Assessment of differences in ascomycete communities in the rhizosphere of field-grown wheat and potato

Mareike Viebahn a, Christiaan Veenman a, Karel Wernars b, Leendert C. van Loon a, Eric Smit b, Peter A.H.M. Bakker a,*

a Section of Phytopathology, Faculty of Biology, Utrecht University, P.O. Box 80084, 3508 TB Utrecht, The Netherlands
b National Institute of Public Health and the Environment, Bilthoven, The Netherlands

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Abstract

To assess effects of plant crop species on rhizosphere ascomycete communities in the field, we compared a wheat monoculture and an alternating crop rotation of wheat and potato. Rhizosphere soil samples were taken at different time points during the growing season in four consecutive years (1999–2002). An ascomycete-specific primer pair (ITS5–ITS4A) was used to amplify internal transcribed spacer (ITS) sequences from total DNA extracts from rhizosphere soil. Amplified DNA was analyzed by denaturing gradient gel electrophoresis (DGGE). Individual bands from DGGE gels were sequenced and compared with known sequences from public databases. DGGE gels representing the ascomycete communities of the continuous wheat and the rotation site were compared and related to ascomycetes identified from the field.

The effect of crop rotation exceeded that of the spatial heterogeneity in the field, which was evident after the first year. Significant differences between the ascomycete communities from the rhizospheres of wheat in monoculture and one year after a potato crop were found, indicating a long-term effect of potato. Sequencing of bands excised from the DGGE gels revealed the presence of ascomycetes that are common in agricultural soils.

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1. Introduction

Fungi are a diverse group of microorganisms, playing a fundamental role in terrestrial ecosystems. They are involved in nutrient cycling, transportation of nutrients to plants, plant growth stimulation and antagonism against plant pathogens [1,2]. The majority of plant-associated fungi show a beneficial or neutral interaction with the host plant. Additionally, some fungi are used as biological control agents against plant pathogenic fungi.

On the other hand, they also comprise a large number of plant pathogens [3]. It is estimated that only 5% of all fungal species are known [4] and that only 17% of the known species can be grown in culture [5]. This implies that the diversity of fungi is largely unrecognized. Moreover, cultivating fungi from soil is not without bias, because they can be dormant, occur as spores, as mycelium, or both, and are often closely associated with other organisms [5]. Therefore, in recent studies cultivation-independent molecular approaches have been used to determine the diversity of fungal communities through analysis of ribosomal (r) RNA genes [6–8]. The rRNA genes are well suited for analyzing microbial diversity, because they are present in all known...
organisms, contain conserved as well as variable regions and their sequences are collected in large public databases [9]. While comparison of the small subunit (SSU or 16S) rRNA is a well-accepted tool in bacterial community studies, the SSU or 18S rRNA genes of fungal communities are less informative and therefore less suitable for fungal community studies [10].

Previous research has shown that the internal transcribed spacers (ITS) of the nuclear rRNA genes are better targets for the molecular analysis of fungal communities than the 18S rRNA genes [11,12]. The ITS are variable, non-coding regions located between the 18S and 5.8S subunit (ITS1) and between the 5.8S and 28S subunit (ITS2) of the rRNA [13]. Ribosomal RNA operons in fungi are often found as tandem repeats of up to a hundred copies, which makes it possible to amplify specific fragments even from small environmental samples [14]. Several primers have been designed to target this region. Primers ITS1 to ITS5 cover the two spacer regions and partial sequences of the 18S and 28S rDNA [12]. Recently, Larena et al. [15] developed a primer with enhanced specificity for ascomycetes. With this primer, in combination with ITS1 or ITS5, only the ascomycetes were successfully amplified from a mixture of ascomycetes, basidiomycetes, oomycetes, zygomycetes and mitosporic fungi.

Ascomycetes are the largest group of the true fungi [15]. Most of them are saprophytic and live on dead organic material, which they help decompose. However, ascomycetes also cause plant diseases, varying from powdery mildews to rots, cancers and vascular wilts [3]. In the present study, we compared the rhizosphere microflora in a long-term field trial between a crop rotation of wheat and potato and continuous wheat cultivation. In our first attempt, we tried to analyze the total fungal community. However, polymerase chain reaction (PCR) amplicons of 18S rDNA could not be adequately separated on a denaturing gel. Therefore, we decided to analyze a subgroup. The ascomycete communities were compared by performing an rDNA fingerprint analysis of the ITS region of the rRNA operon by denaturing gradient gel electrophoresis (DGGE) [16,17]. Ascomycetes were chosen because of their preponderance in the soil and because of the availability of specific primers. Finally, some of the predominant ascomycete bands revealed by DGGE were identified by cloning and sequencing.

2. Materials and methods  

2.1. Ascomycete isolates

Ascomycete isolates used to investigate the specificity of DGGE were grown on potato dextrose agar at 25 °C for 3–7 days. Alternaria brassicicola was kindly provided by Dr. J. Ton (Utrecht University, The Netherlands). Gaeumannomyces graminis var. tritici was kindly provided by Dr. J.M. Raaijmakers (Phytopathology, Wageningen Agricultural University, The Netherlands). Genomic DNA of Trichoderma harzianum B28 and Verticillium longisporum was kindly provided by Dr. J. Postma (Plant Research International, Wageningen, The Netherlands). All other isolates were derived from the experimental field and have been identified by Dr. W. Gams (Centraal Bureau voor Schimmelcultures, Utrecht, The Netherlands).

2.2. Rhizosphere soil sampling

Rhizosphere soil was sampled from an experimental field located at the Botanical Gardens of Utrecht University, the Netherlands, as described earlier [18–20]. The soil contains 12% clay, has an organic matter content of 4% and a pH (KCl) of 5, and was a grassland for more than 20 years. From 1999 to 2002 wheat (Triticum aestivum cv. Baldus) was grown continuously in six 1 m 2 plots, while in another six plots wheat was grown in 1999, followed by potato (Solanum tuberosum L. cv. Modesta) in 2000, wheat in 2001, and potato in 2002. Wheat was sown and potatoes were planted in early spring every year, and samples were taken 11, 25, 39 and 54 days later [19,20]. The field was designed in such a way that three replicates of each treatment were located on the left half of the field, the other three replicates on the right half. From each plot, three random samples of plant roots with adhering soil were taken at each time point, and excess soil was removed. The upper 10 cm of the roots (3–5 g), with tightly adhering soil, were mixed with 10 ml sodium phosphate buffer (120 mM, pH 8).

2.3. Total DNA extraction

One gram of gravel was added and samples were vortexed for 30 s. The supernatant was decanted into a new tube. One milliliter of the supernatant was used to extract total DNA with the FastDNA® SPIN Kit for Soil (Bio 101, Biogene, Vista, CA, USA) in combination with a Ribolyser (Hybaid, Ashford, UK) [21]. The extracts were diluted 1:100 with Millipore-filtered distilled water before purification with the Wizzard® DNA Clean-Up System (Promega, Madison, WI, USA), according to the manufacturer’s protocol. DNA from the ascomycete isolates was extracted by using the Fast DNA® Spin Kit (Bio 101) according to the supplier’s manual, and subsequently purified with the Wizzard® DNA Clean-Up System.

2.4. PCR amplification of the ITS region

Polymerase chain reaction on purified DNA extracts was performed with primers specific for ascomycetes, amplifying the ITS1, 5.8S, and ITS2 region of the fungal
ribosomal RNA operon. The forward primer ITS5 (5’GGAAGTAAAAGTGTAACAGG-3’) [12] with the reverse primer ITS4A (5’GGCCGTTACTGG-GGCAATCCCTG-3’) [15] generates amplicons of about 700 bp. ITS4A was extended with a 40-base-pair G+C rich sequence to stabilize the melting behavior of the DNA fragments for DGGE analysis [22]. Primers were synthesized by Eurogentec, Maastricht, The Netherlands. The 50 µl reaction mixture contained 1 µl of appropriately diluted DNA extract, PCR buffer 2 (pH 9.2) containing 2.2 mM MgCl₂ (Roche Diagnostics, Mannheim, Germany), 200 µM each of dATP, dTTP, dGTP and dCTP, 200 nM primers ITS5 and ITS4A, and 1.5 U Expand Long Template enzyme (Roche Diagnostics, Mannheim, Germany). Amplification was carried out in a Hybaid Thermocycler (Thermo Hybaid, Ashford, UK). The initial denaturing step was done at 94 °C for 5 min, followed by 35 cycles of 94 °C for 1 min, 60 °C for 1 min and 72 °C for 3 min. The reaction was terminated at 72 °C for 10 min. To check the size of the amplicons, PCR products were separated on a 1% agarose gel in TAE buffer (0.04 M Tris–acetate, 0.001 M EDTA), run for 40 min at 150 V, and stained with the nucleic acid stain SYBR Green (Molecular Probes Inc., Leiden, The Netherlands) for 30 min. The gels were viewed under a blue light transilluminator (Clare Chemical Research, Dolores, CO, USA), and digitalized using a charged-coupled device (CCD) camera (Syngene, Cambridge, UK).

2.5. DGGE

PCR amplicons of the expected size from rhizosphere samples and ascomycete isolates were analyzed by DGGE. Denaturing gels were prepared with the gradient former Bio-Rad Model 230 (Bio-Rad Laboratories, Veenendaal, The Netherlands) at a speed of 4.5 ml/min as described earlier [23]. The resulting gels consisted of 8% (wt/vol) polyacrylamide (0.5 × TAE buffer, 37.5:1 ratio of acrylamide/bisacrylamide, 20 × 20 cm) and had a gradient of 20–50% denaturant. A 100% denaturing acrylamide gel contained 7 M urea and 40% formamide. Up to 25 µl of PCR product per lane was loaded and gels were run for 17 h at 80 V at a constant temperature of 60 °C in a DCode™ Universal Mutation Detection System (Bio-Rad Laboratories, Veenendaal, The Netherlands). For comparison of DNA patterns a reference marker was added in triplicate to each gel. After electrophoresis, gels were stained and digitalized as described above for the agarose gels. Gels were analyzed with the BioNumerics program version 3.5 (Applied Maths, Sint-Martens-Latem, Belgium). After normalizing the gel and background subtraction, Pearson’s correlation coefficient was calculated for each lane, followed by the UPGMA cluster analysis (unweighted pair group method using arithmetic averages). Dendrograms were generated from samples at a single time point, or combined from samples taken at three to five time points (composite dendrograms).

2.6. Cloning and sequencing of DGGE bands

Twelve dominant bands from gels loaded with samples taken 11 days after sowing or planting were excised from DGGE gels and eluted in 20 µl Millipore-filtered distilled water. One microliter was used for re-amplification with the primers mentioned above, using the following conditions: one cycle of 94 °C for 5 min, followed by 35 cycles of 94 °C for 1 min, 63 °C for 1 min and 72 °C for 3 min. The reaction was terminated at 72 °C for 10 min. After re-amplification the purity of the excised DNA fragments was checked in a final DGGE under the conditions described above. Appropriate PCR products were ligated into the pGem®-T Easy Vector (Promega, Madison, WI, USA) and transformed into Escherichia coli Ultra Competent Cells (Stratagene, Cambridge, UK) as described by the manufacturers. Clones were directly resuspended in the PCR mixture and amplified with primer pair ITS5/ITS4A. The amplified DNA fragments were identified by DGGE as described. Only clones with a migration comparable to the excised band were used for further sequence analysis. Prior to sequencing, the PCR products were purified with the QIAquick PCR Purification Kit (Qiagen, The Netherlands), according to the manufacturer’s protocol. The sequencing reaction was carried out with the BigDye® Terminator v.3.1 Cycle Sequencing Kit in an AB3700 Sequencer (Applied Biosystems, Nieuwerkerk, The Netherlands). Two clones per band were sequenced in both directions with primers ITS5 and ITS4A, respectively. Sequences obtained were checked for chimeras using the program CHIMERA_CHECK version 2.7 [24]. Sequences that most likely did not contain chimeric regions were compared with known ascomycete sequences of relevant databases with the Standard nucleotide Blast program [25], provided by the National Center for Biological Information (NCBI, USA). Sequences of the clones were submitted to GenBank with Accession Nos. AY615877–AY615884.

2.7. Comparing DGGE gels with a defined marker

Sequencing of the twelve excised DGGE bands revealed matches for ten bands with ascomycete sequences from relevant databases, and these bands were used as a reference marker. DGGE was performed with rhizosphere samples taken 11, 25, 39 and 54 days after sowing wheat in the experimental field in 2001. Prior to PCR, samples from three replicate plots were pooled, yielding two replicates per treatment, and samples from six continuous wheat plots were compared with samples from six wheat–potato–wheat rotation plots. DNA was extracted and DGGE was performed as described above.
3. Results

3.1. DGGE analysis of ascomycete isolates and predominant ascomycete communities in the wheat and potato rhizospheres

To evaluate the specificity of DGGE, DNA extracted from different ascomycete isolates were analyzed. Fig. 1 shows the separation of the ITS amplicons of eight different ascomycetes. Most isolates showed a single discrete band. However, the bands in lanes 2, 8 and 9 are fuzzy.

Total DNA from rhizosphere samples of six wheat plots and six rotation plots was extracted and analyzed by DGGE. Samples of the three plots from each half were pooled for analysis, resulting in two replicates per treatment. Fig. 2 shows the results from the cluster analysis of the four consecutive years (1999–2002). In 1999, wheat was sown in both the control and the rotation plots. Samples from the left half of the field (marked a) clustered apart from samples of the right half (b) at a similarity index of only 30% (Fig. 2A). The similarity of both samples within each cluster was 40% and 52%, respectively. In 2000, with potato planted in the rotation plots, there is a clear differentiation of the ascomycete communities in the rhizospheres of wheat and potato (Fig. 2B). All samples from the potato rhizosphere clustered separate from those of wheat. The 2001 samples clustered in the same way as in 2000, even though now wheat was sown again in the rotation plots (Fig. 2C). The similarity between the two clusters was only 2%. In 2002, the dendrogram from the wheat and potato rhizosphere samples was again comparable, with only about 2% similarity between the potato and the wheat clusters (Fig. 2D).

The pronounced differences in the ascomycete communities of the rhizospheres of wheat and potato led us to a more thorough investigation of samples taken at 11 and 25 days after sowing in 2002. Fig. 3A and B shows...
the dendrograms based on the DGGE patterns of all six replicate samples of the rhizosphere of wheat and potato. After 11 days, three main clusters were defined at a cut-off value of 50% similarity. One cluster contained only the rhizosphere samples from wheat (Fig. 3 A). Five of the six replicates from the wheat rhizosphere were at least 65% similar. The remaining two clusters contained only the samples from the potato rhizosphere. The three replicates from the left half of the field (potato 1, 2, 3) formed one cluster and were at least 63% similar. The three replicates from the right half (potato 4, 5, 6) formed the other cluster and differed by no more than 15%.

Analysis of samples taken at a later time point revealed a largely comparable clustering (Fig. 3 B). Again, overall similarity was less than 10%. At a cut-off value of 50%, five clusters were distinguished. One cluster contained only one replicate from wheat (Fig. 3 A). Five of the six replicates from the wheat rhizosphere were at least 65% similar. The remaining two clusters contained only the samples from the potato rhizosphere. The three replicates from the left half of the field (potato 1, 2, 3) formed one cluster and were at least 63% similar. The three replicates from the right half (potato 4, 5, 6) formed the other cluster and differed by no more than 15%.

Furthermore, DGGE gels profiling the ascomycete communities revealed only a small number of bands per treatment, varying between four and twelve.

3.2. Sequence analysis of ITS fragments recovered from DGGE gels

To further characterize the differences in the ascomycete communities of the wheat and potato rhizospheres, twelve differentially migrating DGGE bands were excised (Fig. 4), re-amplified and cloned into a vector. At least four clones per band were subjected to DGGE under the same standardized conditions, and their migration compared to that of the excised band. Two clones per band with exactly the same position in the DGGE gel were used for further sequence analysis.
Two of the twelve bands were not successfully recovered and excluded from further analysis. Table 1 shows the best match of the ten sequences of the recovered bands with known ascomycete sequences. Two sequences (bands 9 and 11) did not match any of the known sequences and, therefore, could not be identified. The other sequences all represent ascomycetes that are common in agricultural soils [26]. Six bands showed high similarities (at least 97%) with the genera Issatchenka, Plectosphaerella, Podospora and Verticillium. Two bands showed a modest similarity of 95% and 93% to Chaetomium sp. and Ericoid mycorrhiza, respectively. In two cases, two bands with different melting behavior and thus different sequences resembled the same species. Verticillium nigrescens sequences 1 and 2 differed by 1% of their sequence, Plectosphaerella cucumerina sequences 1 and 2 by 3%.

### 3.3. Ascomycete communities of the wheat rhizosphere after monoculture and rotation

Ascomycete communities from the rhizospheres of wheat from the preceding three-year monoculture and from the wheat–potato–wheat rotation were compared to the ten ascomycetes identified from the 2002 samples (Table 1). Given the fact that the samples were derived from the same field, there is a high probability that corresponding bands represent the same species. Based on this assumption, Table 2 shows the different ascomycetes from the continuous wheat and the wheat–potato–wheat rotation plots in the season of 2001 that were identified according to their position in the DGGE gel in comparison with the characterized bands. Two species, Chaetomium sp. and Issatchenka orientalis, were found only in the rotation plots. V. nigrescens sequence 2 and one

<table>
<thead>
<tr>
<th>Band No.</th>
<th>Ascomycetes</th>
<th>Days after sowing</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>cw</td>
</tr>
<tr>
<td>6</td>
<td>p</td>
<td>I. Orientalis</td>
</tr>
<tr>
<td>10</td>
<td>p</td>
<td>Chaetomium sp.</td>
</tr>
<tr>
<td>5</td>
<td>p</td>
<td>P. cucumerina seq. 1</td>
</tr>
<tr>
<td>11</td>
<td>p</td>
<td>unidentified</td>
</tr>
<tr>
<td>7</td>
<td>p</td>
<td>P. cucumerina seq. 2</td>
</tr>
<tr>
<td>2</td>
<td>w</td>
<td>V. nigrescens seq. 1</td>
</tr>
<tr>
<td>12</td>
<td>w</td>
<td>Ericoid mycorrhiza</td>
</tr>
<tr>
<td>3</td>
<td>w</td>
<td>Podospora sp.</td>
</tr>
<tr>
<td>4</td>
<td>w</td>
<td>V. nigrescens seq. 2</td>
</tr>
<tr>
<td>9</td>
<td>w</td>
<td>unidentified</td>
</tr>
</tbody>
</table>

Samples were taken at 11, 25, 39 and 54 days after sowing during the season of 2001 from continuous wheat plots (cw) and from rotation plots of wheat–potato–wheat (wpw) cultivation. w and p indicate that samples, from which the excised DGGE bands were recovered, originated from the rhizosphere of wheat (w) or potato (p) in 2002.
unidentified species did not occur at all prior to 2002. All other species were detected in both treatments. Within the season some changes became apparent. In the continuous wheat plots, *P. cucumerina* sequence 1 was detected only at 11 days, one of the unidentified species (band 9) at the first two sampling dates, and the Eroid mycorrhiza at 11, 39 and 54 days after sowing. In the rotation plots, *Podospora* occurred at the first sampling date only, the Eroid mycorrhiza at the last two sampling dates and *P. cucumerina* sequence 2 and *V. nigrescens* sequence 1 intermittently at 11, 39 and 54 days after sowing.

4. Discussion

Currently, various genetic fingerprint techniques are used in microbial ecology. They have provided patterns of the microbial community diversity in different habitats, but it is not clear which technique is yielding better resolution or sensitivity. Torsvik et al. [27] analyzed microbial diversity by comparing different fingerprinting methods with DNA melting profiles and reassocation kinetic studies. They found that ARDRA (amplified rDNA restriction analysis) must be considered too sensitive for a reliable characterization of complex microbial communities, which contain too much information to be reliably assessed, and that fingerprinting methods such as DGGE are better suited for that purpose. Liesack and Dunfield [28], on the other hand, argued that DGGE is often too sensitive for the determination of total community fingerprints.

Previously, it has been demonstrated that crop species have a pronounced effect on the composition of the microbial communities [29–32]. In a recent study, using ARDRA, we could not detect that a crop rotation from wheat to potato influenced the predominant bacterial and fungal communities [20]. However, by re-examining the samples using DGGE for comparing genetic fingerprints of PCR-amplified ITS regions of ascomycete communities, distinct clusters were evident: one comprising all samples from the potato rhizosphere and one containing all wheat rhizosphere samples. Ascomycete communities were analyzed since the separation of fungal 18S rDNA amplicons on a denaturing gradient did not lead to proper gels. It demonstrates that the two plant species did have an effect on the composition of the rhizosphere communities. It also illustrates that the use of different molecular techniques can lead to different conclusions.

In the present study, using DGGE, we have shown that in the first year of wheat cultivation the ascomycete communities differed according to their location in the field. Apparently, the soil and its environment are not homogeneous, which caused a differentiation between ascomycete communities from the rhizosphere of the same plant species, positioned in the left or the right half of the field. Crop rotation from wheat to potato in the second year led to a marked difference between the ascomycete communities of the two plant species. This effect was still evident after cultivating wheat again in the rotation plots, suggesting that a single change of wheat to potato has a long-term impact on the microflora. Thus, the effect of crop rotation clearly exceeded that of spatial heterogeneity in the field.

In addition, DGGE was performed on six non-pooled replicate rhizosphere samples from wheat and potato taken 11 and 25 days after sowing in 2002. Results from both DGGE analyses were consistent and in agreement with the earlier results. Although DGGE can be used successfully to assess the diversity of fungal communities [7,33–35], the method provides no information about the species composition. This information can be obtained by sequencing individual bands of the DGGE gels and comparison with known sequences from the public databases. Sequencing of DGGE bands revealed only members of the phylum Ascomycota, confirming the specificity of the primers. Ten of the twelve excised bands showed unambiguous results and were included in the analysis. Two of the ten bands could not be identified. One likely explanation is that the number of sequences of the ITS region in the public databases is limited. Two clearly distinct bands in the DGGE gel resembled *V. nigrescens*, whereas two other bands resembled *P. cucumerina*. The sequences differed by 1% and 3%, respectively. This indicates that the described species can be heterogeneous or that the names deposited in the databases are inconsistent.

All identified ascomycetes are common in agricultural soils. Three of these ascomycetes are reported to be potentially pathogenic to plants. *Chaetomium* species are often found as decomposers of herbaceous and lignified plant material and have been recognized as the causal agent of soft rot in wood [26]. *P. cucumerina* has been almost exclusively associated with arable soil and has been isolated from the rhizosphere of flax, grass, potato, sugar beet and wheat [26]. It can produce necrotic spots on potato stalks and cause wilting in beets [26]. *V. nigrescens* is a common soil fungus in arable and non-cultivable soil. It has been found to attack spearmint and peppermint [26].

Using DGGE, we compared patterns of the ascomycete communities of the continuous wheat and the rotation sampling sites with ascomycete reference markers resembling species identified from the same sites, but in different years. Bands that correspond to bands of the markers were assumed to be the ascomycete represented by the marker band. We are aware that this is a very simplified method to assess differences in the microbial communities. However, it seems to be justified, since all material was sampled from the same site, and only
clones with unambiguous positions in the gel before and after cloning and with high sequence identity were used as marker bands.

Two species, *I. orientalis* and *Chaetomium* sp., were detected only in the rhizosphere of wheat after crop rotation, but not in the rhizosphere of wheat monoculture. The other ascomycetes occurred in both sampling sites at one or more time points. *V. nigrescens* sequence 2 and an unidentified strain did not occur in 2001, but were found in 2002. Comparable investigations support the assumption that crop rotation practices in agriculture influence the composition of the microbial communities. Olsson and Alström [32] analyzed the fatty acid profiles of rhizobacterial populations on barley roots from monoculture and from an 8-year crop rotation (fallow, winter rape, winter wheat, peas, spring barley, ley, ley and oat). The fatty acid profiles of the microbial populations from the monocropping were significantly different from that of the rotation treatment. Oehl et al. [31] investigated the impact of land use on the diversity of arbuscular mycorrhizal fungi (AMF). The highest AMF species number and diversity was found in the low input, organically managed arable land under crop rotation. They identified 18 AMF species from land under crop rotation, and 8–13 AMF species from land under maize monocropping using trap cultures. It has also been shown that plants have a selective influence on bacterial populations [36,37]. van Elsas et al. [38] used DGGE analysis to show a clear effect on the bacterial and fungal microflora of arable land under oat–maize rotation, maize monoculture, and permanent grassland. The microbial diversity was higher in arable land under crop rotation than under monoculture, and higher in grassland than in arable land.

We report differences in diversity of ascomycete communities in wheat monoculture and in a wheat–potato rotation system in a long-term field trial. This information will aid in assessing the relative importance of possible changes of microbial rhizosphere communities induced by perturbations of ecosystems.

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