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Title: Shifts in rhizosphere fungal community during secondary succession following abandonment from agriculture

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Abstract

Activities of rhizosphere microbes are key to the functioning of terrestrial ecosystems. It is commonly believed that bacteria are the major consumers of root exudates and that the role of fungi is thought to be limited to that of mycorrhizae and pathogens. In order to test the hypothesis that the role of saprotrophic fungi in rhizosphere processes increases with increased time after abandonment from agriculture, we determined the composition of fungi that are active in the rhizosphere along a chronosequence of ex-arable fields in The Netherlands. Intact soil cores were collected from nine fields that represent three stages of land abandonment and pulse labelled with $^{13}CO_2$. The fungal contribution to metabolizing plant-derived carbon was evaluated using phospholipid analysis combined with stable isotope probing (PLFA-SIP), whereas fungal diversity was analysed using DNA-SIP combined with 454-sequencing. In long-term abandoned fields most of the root derived ^{13}C was found in fungal biomass while in recently abandoned fields most of the root derived ^{13}C was taken up by bacteria. Furthermore, we observed a shift along the chronosequence at the functional level from fast growing stress tolerant to slower growing competitive fungi and from pathogenic to beneficial fungi and consequently a change in network configuration.

Introduction

Studying chronosequences has increased our understanding of plant community succession, which is an important theme in ecology (Tilman, 1988). Most chronosequence studies have focused on plants, but they also offer great opportunities to study soil microbial community responses to primary or secondary vegetation succession and coinciding changes in soil characteristics (Kuramae et al., 2010; Hell et al., 2013; Dini-Andreote et al., 2014). One such a series of sites in the Netherlands represent a secondary succession chronosequence of ex-agricultural fields abandoned at different times. All sites are located in the Veluwe, a region in the centre of the Netherlands and have the same soil type. Since the Middle Ages species-rich, semi-natural grasslands, heathlands and even drifts sands have developed as a consequence of agricultural practices on formerly glacial sand deposits in several parts of the Netherlands. After the introduction of artificial fertilizers in the beginning of the former century, and through intensification of agricultural practice, land use change and increased levels of atmospheric nitrogen deposition, the distribution of species-rich grasslands and heathlands declined dramatically. In order to restore the semi-natural ecosystems, agricultural soils have been taken out of production at different times. This chronosequence in this study (7-30 years of land abandonment) has served as a model system to study the relationship between plant secondary succession and soil abiotic and biotic factors (Kardol et al., 2006; Van der Wal et al., 2006; Holtkamp et al., 2008; van de Voorde et al., 2011).

It is acknowledged that soil microbes are strongly involved in plant community succession (Bardgett and van der Putten, 2014). Microbes control soil carbon and nutrient cycling affecting plant community indirectly (Wardle et al., 2004) but can also have direct effects on plants via mutualistic and pathogenic interactions (Buee et al., 2010). Fungi are assumed to be more important in natural ecosystems than in intensively managed systems that are largely dominated by bacteria (de Boer et al. 2005; de Vries et al. 2006). Thus, systems in transition such as this chronosequence of abandoned arable fields can give

valuable information on the shifts in microbial communities and consequently on their contribution to soil ecosystem function.

It was initially assumed that saprotrophic fungal biomass would increase over time of secondary succession due to changes in substrate quality, and at the same time associations between (arbuscular) mycorrhizal fungi and plants would become more important due to depletion of easily available nutrients (Holtkamp et al. 2008) contributing to the observed changes in plant species composition and cover (Kardol et al., 2006). However, when the fungal biomass and fungal:bacterial biomass ratio were investigated an initial increase during the first 2 years after abandonment was seen without any further effect on fungal abundance or fungal:bacterial biomass ratio for the next 30 years (Van der Wal et al., 2006). Yet, an increase in carbon and nitrogen mineralization rates was observed (Holtkamp et al., 2011; Tardy et al. 2015) indicating an increase in activity of microbial decomposers which, however, did not appear to have a positive effect on plant growth nor change the community (Holtkamp et al. 2011). When the identities of the archaeal, bacterial and fungal communities along this chronosequence were assessed, the microbial communities appeared to differ between soils abandoned for less than 10 years and for over 25 years (Thomson et al., 2015). However, this only partly explained changes in mineralization rates and could not be related to the changes in plant community.

In the current study, we want to relate the secondary succession to the active fungal community in the rhizosphere using $^{13}CO_2$ pulse labeling of plants and subsequent isotope analysis of both fungal lipids and DNA in intact soil cores collected along the Veluwe chronosequence. We focused on the active rhizosphere fungi regulated mainly by the plant community (Strickland et al. 2016) and used the pulse labeling approach to separate the fungi that are actively assimilating recently photosynthesized, plant derived carbon from the inactive fungi or fungi fulfilling other ecological roles. Our main interest was in saprotrophs using root exudates and thus priming SOM decomposition and affecting mineralization rates,

84 plant pathogens and AM fungi. The ITS-region of fungi was sequenced using 454-sequencing and the fungi
85 were divided into functional trait-based categories based on knowledge of the ecology of species
86 (Tedersoo et al., 2014, Nguyen et al., 2016).

87 The research questions of the current study were

88 (i) how does the diversity, functional composition and species interactions of the active
89 rhizosphere fungal community change during secondary succession
90 (ii) what are the main (biotic and abiotic) factors driving these changes.

Commented [HE1]: Consequences as well?

91

Material and methods:

Experimental set-up and sampling

In July 2012 intact soil cores were collected from 9 grassland sites that were taken out of agricultural production 7-30 years prior to sampling. All fields are located in the south Veluwe region, in the centre of the Netherlands (Suppl. Table 1). In three separate sampling locations per field 4 soil cores (12 cm diameter, at least 20 cm deep) were collected with native vegetation growing on it. We selected sites that were dominated by grasses, *Agrostis capillaris*, *Holcus lanatus* and *Plantago lanceolata* to decrease variation due to differences in plant cover. More details on the sampling and sample handling are presented in Morrien et al. (2017) and in supplementary material and methods.

Nine cores per field site were labeled with 99.99 atom-% $^{13}CO_2$ (Cambridge Isotope Laboratories, Andover, MA, USA) in an artificially lit air-tight growth chamber for a total of 8 hours. Three other cores per field site were placed in a similar chamber and kept under identical conditions but with a $^{12}CO_2$ atmosphere, representing the control treatment. Samples were collected 24h, and 7 and 14 days after the start of labeling. Samples from rhizosphere were collected from the upper, root filled part of the cores by brushing the roots. More details on ^{13}C labeling and sampling are presented in supplementary material and methods. Soil chemistry and analysis of soil biota was analyzed and data are given by Morrien et al. (2017). Here these data are used for correlation analysis.

PLFA and NLFA analysis

PLFAs were extracted from freeze dried soil and concentrations and $\delta^{13}C$ values were measured on a Finnigan Delta-S gas chromatograph - isotope ratio mass spectrometer (GC-IRMS) as described in (Boschker, 2004). For more details see Suppl. Material and Methods.

115

116 *Molecular and bioinformatic analysis*

117 DNA was extracted from rhizosphere soil using MoBIO PowerSoil Kit according to manufacturer's
118 instructions. The quantity of DNA was inspected using Quant-iT™ PicoGreen® dsDNA Assay Kit (Thermo
119 Life sciences) using λ-DNA as standard. ^{13}C-enriched ('heavy') DNA was separated from non-labeled ('light')
120 DNA by density-gradient centrifugation and analysed as described in Neufeld et al. (2007). Fungal PCR was
121 performed for combined 'heavy' and 'light' fractions using primers ITS4 and ITS9 (Ihrmark et al. 2012) and
122 subsequent 454-sequencing was performed. Resulted sequences were submitted to ENA under project
123 number PRJEB15250. For more details on molecular and bioinformatics analysis see Suppl. Material and
124 Methods.

125

126 The fungi were divided into functional groups when possible. This approach is similar to the one taken
127 earlier by Tedersoo et al. (2015) and refined by Nguyen et al. (2016). The functional groups used in this
128 study were: AMF, ericoid mycorrhizae, coprotrophic fungi, endophytes and potential plant pathogens,
129 molds, nematophagous fungi, saprotrophic fungi, wood parasites and - decomposers, yeasts,
130 entomopathogens, animal pathogens, lichens, ectomycorrhizae and mycoparasites. The groups not
131 expected to have functions in rhizosphere that also had little reads/OTUs assigned to them (incl.
132 entomopathogens, animal pathogens, lichens, ectomycorrhizae and mycoparasites) were later grouped
133 into a group 'other'. The types that could not be identified into functional groups were categorized as
134 unclassified Chytridiomycota, unclassified Mucoromycota, unclassified Ascomycota, unclassified
135 Basidiomycota and unclassified fungi. Species could belong to multiple functional groups, especially yeasts
136 and molds (lifestyles) were also members of other groups (i.e. saprotrophs and pathogens). Both data on

OTUs presence-absence and OTUs percentage of total reads were used in the analysis. For each analysis, the data used is highlighted.

The relative labeling of each OTU was determined as the ratio of the abundance of that OTU in the heavy ^{13}C fraction to that of the abundance in the light ^{12}C fraction

Statistical analysis

The difference in the amount of reads per sample was standardized by using % of total OTUs in a sample as a measure for the relative abundance of each OTU (McMurdie & Holmes 2014). Absolute values were used for diversity and rarefaction calculations. The effect of treatment (recent, mid- and long-term abandonment) on fungal community composition at the level of phylum, class, order and OTU was estimated using ANOVA combined with Tukey's pairwise comparisons as the post-hoc test. When the distribution of data was not in accordance with assumptions of ANOVA (i.e. due to complete absence of a group in one or more treatments) Kruskal-Wallis test was used in combination with Mann-Whitney as a post-hoc test. The effect of abandonment time was evaluated using Pearson's correlation coefficient.

The OTUs explaining most differences between treatments were analyzed using SIMPER in program PAST (Hammer et al., 2001) and their statistical significance confirmed with ANOVA. Cluster analysis (using WARD linkages) was performed to analyze the similarity between ' heavy' and ' light' fractions from the same sample. NMDS using both presence-absence data and the % data was applied to visualize the effects of time since labeling, the fraction (i.e. presence in 'heavy' or 'light' fraction) and the agricultural abandonment time on community composition. Differences in beta-diversity between abandonment categories were evaluated using ANOSIM with Jaccard as a distance measure in PAST.

In order to assess α-diversity both Simpson and Shannon-H indexes were calculated using the non-transformed raw data and resulting values were subjected to ANOVA to compare treatment effects.

159 Rarefaction curves of the observed richness were calculated in PAST using 1000-fold resampling without
160 replacement.

161 Variance partitioning was performed in CANOCO (ter Braak and Smilauer, 1998) using both forward
162 selection of the variables and canonical correspondence analysis (CCA) with data derived from Morrien et
163 al. (2016).

164 In order to explore which species are most connected to others in different abandonment categories, co-
165 occurrence networks were used (Barberan et al. 2012). Data on only labeled (i.e. 'heavy' fractions) OTUs
166 were used and time points were combined to get a better view on the system making the total number of
167 samples used for correlations 3 (replicates) x 3 (time points sampled) x 3 (soils in the land abandonment
168 category) = 27 . Only species present in more than 3 samples per abandonment category and with relative
169 abundance of over >1 % were used in analysis. Significant correlations (Pearson's rho >0.5, $p<0.05$,
170 adjusted using the false discovery rate method in R) were used to plot the networks in Cytoscape (Shannon
171 et al., 2003).

172 Data from labeling status of fungal feeding soil biota was taken from another data set (Morrien et al. 2016)
173 and correlated with the fungal data in R using Pearson's correlation coefficient.

174

175 **Results:**

176 The total number of good quality reads was 1.8 10^6 which were divided into 3562 OTUs. The average
177 number of reads per sample was 10 123 and the average number of OTUs per sample was 412. There were
178 no significant differences in total number of reads or numbers of identified OTUs between heavy and light
179 fractions, time points (incubation) or time of abandonment. The rarefaction curves of all samples are
180 presented in Supplementary Figure 1.

Of the total raw reads, 80.5 % was certainly identified using ITSx (Bengtsson-Palme et al., 2013) to be ITS sequences from Fungi. The remaining reads were categorized mainly as plant derived (14 %) and *Alveolates* (superphylum of protists) (4 %). These sequences were removed from the dataset and are not presented nor discussed here. Approximately 10 % of the reads (and 20 % of the OTUs) assigned as fungi could not be assigned to finer taxonomic level than 'unknown fungi'. The fungal communities across the samples in these abandoned ex-arable ecosystems consisted predominantly of Sordariomycetes (making up 25.1 % of all reads and 19.5 % of all OTUs) and of Dothideomycetes (21.7 % and 12.0 %, respectively). According to the functional classification, the majority of reads (70.1 %) and OTUs (52.7 %) were classified as 'saprotrophic'.

There were 18 OTUs with average abundance of >1 % across the samples and fractions, and 141 OTUs with an average abundance of >0.1%. These 141 OTUs are referred to as the dominant OTUs in the dataset. The most commonly found OTU with an average abundance of 7.12 % was a *Pleosporales* sp. (OTU 5302), which was also present in all samples.

Fungal contribution to the carbon flow

Neither the amount of fungi as based on the PLFA signature of 18:2ω6c nor the fungi to bacteria ratio (F:B ratio) changed during the secondary succession following land abandonment ($F_{2,24}$=1.19, p=0.32). However, when the excess ^{13}C in fungal and bacterial PLFAs (calculated compared to PLFAs in ^{12}C labeled control samples) was used to calculate the 'active F:B ratio', we observed that fungi were receiving relatively more carbon from the plants in the long-term abandoned fields than in recent and mid-term abandoned sites (Fig. 1). Furthermore, the 'active F:B ratio' was positively correlated with time since abandonment from agriculture (R^2=0.48, p<0.05).

203

204 *Active vs. total fungi in the rhizosphere*

205 Heavy and light fractions (i.e. fungal communities enriched in ^{13}C and ^{12}C) were similar to each other in the
206 general composition of the fungi when all samples were included in the analysis (data not shown). ANOSIM
207 analysis confirmed that the fraction 'heavy' or 'light' was not a significant factor affecting the fungal
208 community structure ($R<0.20$, $p>0.20$). Furthermore, the Ward linkage analysis revealed that in 10 of the
209 18 soil samples tested at the first time point, heavy and light fractions from the same sample clustered
210 together. However, the light fractions were significantly more diverse (measured by Shannon-H index)
211 than the heavy fractions when all the samples were combined ($t=2.6$, $p<0.05$), which was mainly explained
212 by observed differences between the fractions at the first sampling point 24h after labeling (Suppl. Fig. 2).

213

214 *Active rhizosphere communities in soils and between treatments*

215 Land abandonment time had no significant effect on the α-diversities of the fungi in the heavy fraction at
216 any time point after labeling (ANOVA: $F=0.74$, $p= 0.48$) (Suppl. Fig. 3). A significant effect of the
217 abandonment time on fungal community structure in the 'heavy' fraction was found in samples that were
218 taken 1 day after labeling (ANOSIM: $R=0.42$, $p=0.024$, Suppl. Fig. 4). No effect of time since abandonment
219 was detected for the two other sampling time points or in the 'light' fractions. For this reason, a more
220 detailed analysis was made for fungi assimilating recently photosynthesized carbon (i.e in the ^{13}C labeled
221 'heavy' fraction in the first time point).

222 At the level of classes, using the proportional data, some significant directional changes in the composition
223 of fungi using recently photosynthesized carbon were detected despite the large variation between soils
224 (Fig. 2). The classes most affected by the time since land abandonment were: dothideomycetes ($F_{2,27}$13.2,

Commented [HE2]: Make a supplementary figure?

225 $p<0.01$, $R^2=0.46$, $p<0.05$) and agaricomycetes ($F_{2,27}=4.5$, $p=0.06$, $R^2=0.40$, $p=0.07$) which decreased in
226 abundance with increasing abandonment time, leotiomycetes ($F_{2,27}=5.4$, $p<0.05$) which showed the
227 highest abundance in mid-term abandoned fields and lecaronomycetes ($H=7.6$, $p=0.06$) which were
228 completely absent in mid- and long-term abandoned fields (Suppl. Fig. 5). When looking at a more detailed
229 level, the orders affected by the treatment were Capnodiales ($F_{2,27}=12.5$, $p<0.01$), Pleosporales ($F_{2,27}=12.0$,
230 $p<0.01$) and unclassified Dothideomycetes ($F_{2,27}=5.5$, $p<0.05$).

231 When OTUs were divided into functional categories, significant effects of the time since land abandonment
232 were detected: 'potential plant pathogens and endophytes' and 'yeasts' were proportionally more
233 abundant in recently and mid-term abandoned fields than in long-term abandoned fields ($F_{2,18}=5.5$, $p<0.05$
234 and $F_{2,18}=11.88$, $p<0.05$, respectively) and also decreased in proportion as a function of time since
235 abandonment ($R^2 = 0.52$ and $R^2 = 0.48$, for both $p<0.05$) (Fig. 3). Saprotrophic fungi were most abundant
236 in the mid-term abandoned fields ($F_{2,18}=11.60$, $p<0.01$) and the proportion of 'molds' was highest in long-
237 term abandoned fields and increased with time since abandonment ($F_{2,18}=5.68$, $p<0.05$, $R^2 = 0.62$, $p<0.05$)
238 (Fig. 3). Furthermore, there was a trend that the proportion of AMF of total fungi receiving carbon from
239 the plants increased with time since abandonment ($F_{2,18}=2.64$, $p=0.15$). When a ratio between AMF to
240 potential pathogens was calculated, a significant effect of the treatment ($F_{2,18}=9.97$, $p=0.01$) and time since
241 abandonment ($R^2= 0.51$, $p<0.05$) was found (Fig. 4).

242 Furthermore, some of the soils had a significantly larger proportion of certain functional groups: for
243 example long-term abandoned field BB had significantly more OTUs categorized as wood decomposers or
244 - parasites and nematophagous fungi than other fields (Fig. 3). These differences were not consistent over
245 abandonment time but rather related to unique attributes to the field probably related to the abiotic or
246 biotic factors in that specific field. Similarly, each soil had several OTUs that were unique: they were
247 present in at least two soil samples from that site but absent in other sites. One of the sites abandoned

for 30 years (BB) had the most unique OTUs (64) while a recently abandoned site REY had no unique OTUs at all (Fig. 5).

There were 816 OTUs present in at least two sites for each abandonment category. 58 OTUs were found in at least two long-term abandoned fields but were not found in earlier abandoned fields. Of these, only 2 OTUs were consistently found in all long-term abandoned fields but not in any other field (Suppl. Table 4). The recently abandoned fields had 33 OTUs that were not present in older abandoned sites and only 4 of them were present in all three recently abandoned soils (Suppl. Table 4). When the number of shared OTUs between fields was related to the time difference since abandonment, a significant negative correlation was found ($R^2 = 0.25$, $p<0.01$) indicating that the there is a directional succession in the fungal community composition.

The contribution of individual OTUs to the observed differences between the abandonment times was evaluated using SIMPER and the significance in presence of OTUs between treatments was confirmed using ANOVA. The top 20 most abundant OTUs explaining 32.4 % of the observed differences between abandonment time categories are shown in Table 1. Due to large variation between soils, only 4 out of the 20 OTUs selected by SIMPER were significantly different between abandonment categories when evaluated with ANOVA (Table 1). These OTUs were mostly categorized as ascomycetes with unknown function.

To reveal the connectedness of the species and possibly find more indicator species of land abandonment time, co-occurrence networks were constructed for the different abandonment stages (Fig. 6). Network topology revealed that the highest density of networks was at mid-term abandoned fields (0.13) and lowest at long-term abandoned fields (0.05). Species in the networks from mid-term fields had also highest number of direct neighbors (average of 14 per OTU). Interestingly, we found a split of network into two

subclusters ('hubs') starting in mid-term abandoned fields and continuing to long-term fields (Fig. 6) where the two 'hubs' were completely separated.

We looked in more detail to the OTUs with most neighbors in each of the three land abandonment categories in order to identify potentially important species in the systems. In recent fields the most connected OTUs were two unclassified Ascomycota, OTU4843 and OTU5273, both with 24 neighbors. In mid-term abandoned fields the most connected OTU was identified as *Coniochaetales* sp. with 36 neighbors. In long-term fields the OTU with most neighbors was *Lecythophora* sp. with 23 neighbors (Suppl. Table 5). Furthermore, some OTUs identified as indicators by SIMPER analysis were among the top connected OTUs in respective fields (table 1). Notably, OTU5177, unclassified ascomycete, was more abundant and also among the top connected species in the long-term fields making it a good candidate for further analyses as a keystone OTU.

Abiotic factors affecting rhizosphere fungi

Variance partitioning was performed in order to find out which abiotic and biotic factors affect the fungi in the 'heavy' fraction the most. Factors tested were: soil pH, N-mineralization, soil C, total N, total P and C:N ratio. Biotic factors included were: $\delta^{13}C$ in root, fungal PLFA and fungal feeding groups, and dominant plant species in the core. Abiotic variables explained 23.7 % of the total variation in CCA ($F=1.2$, $p=0.012$) but the measured biotic variables failed to explain significant variation alone or in combination. Of the abiotic variables, pH and organic carbon in the soils were factors significantly explaining most of the active rhizosphere fungal composition (Table 2). Time since abandonment in years (explaining 3.6 % of the variation, $F=1.3$, $p=0.049$), time category (4.0 % of the variation, $F=1.4$, $p=0.016$) and soil (4.3 % of the variation, $F=1.5$, $p=0.012$) were found to effect OTU composition when constrained and unconstrained CCAs were compared (Table 2). The sampling time (1 day, 1 week or 2 weeks) did not significantly affect the overall OTU composition in the heavy fraction.

293 *Fungal feeding organisms*

294 Data on the labeling status of morphologically identified soil animals collected one week after labeling was
295 correlated with fungal orders and functional groups. This revealed that relative abundance in ^{13}C fraction
296 of groups such as ericoid mycorrhizae, molds, wood decomposers and parasites and nematophagous fungi
297 was related to ^{13}C carbon detected in fungal feeding cryptostigmatic mites one week later (Suppl. Table
298 7). Labeling of fungivorous nematodes with ^{13}C was correlated with labeling of coprophilic,
299 entomopathogenic and potential plant pathogenic fungi. Labeling of fungivorous prostigmatic mites was
300 related to general saprotrophic fungi (Suppl. Table 7) and amount of ^{13}C in protist PLFA was positively
301 correlated with % of Saccharomycetes (yeasts) and Chytridiomycetes in the heavy fractions of the samples
302 (Suppl. Table 6).

303

304 **Discussion:**

305 Fungal α- and β-diversity was determined for a total of 9 ex-arable fields that were abandoned 7-30 years
306 prior to sampling. As expected, we observed that a large proportion of the variation in the diversity of
307 fungal communities could be explained by abiotic factors and a lesser part by abandonment time. High
308 explanatory values based on abiotic variables is more rule than exception for soil fungal community
309 composition (Tedersoo et al. 2015; Thomson et al. 2015). With the current set-up, we could, however,
310 separate the effects of abandonment time from the abiotic effects and found a shift in the active
311 community of fungi consuming recently fixed (^{13}C labeled) carbon in relation to abandonment time. We
312 showed that fungi become a more important sink of recently photosynthesized carbon during secondary
313 succession (Fig. 1), although the fungal:bacterial ratio or fungal biomass did not increase (van der Wal et
314 al. 2007; Morrien et al. 2017). Earlier it has been shown that shifts in flow of carbon and relative
315 contribution of fungi compared to bacteria has direct consequences for the food-web architecture and

influence through microbial community function ecosystem functions such as carbon and nitrogen mineralization (Holtkamp et al. 2011; Malik et al. 2016; Morrien et al. 2016). Recently, it has been proposed that fungi in many systems might be more important consumers of labile organic compounds, such as root exudates, than thought (Hannula et al. 2012; Morrien 2016; de Vries & Caruso 2016). However, the identity and functionality of these rhizosphere fungi was unknown. We identified these fungi and could show that active fungal community using these label compounds is very diverse and largely influenced by soil and plant.

The α-diversity of fungi actively assimilating recently photosynthesized carbon was not significantly affected by abandonment time. Lowest diversity of active fungi was found in mid-term abandoned fields, which is contrary to findings of studies done on same soils on total fungal community in bulk soil where 'hump-shaped' diversity patterns with highest diversity in mid-term abandoned fields were observed (Tardy et al. 2015; Morrien et al. 2016). This discrepancy in the results can only be explained by the presence of a high number of OTUs not actively using recently photosynthesized carbon present in mid-term abandoned fields.

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The division of fungi into functional guilds based on phylogeny and literature is becoming a common practice in fungal ecology (Nguyen et al. 2016). We acknowledge that such division into functional guilds is rather artificial as in different circumstances fungi can have different roles, for example along the continuum pathogen-endophyte-saprotroph (Arnold 2007; Aguilar-Trigueros et al. 2015). However, when done conservatively and based on available literature, it will give a more complete picture on the function soil fungal communities. Following this approach, it appeared that abandonment time had the strongest effect on communities of potential endophytes and - plant pathogens and yeasts (decrease with abandonment time), AMF and molds (increase with abandonment time) and saprotrophic fungi (hump-shaped pattern) (Fig 3). Furthermore, there was an increase in the AMF:pathogen ratio with abandonment

time (Fig 4). Our study is the first to demonstrate pathogen enrichment in early and AMF enrichment in late-secondary succession stages. The confinement of pathogens to recent abandonment stages fits well with observations that plant species in recently abandoned soils have negative feedback interactions through soil (Kardol et al. 2006). In that study, long-term abandoned field soil provided more positive feedback effects to the plant species that are typical for that succession stage (Kardol et al. 2006), which corresponds well with our finding that AMF are more prominent in long-term abandoned soil. Furthermore, we found a significant negative relationship between agricultural abandonment time and abundance of yeasts indicating that there is a shift from fast growing, single cell fungi to more resilient, hyphal growing fungal community using root exudates. Yeasts are more common in more disturbed systems growing on labile carbon sources such as root exudates efficiently contributing probably very little to the maintenance of the soil structure and nutrient cycling as compared to hyphal growing fungi that are usually better at producing extracellular enzymes (Mestre et al. 2011; Hannula et al. 2012; Treseder and Lennon 2015).

Besides observed changes in the proportion of functional groups and classes with abandonment time, changes at Order and Taxa level were detected and even individual OTUs were significantly affected by treatment. Furthermore, highly connected species, specific to certain abandonment stages (so called keystone OTUs; for synthesis see van der Heijden & Hartmann 2016) could be identified and may be of interest for speeding up nature restoration (Wubs et al. 2016). A hypothesis presented recently is that plant performance in a given ecosystem depends on the presence of keystone species that make the system more stable by protecting against invasion (Banerjee et al. 2015; Agler et al. 2016; van der Heijden & Hartmann 2016; Wubs et al. 2016). Here, the fungal taxa we found to be differentially present and highly connected during secondary succession belong mainly to Ascomycota and more specifically to orders *Pleosporales* and *Eurotiales*. Also a species of yeast from basidiomycete class *Tremellomycetes* was indicated as a significant keystone species by network analysis.

We explored the co-occurrence networks (Barberan et al. 2012) in order to assess how the connectedness of fungi is influenced by time since abandonment. These analyses revealed that the highest network densities of fungi using plant-derived carbon in terms of species connectedness were not found in later successional stages but in the mid-term abandoned fields. In long-term abandoned fields, the network configuration was different than in recently abandoned fields: there was a clear split of the network into two 'hubs' instead of one tightly clustered hub. This increase in modularity and in number of ' hubs' has been recently proposed to result in increased benefits to the plant and soil health through recruitment of species and prevention of pathogen invasion through highly connected hubs (Agler et al. 2016; van der Heijden & Hartmann 2016). If modularity increases over time, this is indicative that community development follows a non-random succession pattern (autogenic succession) which is driven by biotic interactions (Frankland 1998). In our system the second 'hub' started to form in the mid-aged fields indicating that it took some time after agriculture for the system to gain its modularity by recruiting new taxa and creating new connections. This coincided with increase in AMF receiving carbon from the plant and decrease in potential pathogenic fungi.

Last we explored the effects of changes in fungal community on fungal feeding organisms. We could show, based on correlations of the labeling data in time, that in recently abandoned fields, protists and fungivorous nematodes both seem to be using yeasts, usually fast growing and stress tolerant fungi (Treseder and Lennon, 2015) as a primary source of carbon. This can be due to the shorter generation times and relatively small body size of the animals and fungi compared to, for example, mites (Yeates et al. 1993) and filamentous fungi (Treseder and Lennon, 2015). In later successional fields the fungal feeders receiving most of the carbon were cryptostigmatic mites. In microcosms, it has been shown that fungal feeders do have preferences on the fungal species and groups they feed on (Crowther et al. 2015). Here, we provide preliminary evidence that preference of fungal feeders to certain fungal taxa also affects the communities in soils.

In conclusion, we show that thirty years of land abandonment result in shifts of active plant associated communities from bacterial dominated to fungal dominated communities. The composition of active functional fungal community changed from one composed of fast growing and pathogenic species to one consisting of beneficial and slower growing fungal types affecting the carbon flow through the soil food web and consequently the nutrient cycling and the plant succession (model fig).

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Conflict of interest statement

Authors declare no conflict of interest

Figure legends

Figure 1. The share of functional groups of fungi incorporating recently photosynthesized carbon by vegetation on soil cores of 3 land abandonment categories. The total amount of carbon from plants to fungi is calculated as an average of 3 soils for each of the 3 time categories using the excess ^{13}C labeling information of the PLFA marker 18:2ω6c and set to 1 for the long-term soils. For assignment of ITS sequences to functional groups, see text.

Figure 2. Effect of the time since abandonment from agriculture on fungal classes in the samples calculated from the % data. For significant differences between treatments see figure S5.

Figure 3. Fungal functional groups using recently photosynthesized carbon from plants affected by the time since agricultural abandonment. The recently abandoned fields are represented by red bars, mid-term abandoned fields by blue and long-term abandoned fields by green. The bars represent averages and error bars represent standard error. The significant trends in time are shown in the figure, for significance between treatments, see text. The groups are ordered based on their abundances in samples starting from the most abundant group.

Figure 4. The AMF/pathogen ratio changing in time since agricultural abandonment. The recently abandoned fields are marked as red bars, mid-term abandoned fields as blue and long-term abandoned fields with green. The bars represent averages and error bars represent standard error.

Figure 5. Number of unique OTUs present in the heavy fractions and the OTUs shared between treatments and soils. See text for definition of OTU present in a soil.

Figure 6. Development of network topology abandonment time. Only significant ($p<0.05$) positive correlations are visualized. The node size is proportional to the % abundance of the organism and edge darkness is scaled to the strength of the correlation (Pearsons rho). OTUs presented in the figure are grouped to functional groups (see text) and colored based on function. Network parameters such as clustering coefficient, number of nodes, density and number of neighbors are presented in the figure.

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