excluding unique observations), were shared with the microbiome of the air samples (Fig. 5e), supporting the hypothesis that the majority of the endophytic phyllosphere microbiome is airborne. Even though a large share (50%) of the phyllosphere ASVs overlapped with the seed stocks, it is not possible to conclude that endophytic fungi were seed transmitted. Since the overlap of the microbiome of leaves with seed stock differed between locations, this indicates that those ASVs are probably derived from air or soil and in fact, 25% of ASVs in seed stock were also present in air or soil. Therefore, the common ASVs between phyllosphere and seed stock likely reflect the specialisation of omnipresent fungi to wheat phyllosphere colonisation, rather than vertical transmission, as in systemic colonisation of the plant by seed-borne fungi.

All plant tissues were dominated by relatively few taxonomic groups representing genera with many characterised pathogenic and saprotrophic fungi, and fewer genera with species with described neutral or mutualistic endophytic lifestyle. Some of the major fungal genera observed as endophytic colonists of wheat in this study were Fusarium, Alternaria, Cladosporium, Microdochium, Zymoseptoria and Blumeria and these were also found to be dominating in previous studies of the fungal communities on wheat (Nicolaisen et al., 2014; Rojas et al., 2020b). Furthermore, species belonging to the genera Clonostachys, Penicillium, and Acremonium prevalent in this study and have previously been reported as potential biocontrol agents of wheat diseases such as Fusarium head blight and Septoria tritici blotch (Hue et al., 2009; Latz et al., 2020; Rojas et al., 2020a). Therefore, the presence of these genera is of potential interest for future studies. Surprisingly, one cultivar displayed relatively high abundance of the foliar pathogen Blumeria in the roots, which was likely the result of a contamination during sample processing.

Several other factors are known to influence the wheat microbiome, but were not addressed in this study. These include cultivation methods or management strategies such as different tillage practices (Granroz et al., 2017; Karlsson et al., 2017; Simonin et al., 2020), nitrogen fertilisation, plant developmental stage (Chen et al., 2018; Hertz et al., 2016) and diseases such as Fusarium head blight (Rojas et al., 2020b).
5. Conclusions

Understanding the shaping factors of the plant-associated fungal communities is of great relevance for modern agriculture, in the context of sustainability and increasing productivity. We argue that results from experiments with model plants, controlled environments, and synthetic microbial communities are transferable only to a limited extent to crop plants in an agricultural context, because the latter have gone through a long process of domestication and inevitable coevolution of highly specialised pathogens. In order to gain a holistic understanding of plant genotype effects on the microbiome, we suggest to conduct such studies under both field and controlled environmental conditions simultaneously, to elucidate the complex network of contributing biotic and abiotic factors. Metatranscriptomics can further aid in understanding responses of host and associated microorganisms to one another and to changes in the environment, by revealing subtle changes in gene expression. Furthermore, genome-wide association studies (GWAS) have the potential to link host plant genetic variation to microbiome characteristics (Beilsmith et al., 2019; Horton et al., 2014). GWAS on a group of genetically characterised and inbred wheat genotypes like the MAGIC population (Huang et al., 2012) can potentially unravel the genetic base of wheat genotype-associated differences in the microbiomes. GWAS would not be possible in the current study since only 12 cultivars were included, whereas 12 cultivars in a microbiome study is a quite high number. Understanding the shaping of the endophytic plant microbiome under host genetic control will benefit the efforts of crop plant breeding (Bakker et al., 2012; Berg et al., 2017; Cöpal and Gupta, 2016), but also development of microorganism-based biocontrol agents. However, breeding for a healthy plant microbiome may be a challenge for at least a few diseases since we show that the phyllosphere microbiome is shaped by the air microbiome to a large extent and therefore such breeding strategies needs to be carefully considered. Application of bacterial biocontrol agents on the flower of the parent was shown to shape the microbiome of the progeny seed microbiomes (Mitter et al., 2017) if such bacteria are vertically transmitted. Therefore, knowledge about the natural source of inoculum for the microbiome of different tissues can also spur development of biocontrol agents.

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Credit authorship contribution statement

Meike A.C. Latz: Conceptualization, Methodology, Data curation, Formal analysis, Investigation, Project administration, Visualization, Writing - original draft, Writing - review & editing. Mads Herbert Kern: Formal analysis, Writing - review & editing. Helle Sørensen: Formal analysis, Supervision, Writing - review & editing. David B. Collinge: Conceptualization, Writing - review & editing, Funding acquisition. Birgit Jensen: Conceptualization, Writing - review & editing. James K.M. Brown: Conceptualization, Resources, Writing - review & editing. Anne Mette Madsen: Resources, Writing - review & editing. Hans Jørgen Lyngs Jørgensen: Conceptualization, Investigation, Project administration, Supervision, Writing - review & editing, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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