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Strong conditionality in plant-fungal community assembly after soil inoculation in post-agricultural grasslands

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Supporting information:1 table and 8 figures

Abstract

Soil inoculation from plant species-rich into species-poor grasslands may enable the establishment of self-facilitating networks between microbes and vegetation, thereby steering ecosystem development.

We conducted a three-year experiment that covered a wide range of post-agricultural grasslands to determine how succession is affected by the interactive effects of edaphic properties, grass layer removal and hay or soil fragment transfer from a late-successional donor grassland.

Soil inoculation generally impacted community assembly of vegetation and fungi, but not of prokaryotes. Effects were strongest when preceded by removal of the grass layer, indicating the importance of priority effects and dispersal limitation. Inoculation enabled the establishment of putative rhizosphere-associated fungal taxa, particularly from the families Helotiaceae, Glomeraceae and Archaeorhizomycetaceae. Nonetheless, effect sizes were mostly small, as was overall resemblance of the receptor grasslands to the donor. Fungal communities were primarily shaped by environmental filters and only reached a high resemblance to the donor in nutrient-poor sites. Shifts in the vegetation were strongest in those grasslands where the mycobiome more closely resembled that of the donor.

Soil inoculation generally facilitates colonization by target plant and fungal communities, where establishment success of the former can be predicted by the latter, but the final outcome of succession is environmentally determined.

Key words: dispersal limitation, environmental filtering, grassland restoration, plant-microbial interactions, priority effects, soil microbiome

1. Introduction

Species-rich grasslands are a globally important reservoir of biodiversity. However, widespread conversion to agricultural land and concomitant fertilization with nitrogen (N) and phosphorus (P) has led to a decrease in grassland cover and diversity (Wassen et al., 2005; Ceulemans et al., 2014). It is difficult to reverse this loss, which stems from a lack of understanding of how the local environment and biotic community pools interact to determine plant community assembly and biodiversity. Hence, a key question is if and how succession in degraded grasslands can be manipulated in order to restore diversity.

First, the succession of post-agricultural land towards species-rich grasslands typically requires soil nutrient depletion by sustained haymaking or by complete removal of the nutrient-enriched topsoil (Walker et al., 2004; Torok et al., 2011; Resch et al., 2019), thereby exposing bare soil. However, nutrient depletion alone is rarely sufficient: plant-interspecific differences in (i) local historic propagule persistence and (ii) regional dispersal capacity steer priority effects (i.e. community dependence on the order or timing of species arrival), and therefore ultimately determine vegetation community assembly upon abiotic restoration (Bakker and Berendse, 1999; Verhagen et al., 2001; Oster et al., 2009). In grasslands, this persistence and dispersal filter correlates with the establishment of widespread and persistent wind dispersers such as common grasses (Werner et al., 2016), which may, once established, compromise the re-colonization potential of rare target species (Partzsch et al., 2018). Removal of the existing grass layer followed by the manual transfer of propagules of target species, for example by seed or hay transfer from a species-rich reference site, can be a meaningful tool to bypass colonization restrictions (Donath et al., 2007; Klimkowska et al., 2007; Pywell et al., 2007; Torok et al., 2011).

Emerging evidence now suggests that ecosystem development can also be steered by manipulating soil microbiota, e.g. through soil fragment transfer or soil inoculation (Middleton and Bever, 2012; Wubs et al., 2016; Radujkovic et al., 2020). It is hypothesized that the soil community is an important driver of vegetation community assembly, and that the lack of an "appropriate" microbiome (e.g. due to the past loss of characteristic communities or microbial dispersal limitation) may hamper restoration (Koziol et al., 2018). This is because some microbes engage with plants as mutualists or pathogens, and they regulate pivotal ecosystem processes

including organic matter turnover, nutrient cycling, and soil stabilization through aggregate formation (Ohsowski et al., 2012; Semchenko et al., 2018), thereby (dis-)favoring some plant species over others.

Although promising effects of inoculation, e.g. with arbuscular mycorrhizal (AM) fungi, on grassland or heathland community assembly have been reported (van der Heijden et al., 1998; Torrez et al., 2016; Wubs et al., 2016; van der Bij et al., 2018; Neuenkamp et al., 2019; Wubs et al., 2019a; Vahter et al., 2020), there are still important knowledge gaps concerning the mechanisms and wide-scale validity, which we detail below.

First, past inoculation studies were either largely restricted to controlled laboratory and mesocosm experiments (Wubs et al., 2019a; Li et al., 2020), or they lacked replication across a wider environmental gradient (Kardol et al., 2009; Torrez et al., 2016; van der Bij et al., 2018; Radujkovic et al., 2020). These restrictions hamper the possibility to generalize findings beyond a single grassland or (artificial) environment. In other words: what may seemingly work well in one grassland, may not work in another. We hypothesize that it is unlikely that soil inoculation is equally effective under any given set of abiotic –or biotic– conditions, and that there is a multi-level complexity and site dependence in plant-microbial community assembly upon inoculation. It is for instance well known that vegetation and microbial community assembly are linked to local site characteristics such as edaphic properties (Fierer, 2017; Van Geel et al., 2018; Hulshof and Spasojevic, 2020), and that associations between microbes and plants can be context-dependent (Collins and Foster, 2009; Harris, 2009; Fierer, 2017).

Second, positive effects of soil inoculation in grasslands are often attributed to arbuscular mycorrhizal fungi (AMF) (Torrez et al., 2016; Neuenkamp et al., 2019; Vahter et al., 2020), which may indeed facilitate late-successional plant species (Koziol and Bever, 2017). However, AMF are only a subset of the total mycobiome, whereas other cryptic but potentially key taxa may be overlooked. Therefore, previously unknown links may be discovered by assessing a wider range of soil-dwelling microbes, possibly leading to novel insights.

In this study, we investigated whether priority effects and dispersal limitation of plants and plant-associated fungal taxa are important determinants of grassland succession, and how these factors interact with local environmental conditions. We focused on the succession of species-poor post-agricultural grasslands towards species-rich *Nardus* grasslands, which are NP-co-limited

grasslands that harbor a mixture of ericoid heathland species as well as many slow-growing and currently endangered grassland species (De Graaf et al., 2009; Schelfhout et al., 2017). We addressed the abovementioned knowledge gaps by quantifying the interactive effects of removal of the grass layer and hay and soil fragment transfer (= soil inoculation) from a species-rich and late-successional donor site, which we replicated in 16 restoration grasslands that differed in fertilization history and edaphic properties. We show that there is a high conditionality and site-dependence in succession, and we discuss the underlying plant-microbial correlations.

2. Materials and methods

2.1. Study sites and experimental design

Our study area, nature reserve "Landschap de Liereman", is situated in the Campine area of Northeastern Belgium. About a century ago, the region was characterized by a relatively high abundance of species-rich *Nardus* grasslands, most of which have disappeared due to land use intensification and concomitant eutrophication.

We established a field experiment in the area in October 2016, which was monitored for the duration of three consecutive growing seasons. The study included 16 post-agricultural receptor grasslands in which the development of *Nardus* grassland is the target. The grasslands covered a gradient from relatively nutrient-rich to nutrient-poor (due to differences in fertilization history) and from moist to relatively dry (Site coordinates and edaphic properties are summarized in Table S1). Management strives towards gradual nutrient depletion through yearly haymaking; at some sites this has already been ongoing for nearly four decades (Table S1). Despite these efforts, all grasslands have remained remarkably species-poor and dominated by common perennial grasses including *Holcus lanatus* L., *Anthoxanthum odoratum* L. and *Agrostis capillaris* L..

We installed six experimental plots in each of the 16 grasslands (2.5 m x 2.5 m, Fig. **S1**), adding up to a total of 96 plots. In three plots per site, we manually removed the existing grass layer by shallow sod stripping (depth of max. 5 cm to expose bare soil, treatment "grass removed"), while the grass layer of the remaining three plots was trimmed but not stripped (treatment "grass intact"). Next, two plots at each site were inoculated with whole soil fragments collected in a well-developed and nearby donor *Nardus* grassland, two plots received hay (with seeds) from the

same donor grassland, and the two remaining plots received nothing (control plots). These treatments were always replicated in one plot with and one plot without prior grass layer removal. Our set-up implied that we had a lot of between-site variation but no true within-site replication of each unique treatment combination. This was intentional, as our aim was to primarily focus on the wide environmental gradient provided by the 16 different sites.

The donor grassland harbors the last patch of late-successional and well-developed *Nardus* grassland in the area (Site characteristics in Table S1). The grassland is classified as Natura2000 priority habitat (H6230) and contains many key species such as *Nardus stricta* L., *Erica tetralix* L., *Calluna vulgaris* (L.) Hull, *Potentilla erecta* (L.) Raeusch., *Danthonia decumbens* (L.) DC., *Polygala serpyllifolia* (Hosé), *Gentiana pneumonanthe* L., and *Pedicularis sylvatica* L.. Three additional untreated plots were established within this donor grassland to serve as target reference plots for comparison.

Hay (containing seeds) was collected at the donor site in September 2016 using a lawn mower. All hay was first manually homogenized, and then spread out evenly on the experimental plots within the same day in a 2:1 ratio, i.e. the hay of 2 m^2 of the donor grassland was added to 1 m^2 of experimental plot. For soil inoculation, sods (= top 5 cm of the soil) were collected at the donor site one month later during a wet and chilly period, this to prevent drying and warming of the soil during manipulation and transport. Microbiota, particularly fungi, can be sensitive to prolonged drought or warmth, and proper timing is probably important to maximize the chances of successful microbial colonization upon inoculation. Sods were manually fragmented into small chunks using a shovel, homogenized, and were within the same day spread out thinly but evenly on the experimental plots in a 1:15 ratio, i.e. fragments of 0.067 m^2 of the donor grassland were added to 1 m^2 of experimental plot.

Management of the experiment consisted of yearly manual mowing (which was done meticulously to prevent cross-contamination) and hay removal at the end of each growing season, i.e. from September to October. Grazers were excluded by a fence.

2.2. Vegetation community assembly

Vegetation community composition (i.e. presence and relative abundance (% cover) of all vascular plant species) was monitored every summer at the height of the growing season. This was repeated in each plot for three consecutive years (2017-2019).

2.3. Edaphic properties

Abiotic conditions were characterized in summer of 2017, i.e. during the first growing season. Fresh samples of the top 0-5 cm of soil were collected using Kopecky rings (volume 100 cm^3) in each of the two control plots (i.e. in the plots with and without grass layer removal, but without addition of hay or soil) at each grassland, as well as in the three plots of the late-successional donor site. Samples were composed of three homogenized subsamples per plot, and were stored at 4°C until chemical analysis within the same week.

Simultaneously, we collected a second pairwise batch of samples for calculation of soil bulk density, soil organic matter and moisture content. Soil bulk densities and moisture contents were determined by drying samples of known volume (100 cm^3) at 105°C, after which organic matter contents were determined gravimetrically by loss-on-ignition (4h at 550°C).

Concentrations of nitrate (NO_3^-) and ammonium (NH_4^+) were determined on fresh soil subsamples by extraction with KCl (2h shaking in 1M KCl; 1:5) and subsequent analysis on an auto-analyzer system (SAN++, Skalar). Soil acidity (pH_{KCl}) was determined in the extracts using portable equipment (Hannah Instruments, HI99121 with HI12923 electrode).

To determine the acid-neutralizing capacity of the soil, cation exchange capacity (CEC) and base saturation (%) were determined by adding 50.0 ml of 1M ammonium acetate (brought to pH 7 with NH_3OH) to 5 g of soil subsample. The mixture was then shaken for 1 hour and filtered, after which cations were measured using ICP (iCAP 6300 Duo, Thermo Scientific). Exchangeable H^+ was determined by titration following Brown (1943). Total CEC was calculated as the cumulative sum of all exchangeable cations (Ca^{2+}, Mg^{2+}, K^+, Na^+, Fe^{2+}, Al^{3+}, Mn^{2+}, H^+), and base saturation was calculated as the sum of the base cations (Ca^{2+}, Mg^{2+}, K^+, Na^+) divided by total CEC.

Bioavailable phosphorus (P_{Olsen}) was determined by $NaHCO_3$ extraction (Olsen, 1954) and P was measured on an auto-analyzer system (SAN++, Skalar).

Contents of all soil elements were multiplied with the corresponding sample bulk densities to calculate total soil pools (in mmol per L soil).

2.4. Molecular analyses

Fresh soil samples for molecular analyses were collected from each plot exactly three years after the start of the experiment (October 2019). As such, the soil microbial community was given ample time to develop and stabilize before sampling. Composite samples of the topsoil (0-5 cm) were collected using a soil corer of 1 cm width, and each sample was composed of five randomly placed subsamples per plot. Sampling equipment was thoroughly rinsed with demineralized water between each sampling round to prevent contamination. All samples were homogenized and lyophilized, and DNA was extracted from a 0.1-0.2 g subsample using the PowerSoil DNA Isolation Kit (MO BIO, Carlsbad, California), following the manufacturer's protocol. Isolations were additionally cleaned using one-step PCR inhibitor removal kit (Zymo research, Irvine, CA, USA) and DNA was quantified using the dsDNA Assay Kit and Qubit fluorometer (Invitrogen).

2.4.1. qPCR

Total abundance of fungi and prokaryotes was determined with quantitative PCR (qPCR). Fungal 18S rDNA gene abundance was measured using the primer pair FF390.1 (CGA TAA CGA ACG AGA CCT)/FR1 (AIC CAT TCA ATC GGT AIT) (Vainio and Hantula, 2000), and total prokaryote 16S rRNA gene abundance was measured using the primer pair 515F (GTGYCAGCMGCCGCGGTAA)/806R (GGACTACNVGGGTWTCTAAT) (Caporaso et al., 2011). qPCR was performed in duplicate reactions using KAPA SYBR fast (Roche, Basel, Switzerland) on a Bio-Rad CFX96 Real-Time Thermal Cycler (Bio-Rad, Hercules, CA, USA). Each reaction contained 4 µl of sample, 10 µl KAPA SYBR FAST qPCR Master Mix, 3.8 µl water, 0.4 µl of each primer (1:10 diluted), 0.4 µl ROX high and 1 µl BSA. Control reactions contained water instead of sample. Cycling conditions were as follows: 95°C for 180 sec followed by 40 cycles at 95° C for 3 sec, 57°C (16S) or 52 °C (18S) for 20 sec and 72°C for 12 sec. Standard curves were created using a 10-fold dilution of cleaned-up PCR product for which

the concentration was determined in advance. It was ascertained that accuracy was high through a linear model of log-transformed concentrations on Cq's ($R^2 > 0.98$).

2.4.2. High-throughput amplicon sequencing and bioinformatics

The fungal ITS1 region was amplified using general fungal primers ITS1f (Gardes and Bruns, 1993) and ITS2 (White et al., 1990). For prokaryotes, an approximately 250 bp stretch within the V4 region of the 16S rRNA was amplified with the F515/R806 primer set (Caporaso et al. 2011). Each primer contained Illumina adapters at the 5' ends to facilitate adding barcodes and sequencing adapters in a second PCR ("Nextera" procedure). Each 25 µl reaction mixture contained 2 µl of the sample, 16.3 µl of H_2O, 0.2 µM of each forward and reverse primer, 5 µl PCR buffer, 0.5 µl dNTPs and 0.2 µl Phusion High-Fidelity DNA polymerase (New England Biolabs, Ipswich, MA, USA).

PCR conditions were as follows: initial denaturation at 98 °C for 60 s, followed by 35 (fungi) or 30 (prokaryotes) cycles of denaturation at 98 °C for 30 s, annealing at 55 °C for 30 s, extension at 72 °C for 30 s; and an additional extension of 72 °C for 10 min.

PCR products were run on a 1.5 % agarose gel to confirm successful PCR amplification and to confirm that negative controls were empty (and in case of failure the procedure was repeated). Successful PCR products were diluted 50 x and a second PCR was performed using dual barcoded primers with Illumina adapters in an identical PCR mix as above except using 3 µl of template and adjusting the water content accordingly.

The conditions were: 98 °C for 60 s, 12 cycles: at 98 °C for 10 s, 63 °C for 30 s, 72 °C for 30 s; and 72 °C for 5 min. PCR products were run on an agarose gel and successful amplicons were pooled into a single library. The library was purified using Machery Nagel gel purification kits QIAquick Gel Extraction Kit (Qiagen, Venlo, the Netherlands) and quantified using qPCR (KAPA Library Quantification Kits, Kapa Biosystems, Wilmington, MA, USA). The sequencing was performed using the Illumina MiSeq platform (Illumina Inc; San Diego, CA, USA) with 2x300 cycles for forward and reverse reads.

Sequences were analysed using the USEARCH (v8.1.1861) and VSEARCH (Rognes et al., 2016) software following the UPARSE pipeline (Edgar, 2013). After trimming to 230 bp (fungi) and 260 bp (prokaryotes), the paired-end reads were merged and primers were removed. This trim length was chosen because it was the optimal length for merging paired reads by removing low-quality bases at the end. Merged sequences were quality filtered using the expected number of errors (E) as a measure of read quality, with a threshold of $E_{max} = 0.5$. Following singleton removal, the sequences were clustered into OTUs (operational taxonomic units) based on 97% similarity using the UPARSE-OTU algorithm (Edgar, 2013), which automatically detects and filters out chimaeras. Filtered reads were then mapped to the OTUs with an identity threshold of 0.97, yielding one OTU table for fungi and one for prokaryotes.

For fungi, representative OTUs were then aligned to fungal sequences in the UNITE database (Kõljalg et al., 2005)(02.02.2019 update), and non-fungal sequences were removed. To annotate fungal sequences to known genera, we used NCBI's BLAST algorithm with default settings. OTUs were then assigned to particular taxa if they had a maximum E-value of 10^{-36} and from this, the lowest E-value hit with a known genus was selected. If there were none, the genus level was left unassigned. OTUs were subsequently assigned to functional groups if the genus was successfully matched with one of the genera with known lifestyles in Tedersoo et al. (2014) and Liang et al. (2016).

For prokaryotes, taxonomy was assigned to representative OTUs using the SINTAX function of USEARCH v10 against the RDP database v16. Non-prokaryotic 16S sequences (unassigned or assigned to chloroplasts or mitochondria) were parsed from the OTU table. Fungal and prokaryote data were respectively rarefied to 7,000 and 20,000 reads per sample. Samples with less reads (n = 15 for fungi and n = 25 for prokaryotes) were normalized to allow for a meaningful comparison of relative OTU abundances and community composition between samples.

2.5. Statistical analyses

All analyses were performed in R 4.0.4 and significance for statistical tests was accepted at $P < 0.05$.

We calculated Shannon-Wiener diversity indices of vegetation and microbial communities (using the “Diversity” function on untransformed data in Vegan (Oksanen et al., 2007)). For the vegetation, we discriminated between total plant diversity and diversity of target species only (i.e. species that occurred in the donor grassland). Linear-mixed effect models were used to test for the effects of grass layer removal (fixed factor: yes or no) and the addition treatment (fixed factor: control, hay transfer or soil inoculation) on these diversity indices as well as on total fungal and prokaryote abundance (copy numbers). “Site” was included in the model as a random factor to account for between-site variation. If the interaction term in the models were non-significant, we re-ran the tests including main effects only.

Vegetation, fungal and prokaryote community data were then square root-transformed prior to subsequent analyses to downweigh abundant taxa as well as to give relatively more weight to rarer species, which are often indicative for well-developed *Nardus* grasslands. To test if the experimental treatments led to differences in community compositions, we performed nonmetric multidimensional scaling (NMDS, $k = 2$) based on Bray-Curtis dissimilarity indices for the sample*species and sample*OTU matrices. Differences in plant and microbial community composition across experimental treatments (excluding the donor site) were tested with permutational analysis of variance (permanova) with 999 iterations, using the function ‘adonis’ in which we included “site” as a blocking factor (‘strata’) to account for between-site variation.

For communities that responded significantly to any of the addition treatments, we then analyzed which parameters most accurately predicted the dissimilarity in vegetation and fungal communities of the experimental plots to the late-successional donor site, in which a lower dissimilarity indicates convergence towards the target donor grassland. We first calculated community dissimilarities by averaging all Bray-Curtis distances from each experimental plot to each of the three plots of the late-successional donor site, thereby obtaining one dissimilarity measure per plot. We then constructed two separate regression trees with (1) vegetation community dissimilarities [to the donor] and (2) microbial community dissimilarities [to the donor] as dependent variables. To avoid potential overfitting, the complexity parameters were set

to a value of 0.05, while the bucket sizes (= the minimum number of plots included in each terminal node) were set at a minimum of five plots. We included the following explanatory variables: grass layer removal (yes or no), addition treatment (control, +hay or +sods), soil-pH_{KCl}, $NO_3^-{}_{KCl}$, $NH_4^+{}_{KCl}$, bioavailable P, soil base saturation (%), organic matter and moisture content (%). Since vegetation community composition may also strongly depend on fungal community composition and vice versa, we additionally included both community dissimilarity vectors as potential explanatory factors for each other. If variables were selected in this analysis we considered them important determinants of community convergence towards the target donor site.

Finally, we performed an indicator species analysis, using the multipatt function (999 iterations) from the indicspecies package (Cáceres and Legendre, 2009), to identify which fungal OTUs were "indicator species" of the six unique experimental treatment combinations. We allowed for overlap between these combinations (i.e. a unique OTU can be an indicator of more than one treatment level), and we only retained significant ($P < 0.05$) indicator OTUs with a minimum of five cumulative reads across samples. For each of the identified taxa, we then marked whether it also occurred in the donor grassland (as an indication of convergence or divergence towards the donor grassland). For data simplification, visualization and subsequent ecological interpretation, we then grouped the indicator OTUs per fungal family. Data were visualized as divergence bar charts in which we distinguished between indicator OTUs that did (blue color) or did not (red color) also occur in the donor site. Indicator OTUs that could not be assigned to a family were excluded from visualization. Finally, we performed a chi-square test to test whether the observed distribution of all indicator OTUs (also including OTUs of unknown families) that were either present or absent in the donor site differed from the expected distribution in the experimental treatments, i.e. to test whether the plots with soil inoculation were significantly overrepresented by indicator OTUs that also occurred in the donor grassland.

3. Results

3.1. Treatment effects on vegetation and microbial community structure and composition

We counted a total of 127 vascular plant taxa over three growing seasons. In 2019, i.e. in the final growing season of the experiment, 81 vascular plant taxa were registered, together with 4,931 unique fungal and 14,084 prokaryote OTUs. Highest vegetation diversity was found in the plots with soil inoculation and prior grass layer removal, while lowest diversity was found in the control plots (Fig. **1a**). There was a significant interaction between the grass removal treatment and the soil inoculation or hay addition treatment on total vegetation diversity ($F_{2,75} = 4.42$, $P = 0.015$), with stronger effects of hay addition and soil inoculation in the plots with prior grass layer removal (Fig. **1a**). Results were similar and more pronounced when we only included the target species (i.e. the species that occurred in the donor grassland; $F_{2,75} = 11.80$, $P < 0.001$, Fig. **S2**). The soil and hay inoculation plots, especially those with prior grass layer removal, contained many characteristic *Nardus* grassland species that were generally absent in the control plots. Most frequently encountered species included *Calluna vulgaris, Erica tetralix, Potentilla erecta, Danthonia decumbens, Succisa pratensis* and *Nardus stricta.*

The experimental treatments did not affect fungal diversity of the topsoil (Addition treatment: $F_{2,77} = 0.82$, $P = 0.441$; Grass removal treatment: $F_{1,77} = 0.44$, $P = 0.508$, Fig. **1b**), nor fungal abundance (Addition treatment: $F_{2,76} = 2.43$, $P = 0.095$; grass removal treatment: $F_{1,76} = 1.01$, $P = 0.318$, Fig. **1c**). Vegetation diversity and fungal abundance tended to be higher in the donor site than in the control plots, while fungal diversity was nearly twice as low at the donor site (Fig. **1b**). The low fungal diversity in the donor site was due to the hyperabundance of a handful of OTUs of the Archaeorhizomycetaceae family, which on average comprised 80% of all fungal reads at the donor (Fig. **S3**). Diversity of prokaryotes was also not affected by any of the experimental treatments (Addition treatment: $F_{2,77} = 0.82$, $P = 0.441$; Grass removal treatment: $F_{1,77} = 0.10$, $P = 0.749$, Fig. **S4a**), and neither was prokaryote abundance (Addition treatment: $F_{2,76} = 0.14$, P = 0.867; grass removal treatment: $F_{1,76} = 0.78$, P = 0.380, Fig. **S4b**).

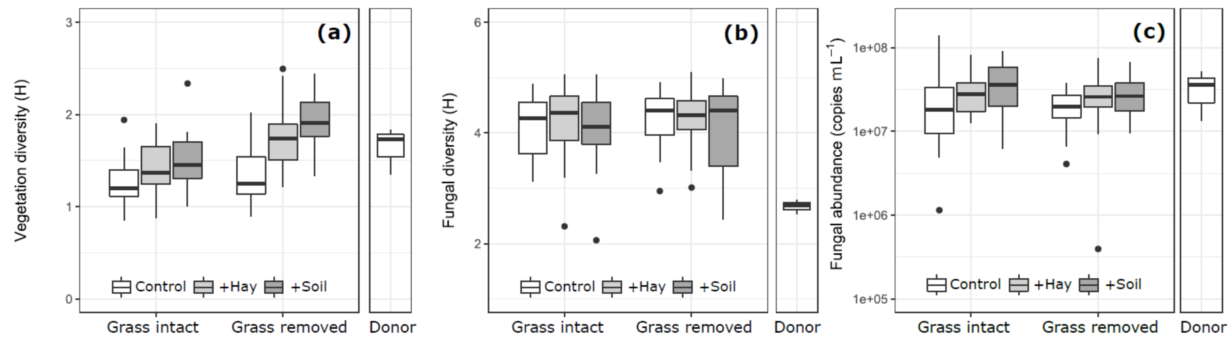

Fig. 1: Effects after three growing seasons of different treatments on (a) vegetation diversity, (b) fungal diversity and (c) fungal abundance (log10-scale) in the topsoil of 16 post-agricultural grasslands, with the late-successional donor site as comparison. Experimental treatments included initial removal of the grass layer (yes or no), and nested within were addition treatments of nothing (control), soil inoculation or hay transfer (both collected in the donor site). Diversity was calculated as the Shannon-Wiener diversity index (H). Fungal abundance was calculated as fungal 18S rDNA gene copy numbers per milliliter of soil.

In line with results on vegetation diversity, we found a significant interaction effect between the grass removal treatment and the addition treatments on vegetation community composition (Permanova $F = 0.956$, $R^2 = 0.02$, $P = 0.001$), with strongest effects of the addition treatments after grass layer removal. The main effects of the grass removal treatment and the addition treatment were also significant (Grass removal treatment: Permanova $F = 3.53$, $R^2 = 0.04$, $P < 0.001$; Addition treatment: Permanova $F = 2.61$, $R^2 = 0.05$, $P < 0.001$). Overall, the vegetation of the plots with grass layer removal and soil inoculation most closely resembled the vegetation of the donor site (Fig. **2a** for the final monitoring year; all previous years are plotted in Fig. **S5**).

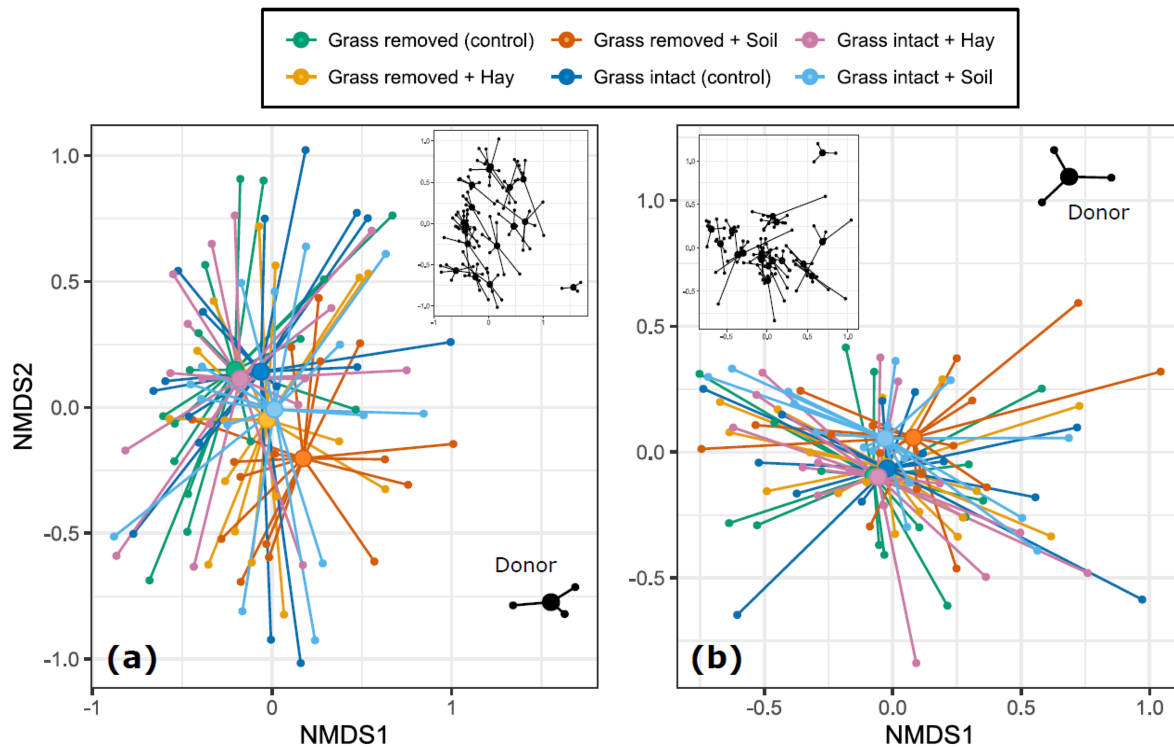

Fig. 2: Effects after three growing seasons of different treatments on (a) vegetation (stress = 0.18) and (b) fungal (stress = 0.17) community composition in 96 experimental plots distributed over 16 post-agricultural grasslands and one late-successional donor grassland, using nonmetric multidimensional scaling (NMDS, k = 2). The main (colored) panels show plot grouping by experimental treatment, the embedded (black) panels show the same ordination but with plot grouping by grassland site. The analyses are based on Bray-Curtis dissimilarity indices of the sample*species and sample*OTU matrices. Groups are joined by a central "circle" representing group centroids, whereas the small dots represent the individual sample scores.

Fungal community composition was also affected by the grass removal and addition treatments (Grass removal treatment: Permanova F = 1.62, $R^2 = 0.02$, P < 0.001; Addition treatment: Permanova F = 0.88, $R^2 = 0.02$, P < 0.001), but there was no significant interaction effect between treatments (Permanova F = 0.60, $R^2 = 0.01$, P = 0.726). Overall, fungal communities of the plots with soil inoculation most closely resembled the community of the donor site (Fig. **2b**), although the overall magnitude of treatment-induced shifts in fungal community composition

towards the donor grassland was relatively small. Fungal communities of plots with hay addition did not shift in the direction of the donor grassland.

Prokaryote community composition was not altered by any of the addition treatments (Permanova F = 0.55, R^2 = 0.01, P = 0.208), and we only observed a minor shift in composition in response to the grass removal treatment (Permanova F = 1.97, R^2 = 0.02, P < 0.001, Fig. **S6**).

Site effects strongly shaped microbial and vegetation community composition, as evidenced by a strong within-site plot clustering (Embedded figures in Fig. **2a,b** and Fig. **S6**).

3.2. Determinants of ecosystem development

Regression tree analysis indicated that fungal community (dis)similarities of the experimental plots to the late-successional (target) donor site were primarily explained by soil pools of bioavailable P, with lowest fungal dissimilarities (= highest similarities) to the donor site in P-poor plots (P_{Olsen} < 0.44 mmol L^{-1}, Fig. **3a**, first branch). In P-rich plots (P_{Olsen} ≥ 0.44 mmol L^{-1}), fungal communities more closely resembled the fungal communities of the donor site only when the soil had been inoculated and when pools of NH_4^+ were low (< 0.13 mmol L^{-1}) (Fig. **3a**, branch 3 and 4).

In contrast, vegetation community (dis)similarity of the experimental plots to the late-successional donor site seemed largely unaffected by any of the measured environmental variables. The strongest predictor of vegetation community (dis)similarity was fungal community (dis)similarity to the donor site (Fig. **3b**, first branch): the vegetation was more likely to resemble the vegetation of the donor grassland wherever fungal communities were more similar to the donor grassland. In plots with a high fungal dissimilarity to the donor site (≥ 0.82), soil inoculation triggered a significant decrease in vegetation community dissimilarity (Fig. **3b**, branch 3), especially when preceded by removal of the pre-existing grass layer (Fig. **3b**, branch 4).

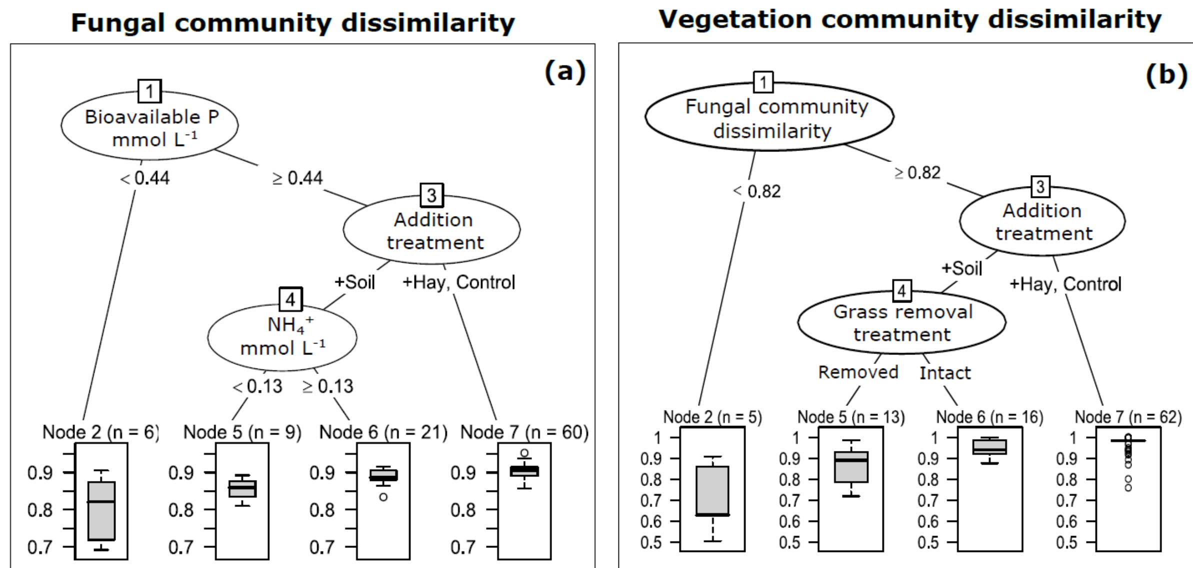

Fig. 3: Regression trees showing environmental variables that are associated with (a) fungal community dissimilarities and (b) vegetation community dissimilarities to the late-successional donor site. The study includes 96 experimental plots evenly distributed over 16 post-agricultural grasslands. The trees show successive significant branches and concomitant branching criteria. The final nodes are visualized as boxplots of (a) fungal and (b) vegetation community dissimilarities to the donor site.

3.3. Indicator OTUs of the experimental treatments

A total of 124 fungal OTUs were identified as indicator taxa for one or more of the six treatment combinations, and 82 of these could be assigned to a total of 50 families (the 25 families that also occurred in the late-successional donor site are shown in Fig. **4**; all remaining families are shown in Fig. **S7**). Overall, the number of indicator OTUs per treatment combination was lowest in the intact control plots (37 indicator OTUs) and highest in the soil inoculation plots with prior grass layer removal (71 indicator OTUs). The majority of the indicator OTUs that were found in the experimental plots with soil inoculation also occurred in the donor grassland (Fig. **4e-f** and Fig. **S7e-f**, blue bars), whereas the control plots and the plots with hay transfer had a higher proportion of OTUs that did not occur in the donor grassland (Fig. **4a-d** and **Fig. S7a-d**, red bars). In line with these observations, presence or absence counts of the indicator OTUs [that also occurred in the donor] were significantly associated to the experimental treatments (chi-square = 32.477, df = 5, P < 0.001): the plots with soil inoculation were significantly

overrepresented by indicator OTUs that also occurred in the donor grassland, whereas the plots without soil inoculation had significantly more OTUs that did not occur in the donor (Fig. **S8**).

Soil inoculation plots were, amongst others, relatively overrepresented by indicator OTUs of the families Helotiaceae (with dominance of the genera *Meliniomyces, Collophora* and *Hymenoscyphus)* and Archaeorhizomycetaceae (represented only by the genus *Archaeorhizomyces*). AMF indicator OTUs (family Glomeraceae, here represented by the genera *Rhizophagus* and *Funneliformis*) were found only in the soil inoculation plots with prior grass layer removal, while absent in all other plots.

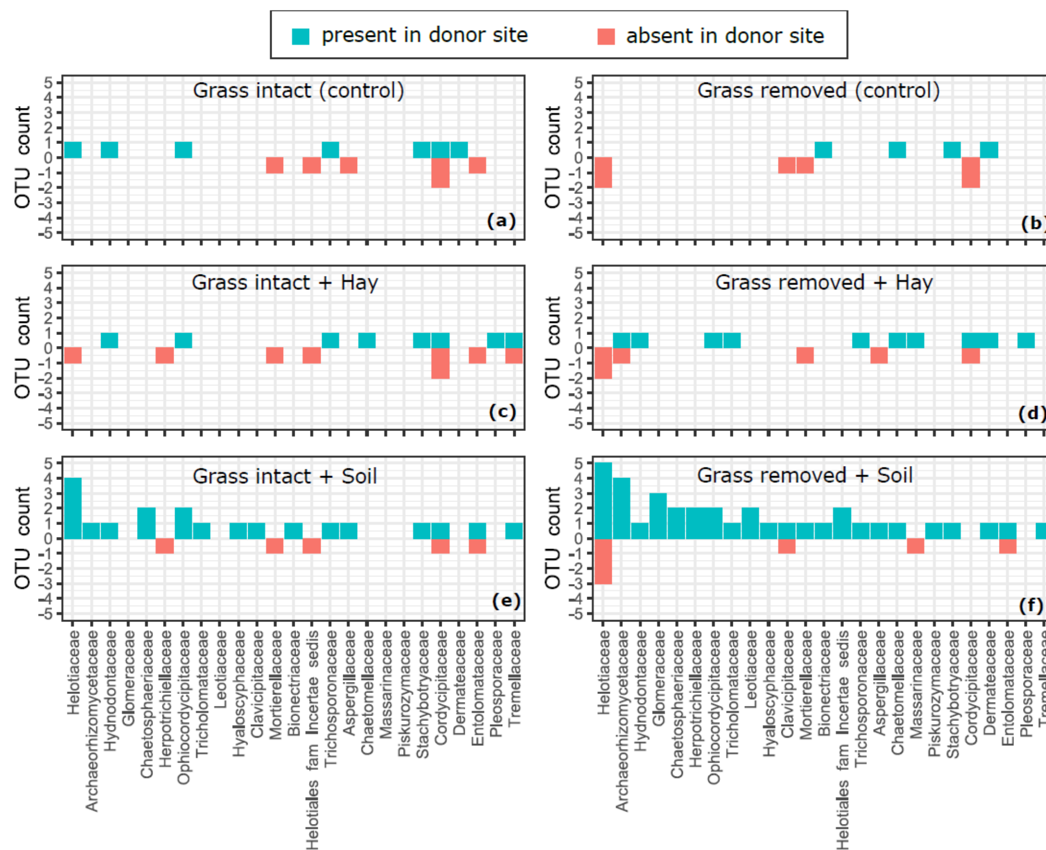

Fig. 4: The number of fungal OTUs, grouped per fungal family, that were identified as indicators for the corresponding treatment. Only OTUs that could be attributed to a family and only the 25 families that were also present in the late-successional donor grassland are shown here, which are ranked from highest (left on x-axis) to lowest (read) abundance (right on x-axis) of the corresponding indicator OTUs in the donor grassland. Blue bars represent the cumulative number of indicator OTUs from the corresponding family that were also present in the donor grassland, while red bars represent the number of indicator OTUs from that family that were absent in the donor grassland.

4. Discussion

We investigated whether priority effects and dispersal limitation of plants and plant-associated fungal taxa are important determinants of grassland succession, and how these factors interact with the local environment. Highest levels of grassland community convergence to the donor were observed in bare soil plots with soil inoculation, which we relate to the manipulation of vegetation-mediated priority effects. The simultaneous transfer of dispersal-limited and putative plant-associated fungal taxa may have had an additional positive effect. However, positive effects were generally limited in magnitude and site-dependent, with strongest signals in nutrient-poor receptor plots.

4.1. General treatment effects on plant-microbial community assembly

The vegetation in the control plots remained species-poor throughout the study, while the plots with hay or soil addition were generally characterized by an instantaneous increase in plant diversity and a community composition that more closely resembled that of the donor *Nardus* grassland. Moreover, significant interaction effects with the grass removal treatment indicated that the hay or soil addition treatments were most successful on bare soil, while hay or soil addition on top of an existing grass layer had negligible effects.

It is well known that manual transfer of plant propagules with hay or soil fragments is an effective method to (i) bypass dispersal limitations of target plant species (Pywell et al., 2007; Torok et al., 2011), and (ii) manipulate priority effects, which imply that the order of species arrival will co-determine competitive outcomes and ecosystem succession (Weidlich et al., 2021). In our experiment, manipulation of priority effects was not only accomplished by manual introduction of the target species (which were thus given a head start compared to non-target species), but also by prior removal of the pre-existing vegetation that appeared highly resistant to change. Hence, a brief "reset" of the vegetation, i.e. by temporarily removing competition from the established vegetation, seems crucial in steering vegetation community assembly (Pywell et al., 2007). Removal of the existing vegetation has very few downsides in species-poor grasslands, apart from the financial cost, and should be considered more widely in grassland restoration. We note, however, that positive effects of removal of the grass layer and propagule transfer could be transient: it is possible that diversity may gradually diminish over time if

dominant grasses are able to reestablish. Although we only investigated succession during the first three growing seasons and therefore cannot extrapolate our results to a longer (i.e. decadal) time span, it has been shown that effects of a single introduction of seeds and soil biota can last decades (Wubs et al., 2019b).

Effects of soil transfer were more pronounced than those of hay transfer, for which we see at least two nonexclusive reasons. First, the number of seeds that are transferred with hay is strongly dependent on the timing of haymaking (Bischoff et al., 2018), and interspecific differences in phenology could mean that some species may be missed altogether. Timing is no issue with soil inoculation, as the whole seed bank is transferred with soil fragments. Second, as we will discuss in more detail later, the co-establishment of target fungal communities with soil inoculation may have facilitated the establishment of target plant communities.

Soil inoculation also triggered a significant (albeit small) shift in fungal community composition towards the donor *Nardus* grassland, regardless of the grass removal treatment (Fig. **2b** and Fig. **4**). In contrast, despite the growing body of evidence that vegetation composition should also co-shape the microbiome (Kardol et al., 2007; Heinen et al., 2020), it was surprising that hay transfer and the subsequent establishment of characteristic plant species did not trigger such fungal community shift towards the donor site within the timespan of our study. This suggests that (i) introduction of the target vegetation alone may be insufficient to promote the establishment of target fungal communities, and (ii) other factors may be more important determinants of fungal community assembly. One of these steering factors may be fungal dispersal limitation. Several studies have suggested that dispersal limitation may not be very relevant for fungi (Hazard et al., 2013; Kivlin et al., 2014), while others have claimed the opposite (Peay et al., 2010; Norros et al., 2012). By using indicator species analysis, we found strong evidence for dispersal limitation of at least some fungal taxa: most fungal OTUs that were indicator taxa of the soil inoculation plots (and that also inhabited the late-successional donor site) remained absent in the neighboring control plots and in the plots with only hay transfer (Fig. **4**). This shows that these specific indicator OTUs originated from the inoculum from the late-successional donor site, and that they have been -to this point- unable to colonize the grasslands on their own account.

In contrast, prokaryote community composition was not affected by the soil or hay addition treatments. This either suggests that the already established prokaryote communities were able to outcompete potentially new colonizers and thereby maintain their equilibrium, or more likely, that bacteria are simply less limited in their dispersal. If the latter hypothesis is true, then prokaryote communities in our plots were probably shaped by local environmental filters only, i.e. “everything is everywhere, but the environment selects” (Baas-Becking, 1934).

4.2. Potential plant-fungal associations after soil inoculation

Vegetation community (dis)similarity to the donor site appeared to be strongly linked to fungal community (dis)similarity (Fig. 3b), but given the partially correlative nature of our field experiment we need to remain cautious with drawing strong conclusions about causality. It is therefore important to additionally investigate which fungal taxa were able to establish with soil inoculation, and how these taxa may interact with the vegetation. Indicator analysis revealed that three fungal families were relatively overrepresented in the soil inoculation plots: Glomeraceae, Helotiaceae and Archaeorhizomycetaceae (Fig. **4**). These three families were also among the most abundant families in the donor grassland (Fig. **S3**), and contain many putative plant-associated taxa. Members of the Glomeraceae are arbuscular mycorrhizal fungi (AMF), and the corresponding indicator OTUs (here from the genera *Rhizophagus* and *Funneliformis*) are well-known plant symbionts that, amongst others, facilitate plant P acquisition or enhance tolerances to environmental stress (Calvo-Polanco et al., 2014; Jiang et al., 2016). Members of the Helotiaceae are more diverse in their functionality, but the dominant indicator genus in the inoculation plots, *Meliniomyces*, is a root-associated taxon that is closely related to the ericoid mycorrhizal fungus *Rhizoscyphus ericae* (Hambleton and Sigler, 2005). Since the genus contains species with ericoid mycorrhizal properties (Vohnik et al., 2007; Vohnik et al., 2013), there could be direct links to the increased establishment rates of ericoid plants in the soil inoculation plots (i.e. *Calluna vulgaris* and *Erica tetralix*). The ecological role of the third main fungal indicator family of the soil inoculation plots, Archaeorhizomycetaceae, is yet to be unraveled. Nonetheless, *Archaeorhizomycetes* species appear to be of pivotal importance for the ecology and functioning of the donor *Nardus* grassland, since -on average- an extraordinary 80% of all fungal reads from this grassland were classified within this group (Fig. **S3**). In general,

Archaeorhizomycetaceae members are known to sometimes dominate belowground communities, for example in heathlands (Radujkovic et al., 2020), and the family contains hundreds of cryptic filamentous species (Rosling et al., 2011). *Archaeorhizomycetes* are tightly linked to the rhizosphere, where they often co-occur with AM Glomerales (Choma et al., 2016), and they may portray host and habitat specificity (Rosling et al., 2011). Hence, *Archaeorhizomycetes* species are at least indirectly associated to plant roots and thus to vegetation assembly, although unfortunately in manners that are yet to be unraveled.

4.3. Nutrient availability as an environmental filter

Despite the generally positive effects of soil inoculation on fungal and vegetation community assembly, e.g. by canceling out dispersal limitations of specific indicator taxa, the overall magnitude of the effect of soil inoculation on [changes in] community composition at all sites combined was rather small and with low R^2 (Fig. **2**). As we had hypothesized, this discrepancy was partially due to the large differences in community assembly between the different grasslands, pointing towards site-dependent filters. In other words: soil inoculation worked well in some grasslands, but not in others.

One would expect highest community convergence [to the donor] in grasslands that abiotically resembled the donor *Nardus* site, and lower convergence in sites with a potentially larger abiotic mismatch. A key trait of *Nardus* grasslands is their low availability of P and NH_4^+ (De Graaf et al., 2009; Schelfhout et al., 2017), which also characterized our late-successional donor grassland (P_{Olsen} < 0.22 mmol L^{-1} soil and $NH_4^+{}_{KCl}$ < 0.1 mmol L^{-1} soil). For fungi, regression tree analysis indeed revealed that communities of the experimental plots were significantly more likely to resemble fungal communities of the donor grassland when bioavailable P pools were smaller than a threshold value of approximately < 0.44 mmol L^{-1} soil (Fig. **3a**), which approximately equates to < 0.35 mmol kg^{-1} soil (i.e. 0.44 divided by a mean soil bulk density of 1.27 kg L^{-1}). This empirical threshold value for bioavailable P is remarkably close to the putative target value for species-rich *Nardus* grasslands (P_{Olsen} < 0.37-0.39 mmol kg^{-1} (= 11.5-12 mg kg^{-1}, (Schelfhout et al., 2017; Schelfhout et al., 2021)). The presence or establishment of typical *Nardus* fungal communities in P-poor soils is not surprising: specific fungal taxa, such as the arbuscular and ericoid mycorrhizal indicator taxa, thrive under P-poor conditions in which they fulfill a pivotal role in plant nutrient acquisition (Read et al., 2004; Ceulemans et al., 2019).

In plots with higher P availability, fungal communities developed in the direction of the donor site only after soil inoculation and under N-poor conditions (NH_4^+ < 0.13 mmol L^{-1}, Fig. **3a**, which equates to approximately 0.1 mmol kg^{-1}). Again, this threshold value of ammonium availability matches nicely with the putative target for species-rich *Nardus* grasslands, which is on average < 0.1 mmol kg^{-1} soil (De Graaf et al., 2009). Taken together, these results show that nutrient limitation is a crucial prerequisite for the recovery of a characteristic *Nardus* grassland mycobiome, and this prerequisite cannot be bypassed by soil inoculation alone.

Finally, regression tree analysis revealed that vegetation community composition did not primarily respond to site-specific edaphic properties. Instead, vegetation composition seemed to closely follow fungal community composition, and it also responded more strongly to the addition treatments. We believe that these patterns reveal two mechanisms: (i) target vegetation can -although to a limited extent- be more easily than fungi "forced" to establish into a seemingly unsuitable abiotic environment through propagule transfer, and (ii) the relationship between vegetation community composition and edaphic properties may to a considerable extent be indirect, i.e. plant-soil interactions may be regulated through the response of the fungal community to [shifts in] edaphic properties.

4.4. Conclusions

We provided evidence for a strong conditionality in the effect of soil inoculation and grass layer removal on the establishment of target plant and fungal, but not prokaryote, communities in grasslands, at least within the short term. Although soil inoculation generally facilitated and accelerated the succession of post-agricultural grasslands towards species-rich grasslands, inoculation was no panacea. Soil fertility imposed a strong environmental filter: clear plant and fungal community convergence [to the donor site] was observed in nutrient-poor receptor grasslands only. We conclude that priority effects and dispersal limitation are pivotal determinants of plant and fungal community assembly, but local environmental filters eventually channel the outcome of succession. A priority for future research is to disentangle causality and feedbacks between community assembly of the mycobiome and of the vegetation over an ever wider range of environmental conditions and on a longer (decadal) time span, thereby focusing on the conditional outcomes of plant-soil community interactions upon soil inoculation.

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Author contribution

W.-J.E. and R.v.D. conceived of the experiments and designed the research; W.-J.E., I.F., Y.L., M.d.W. and E.V. collected the data, W.-J.E., I.F. and E.V. statistically analyzed the data, W.-J.E. led the writing of the manuscript and all authors contributed to drafts and gave approval for publication.

Data availability

Abiotic data are attached as supporting information. Vegetation relevees and fungal indicator species data have been deposited in the Mendeley data repository (DOI: 10.17632/jcbb4bsmzr.1). The raw sequences have been deposited in the SRA-NCBI database (Accession: PRJNA799324).

References

Baas-Becking, L.G.M., 1934. Geobiologie of inleiding tot de milieukunde. WP Van Stockum & Zoon.

Bakker, J.P., Berendse, F., 1999. Constraints in the restoration of ecological diversity in grassland and heathland communities. Trends in Ecology & Evolution 14, 63-68.

Bischoff, A., Hoboy, S., Winter, N., Warthemann, G., 2018. Hay and seed transfer to re-establish rare grassland species and communities: How important are date and soil preparation? Biological Conservation 221, 182-189.

Brown, I.C., 1943. A rapid method of determining exchangeable hydrogen and total exchangeable bases of soils. Soil Science 56, 353-358.

Cáceres, M.D., Legendre, P., 2009. Associations between species and groups of sites: indices and statistical inference. Ecology 90, 3566-3574.

Calvo-Polanco, M., Molina, S., Zamarreno, A.M., Garcia-Mina, J.M., Aroca, R., 2014. The Symbiosis with the Arbuscular Mycorrhizal Fungus Rhizophagus irregularis Drives Root Water Transport in Flooded Tomato Plants. Plant and Cell Physiology 55, 1017-1029.

Caporaso, J.G., Lauber, C.L., Walters, W.A., Berg-Lyons, D., Lozupone, C.A., Turnbaugh, P.J., Fierer, N., Knight, R., 2011. Global patterns of 16S rRNA diversity at a depth of millions of sequences per sample. Proceedings of the national academy of sciences 108, 4516-4522.

Ceulemans, T., Stevens, C.J., Duchateau, L., Jacquemyn, H., Gowing, D.J.G., Merckx, R., Wallace, H., van Rooijen, N., Goethem, T., Bobbink, R., Dorland, E., Gaudnik, C., Alard, D., Corcket, E., Muller, S., Dise, N.B., Dupre, C., Diekmann, M., Honnay, O., 2014. Soil phosphorus constrains biodiversity across European grasslands. Global Change Biology 20, 3814-3822.

Ceulemans, T., Van Geel, M., Jacquemyn, H., Boeraeve, M., Plue, J., Saar, L., Kasari, L., Peeters, G., van Acker, K., Crauwels, S., 2019. Arbuscular mycorrhizal fungi in European grasslands under nutrient pollution. Global Ecology and Biogeography 28, 1796-1805.

Choma, M., Barta, J., Santruckova, H., Urich, T., 2016. Low abundance of Archaeorhizomycetes among fungi in soil metatranscriptomes. Scientific Reports 6.

Collins, C.D., Foster, B.L., 2009. Community-level consequences of mycorrhizae depend on phosphorus availability. Ecology 90, 2567-2576.

De Graaf, M.C., Bobbink, R., Smits, N.A., Van Diggelen, R., Roelofs, J.G., 2009. Biodiversity, vegetation gradients and key biogeochemical processes in the heathland landscape. Biological conservation 142, 2191-2201.

Donath, T.W., Bissels, S., Holzel, N., Otte, A., 2007. Large scale application of diaspore transfer with plant material in restoration practice - Impact of seed and microsite limitation. Biological Conservation 138, 224-234.

Edgar, R.C., 2013. UPARSE: highly accurate OTU sequences from microbial amplicon reads. Nature methods 10, 996-998.

Fierer, N., 2017. Embracing the unknown: disentangling the complexities of the soil microbiome. Nature Reviews Microbiology 15, 579-590.

Gardes, M., Bruns, T.D., 1993. ITS primers with enhanced specificity for basidiomycetes-application to the identification of mycorrhizae and rusts. Molecular ecology 2, 113-118.

Hambleton, S., Sigler, L., 2005. Meliniomyces, anew anamorph genus for root-associated fungi with phylogenetic affinities to Rhizoscyphus ericae (Hymenoscyphus ericae), Leotiomycetes. Studies in Mycology, 1-27.

Harris, J., 2009. Soil microbial communities and restoration ecology: facilitators or followers? Science 325, 573-574.

Hazard, C., Gosling, P., Van Der Gast, C.J., Mitchell, D.T., Doohan, F.M., Bending, G.D., 2013. The role of local environment and geographical distance in determining community composition of arbuscular mycorrhizal fungi at the landscape scale. The ISME journal 7, 498-508.

Heinen, R., Hannula, S.E., De Long, J.R., Huberty, M., Jongen, R., Kielak, A., Steinauer, K., Zhu, F., Bezemer, T.M., 2020. Plant community composition steers grassland vegetation via soil legacy effects. Ecology Letters 23, 973-982.

Hulshof, C.M., Spasojevic, M.J., 2020. The edaphic control of plant diversity. Global Ecology and Biogeography 29, 1634-1650.

Jiang, Q.Y., Tan, S.Y., Zhuo, F., Yang, D.J., Ye, Z.H., Jing, Y.X., 2016. Effect of Funneliformis mosseae on the growth, cadmium accumulation and antioxidant activities of Solanum nigrum. Applied Soil Ecology 98, 112-120.

Kardol, P., Bezemer, T.M., Van Der Putten, W.H., 2009. Soil Organism and Plant Introductions in Restoration of Species-Rich Grassland Communities. Restoration Ecology 17, 258-269.

Kardol, P., Cornips, N.J., van Kempen, M.M.L., Bakx-Schotman, J.M.T., van der Putten, W.H., 2007. Microbe-mediated plant-soil feedback causes historical contingency effects in plant community assembly. Ecological Monographs 77, 147-162.

Kivlin, S.N., Winston, G.C., Goulden, M.L., Treseder, K.K., 2014. Environmental filtering affects soil fungal community composition more than dispersal limitation at regional scales. Fungal Ecology 12, 14-25.

Klimkowska, A., Van Diggelen, R., Bakker, J.P., Grootjans, A.P., 2007. Wet meadow restoration in Western Europe: a quantitative assessment of the effectiveness of several techniques. Biological Conservation 140, 318-328.

Kõljalg, U., Larsson, K.H., Abarenkov, K., Nilsson, R.H., Alexander, I.J., Eberhardt, U., Erland, S., Høiland, K., Kjøller, R., Larsson, E., 2005. UNITE: a database providing web-based methods for the molecular identification of ectomycorrhizal fungi. New Phytologist 166, 1063-1068.

Koziol, L., Bever, J.D., 2017. The missing link in grassland restoration: arbuscular mycorrhizal fungi inoculation increases plant diversity and accelerates succession. Journal of Applied Ecology 54, 1301-1309.

Koziol, L., Schultz, P.A., House, G.L., Bauer, J.T., Middleton, E.L., Bever, J.D., 2018. The Plant Microbiome and Native Plant Restoration: The Example of Native Mycorrhizal Fungi. Bioscience 68, 996-1006.

Li, J., Xie, S., Wilson, G.W., Cobb, A.B., Tang, S., Guo, L., Wang, K., Deng, B., 2020. Plant–microbial interactions facilitate grassland species coexistence at the community level. Oikos 129, 533-543.

Liang, M.X., Liu, X.B., Gilbert, G.S., Zheng, Y., Luo, S., Huang, F.M., Yu, S.X., 2016. Adult trees cause density-dependent mortality in conspecific seedlings by regulating the frequency of pathogenic soil fungi. Ecology Letters 19, 1448-1456.

Middleton, E.L., Bever, J.D., 2012. Inoculation with a Native Soil Community Advances Succession in a Grassland Restoration. Restoration Ecology 20, 218-226.

Neuenkamp, L., Prober, S.M., Price, J.N., Zobel, M., Standish, R.J., 2019. Benefits of mycorrhizal inoculation to ecological restoration depend on plant functional type, restoration context and time. Fungal Ecology 40, 140-149.

Norros, V., Penttila, R., Suominen, M., Ovaskainen, O., 2012. Dispersal may limit the occurrence of specialist wood decay fungi already at small spatial scales. Oikos 121, 961-974.

Ohsowski, B.M., Klironomos, J.N., Dunfield, K.E., Hart, M.M., 2012. The potential of soil amendments for restoring severely disturbed grasslands. Applied Soil Ecology 60, 77-83.

Oksanen, J., Kindt, R., Legendre, P., O'Hara, B., Stevens, M.H.H., Oksanen, M.J., Suggests, M., 2007. The vegan package. Community ecology package 10, 719.

Olsen, S.R., 1954. Estimation of available phosphorus in soils by extraction with sodium bicarbonate. US Department of Agriculture.

Oster, M., Ask, K., Cousins, S.A.O., Eriksson, O., 2009. Dispersal and establishment limitation reduces the potential for successful restoration of semi-natural grassland communities on former arable fields. Journal of Applied Ecology 46, 1266-1274.

Partzsch, M., Faulhaber, M., Meier, T., 2018. The effect of the dominant grass Festuca rupicola on the establishment of rare forbs in semi-dry grasslands. Folia Geobotanica 53, 103-113.

Peay, K.G., Garbelotto, M., Bruns, T.D., 2010. Evidence of dispersal limitation in soil microorganisms: Isolation reduces species richness on mycorrhizal tree islands. Ecology 91, 3631-3640.

Pywell, R.F., Bullock, J.M., Tallowin, J.B., Walker, K.J., Warman, E.A., Masters, G., 2007. Enhancing diversity of species-poor grasslands: an experimental assessment of multiple constraints. Journal of Applied Ecology 44, 81-94.

Radujkovic, D., van Diggelen, R., Bobbink, R., Weijters, M., Harris, J., Pawlett, M., Vicca, S., Verbruggen, E., 2020. Initial soil community drives heathland fungal community trajectory over multiple years through altered plant-soil interactions. New Phytologist 225, 2140-2151.

Read, D.J., Leake, J.R., Perez-Moreno, J., 2004. Mycorrhizal fungi as drivers of ecosystem processes in heathland and boreal forest biomes. Canadian Journal of Botany 82, 1243-1263.

Resch, M.C., Schutz, M., Graf, U., Wagenaar, R., van der Putten, W.H., Risch, A.C., 2019. Does topsoil removal in grassland restoration benefit both soil nematode and plant communities? Journal of Applied Ecology 56, 1782-1793.

Rognes, T., Flouri, T., Nichols, B., Quince, C., Mahé, F., 2016. VSEARCH: a versatile open source tool for metagenomics. PeerJ 4, e2584.

Rosling, A., Cox, F., Cruz-Martinez, K., Ihrmark, K., Grelet, G.A., Lindahl, B.D., Menkis, A., James, T.Y., 2011. Archaeorhizomycetes: Unearthing an Ancient Class of Ubiquitous Soil Fungi. Science 333, 876-879.

Schelfhout, S., Mertens, J., Perring, M.P., Raman, M., Baeten, L., Demey, A., Reubens, B., Oosterlynck, S., Gibson-Roy, P., Verheyen, K., De Schrijver, A., 2017. P-removal for restoration of Nardus grasslands on former agricultural land: cutting traditions. Restoration Ecology 25, S178-S187.

Schelfhout, S., Wasof, S., Mertens, J., Vanhellemont, M., Demey, A., Haegeman, A., DeCock, E., Moeneclaey, I., Vangansbeke, P., Viaene, N., Baeyen, S., De Sutter, N., Maes, M., van der Putten, W.H., Verheyen, K., De Schrijver, A., 2021. Effects of bioavailable phosphorus and soil biota on typical Nardus grassland species in competition with fast-growing plant species. Ecological Indicators 120.

Semchenko, M., Leff, J.W., Lozano, Y.M., Saar, S., Davison, J., Wilkinson, A., Jackson, B.G., Pritchard, W.J., De Long, J.R., Oakley, S., Mason, K.E., Ostle, N.J., Baggs, E.M., Johnson, D., Fierer, N., Bardgett, R.D., 2018. Fungal diversity regulates plant-soil feedbacks in temperate grassland. Science Advances 4.

Tedersoo, L., Bahram, M., Põlme, S., Kõljalg, U., Yorou, N.S., Wijesundera, R., Ruiz, L.V., Vasco-Palacios, A.M., Thu, P.Q., Suija, A., 2014. Global diversity and geography of soil fungi. Science 346.

Torok, P., Vida, E., Deak, B., Lengyel, S., Tothmeresz, B., 2011. Grassland restoration on former croplands in Europe: an assessment of applicability of techniques and costs. Biodiversity and Conservation 20, 2311-2332.

Torrez, V., Ceulemans, T., Mergeay, J., de Meester, L., Honnay, O., 2016. Effects of adding an arbuscular mycorrhizal fungi inoculum and of distance to donor sites on plant species recolonization following topsoil removal. Applied Vegetation Science 19, 7-19.

Vahter, T., Bueno, C.G., Davison, J., Herodes, K., Hiiesalu, I., Kasari-Toussaint, L., Oja, J., Olsson, P.A., Sepp, S.K., Zobel, M., Vasar, M., Opik, M., 2020. Co-introduction of native mycorrhizal fungi and plant seeds accelerates restoration of post-mining landscapes. Journal of Applied Ecology.

Vainio, E.J., Hantula, J., 2000. Direct analysis of wood-inhabiting fungi using denaturing gradient gel electrophoresis of amplified ribosomal DNA. Mycological research 104, 927-936.

van der Bij, A.U., Weijters, M.J., Bobbink, R., Harris, J.A., Pawlett, M., Ritz, K., Benetkova, P., Moradi, J., Frouz, J., van Diggelen, R., 2018. Facilitating ecosystem assembly: Plant-soil interactions as a restoration tool. Biological Conservation 220, 272-279.

van der Heijden, M.G.A., Klironomos, J.N., Ursic, M., Moutoglis, P., Streitwolf-Engel, R., Boller, T., Wiemken, A., Sanders, I.R., 1998. Mycorrhizal fungal diversity determines plant biodiversity, ecosystem variability and productivity. Nature 396, 69-72.

Van Geel, M., Jacquemyn, H., Plue, J., Saar, L., Kasari, L., Peeters, G., van Acker, K., Honnay, O., Ceulemans, T., 2018. Abiotic rather than biotic filtering shapes the arbuscular mycorrhizal fungal communities of European seminatural grasslands. New Phytologist 220, 1262-1272.

Verhagen, R., Klooker, J., Bakker, J., van Diggelen, R., 2001. Restoration success of low-production plant communities on former agricultural soils after top-soil removal. Applied vegetation science 4, 75-82.

Vohnik, M., Fendrych, M., Kolarik, M., Gryndler, M., Hrrselova, H., Albrechtova, J., Vosatka, M., 2007. The ascomycete Meliniomyces variabilis isolated from a sporocarp of Hydnotrya tulasnei (Pezizales) intracellularly colonises roots of ecto-and ericoid mycorrhizal host plants. Czech Mycology 59, 215.

Vohnik, M., Mrnka, L., Lukesova, T., Bruzone, M.C., Kohout, P., Fehrer, J., 2013. The cultivable endophytic community of Norway spruce ectomycorrhizas from microhabitats lacking ericaceous hosts is dominated by ericoid mycorrhizal Meliniomyces variabilis. Fungal Ecology 6, 281-292.

Walker, K.J., Stevens, P.A., Stevens, D.P., Mountford, J.O., Manchester, S.J., Pywell, R.F., 2004. The restoration and re-creation of species-rich lowland grassland on land formerly managed for intensive agriculture in the UK. Biological Conservation 119, 1-18.

Wassen, M.J., Venterink, H.O., Lapshina, E.D., Tanneberger, F., 2005. Endangered plants persist under phosphorus limitation. Nature 437, 547-550.

Weidlich, E.W.A., Nelson, C.R., Maron, J.L., Callaway, R.M., Delory, B.M., Temperton, V.M., 2021. Priority effects and ecological restoration. Restoration Ecology 29.

Werner, C.M., Vaughn, K.J., Stuble, K.L., Wolf, K., Young, T.P., 2016. Persistent asymmetrical priority effects in a California grassland restoration experiment. Ecological Applications 26, 1624-1632.

White, T.J., Bruns, T., Lee, S., Taylor, J., 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. PCR protocols: a guide to methods and applications 18, 315-322.

Wubs, E., van Heusden, T., Melchers, P.D., Bezemer, M., 2019a. Soil inoculation steers plant-soil feedback, supressing ruderal plant species. Frontiers in Ecology and Evolution 7, 451.

Wubs, E.J., van der Putten, W.H., Mortimer, S.R., Korthals, G.W., Duyts, H., Wagenaar, R., Bezemer, T.M., 2019b. Single introductions of soil biota and plants generate long-term legacies in soil and plant community assembly. Ecology Letters 22, 1145-1151.

Wubs, E.R.J., van der Putten, W.H., Bosch, M., Bezemer, T.M., 2016. Soil inoculation steers restoration of terrestrial ecosystems. Nature Plants 2.

Supporting Information

The following Supporting Information is available for this article:

Table S1 Site characteristics

Fig. S1 Experimental setup

Fig. S2 Treatment effects on vegetation diversity of target species only

Fig. S3 Treatment effects on relative abundances of the dominant fungal families

Fig. S4 Treatment effects on prokaryote diversity and abundance

Fig. S5 Vegetation community composition over three years

Fig. S6 Treatment effects on prokaryote community composition

Fig. S7 Indicator OTUs of the different treatments

Fig. S8 Distribution of the number of fungal indicator OTUs of the different treatments in comparison to the donor

Table S1 Characteristics of the 16 experimental grassland sites (as measured in the control plots with (AB) and without (GB) prior grass layer removal) and of the late-successional donor site.

			Latitude	Longitude	KCl-extraction			Gravimetric			$NaHCO_3$-extraction	1M Ammonium Acetate - extraction									
					pH_{KCl}	Ammonium (NH_4^+)	Nitrate (NO_3^-)	Organic matter content	Bulk density	Moisture content	Plant-available P (P-Olsen)	Exchangeable H^+	Calcium	Potassium	Magnesium	Sodium	Iron	Aluminium	Manganese	CEC	Base saturation
Site_Code	**Start nutrient depletion (year)**	**Treatment (Grass layer removal YES or NO)**				**mmol L^{-1}**	**mmol L^{-1}**	**%**	**kg L^{-1}**	**%**	**mmol L^{-1}**	**meq $100g^{-1}$ DW**									**%**
1AB	2009	YES	51.33890	5.00320	4.43	0.185	0.991	3.49	1.27	15.40	1.74	2.07	3.26	0.06	0.34	0.02	0.01	0.01	0.04	5.81	64.1
1GB	2009	NO	51.33890	5.00320	4.93	0.345	0.113	6.52	1.11	23.30	0.98	2.35	5.53	0.10	0.71	0.02	0.01	0.02	0.04	8.78	72.6
2AB	1992	YES	51.34668	5.01918	4.01	0.136	0.081	4.98	1.46	16.10	1.21	4.39	1.68	0.07	0.08	0.01	0.01	0.03	0.04	6.30	29.2
2GB	1992	NO	51.34668	5.01918	3.93	0.304	0.170	6.03	1.47	18.50	0.43	5.99	2.07	0.12	0.13	0.03	0.02	0.03	0.07	8.45	27.9
3AB	1991	YES	51.33550	5.02060	3.97	0.216	0.167	2.06	1.42	6.30	2.34	1.86	1.03	0.07	0.25	0.02	0.00	0.02	0.03	3.27	41.8
3GB	1991	NO	51.33550	5.02060	4.38	0.485	0.071	3.49	1.17	8.20	1.63	2.24	1.92	0.12	0.61	0.02	0.00	0.01	0.03	4.95	54.2
4AB	1990	YES	51.33360	5.02120	3.39	0.608	0.095	7.86	1.31	13.80	0.21	6.90	1.39	0.12	0.18	0.03	0.02	0.03	0.01	8.67	19.8
4GB	1990	NO	51.33360	5.02120	3.59	0.374	0.037	9.29	1.14	15.60	0.45	7.70	1.38	0.18	0.15	0.04	0.06	0.09	0.05	9.66	18.3
5AB	2005	YES	51.33621	5.02868	3.84	0.105	0.153	3.59	1.44	13.90	2.59	4.33	1.18	0.05	0.12	0.02	0.01	0.03	0.02	5.75	23.7
5GB	2005	NO	51.33621	5.02868	3.71	0.203	0.028	4.30	1.12	14.00	1.31	4.16	1.20	0.07	0.18	0.02	0.02	0.03	0.02	5.68	26.0
6AB	1996	YES	51.33165	5.02502	3.37	0.063	0.057	3.86	1.33	10.90	1.12	4.33	0.80	0.05	0.10	0.02	0.01	0.01	0.01	5.33	18.7
6GB	1996	NO	51.33165	5.02502	3.45	0.236	0.058	4.10	1.09	11.40	1.10	4.35	0.92	0.07	0.19	0.03	0.01	0.01	0.01	5.58	22.0
7AB	2009	YES	51.32850	5.01140	3.79	0.077	0.010	2.70	1.43	7.30	4.09	3.00	0.74	0.05	0.08	0.01	0.01	0.01	0.02	3.92	22.8
7GB	2009	NO	51.32850	5.01140	3.75	0.174	0.017	4.89	1.10	9.50	1.88	3.54	1.09	0.09	0.16	0.03	0.02	0.02	0.05	4.99	27.5
8AB	1998	YES	51.32515	5.02175	3.44	0.064	0.027	2.86	1.41	8.60	6.85	4.86	0.14	0.05	0.04	0.02	0.01	0.02	0.01	5.15	4.9
8GB	1998	NO	51.32515	5.02175	3.38	0.203	0.045	3.96	1.37	11.30	5.10	4.70	0.31	0.09	0.11	0.02	0.01	0.04	0.02	5.30	10.1
9AB	1999	YES	51.32940	5.03420	4.07	0.077	0.028	3.17	1.40	10.90	4.58	3.30	1.71	0.03	0.15	0.03	0.00	0.02	0.01	5.26	36.9
9GB	1999	NO	51.32940	5.03420	3.86	0.247	0.012	5.40	1.21	14.00	2.53	4.50	1.75	0.09	0.35	0.03	0.01	0.03	0.03	6.79	33.1
10AB	1996	YES	51.32760	5.03340	3.89	0.111	0.068	3.33	1.43	8.30	2.40	3.06	1.35	0.04	0.15	0.01	0.00	0.01	0.02	4.64	33.5
10GB	1996	NO	51.32760	5.03340	3.78	0.348	0.155	4.11	1.29	12.30	1.88	3.28	1.34	0.07	0.19	0.03	0.00	0.02	0.03	4.96	33.2
11AB	2006	YES	51.31881	5.03456	3.89	0.069	0.013	2.76	1.53	19.10	2.87	2.59	1.08	0.06	0.16	0.03	0.01	0.02	0.01	3.96	33.9
11GB	2006	NO	51.31881	5.03456	3.89	0.159	0.013	4.73	0.97	22.00	1.69	3.18	1.94	0.10	0.43	0.03	0.02	0.02	0.03	5.74	43.6
12AB	2006	YES	51.33132	5.01564	4.07	0.732	1.083	5.76	1.11	14.30	3.47	4.33	3.22	0.07	0.42	0.02	0.01	0.02	0.04	8.13	46.0
12GB	2006	NO	51.33132	5.01564	4.06	0.408	1.630	10.94	0.98	21.00	1.97	6.03	4.73	0.17	0.50	0.02	0.02	0.03	0.08	11.58	46.8
13AB	1999	YES	51.32910	5.03200	3.92	0.062	0.108	2.45	1.53	9.10	4.95	2.98	1.02	0.03	0.11	0.01	0.00	0.02	0.01	4.18	28.0
13GB	1999	NO	51.32910	5.03200	3.98	0.466	0.011	5.11	1.16	9.10	3.16	3.40	1.65	0.08	0.40	0.02	0.00	0.02	0.03	5.61	38.6
14AB	2009	YES	51.34210	5.02760	4.30	0.298	0.484	3.35	1.48	8.70	3.54	3.28	2.45	0.04	0.14	0.01	0.01	0.04	0.02	6.00	44.2
14GB	2009	NO	51.34210	5.02760	4.48	0.296	0.026	7.48	0.93	11.50	1.82	4.13	4.17	0.12	0.56	0.03	0.01	0.03	0.05	9.09	53.8
15AB	2002	YES	51.32970	5.00040	4.34	0.140	1.969	4.56	1.31	16.00	3.44	3.80	3.16	0.06	0.31	0.01	0.01	0.01	0.04	7.40	48.0
15GB	2002	NO	51.32970	5.00040	4.50	0.249	0.159	5.76	1.30	19.30	3.94	3.72	3.72	0.09	0.81	0.01	0.01	0.02	0.04	8.41	55.1
16AB	2006	YES	51.33530	4.98918	4.68	0.168	0.048	6.26	1.29	19.80	1.35	3.19	4.44	0.10	0.57	0.01	0.01	0.03	0.02	8.38	61.5
16GB	2006	NO	51.33530	4.98918	4.95	0.238	0.107	7.99	0.99	23.70	1.08	3.10	5.63	0.13	1.13	0.02	0.01	0.02	0.02	10.06	68.8
REF-A		Donor	51.33940	5.00120	3.29	0.078	0.016	18.88	0.53	41.20	0.11	12.86	1.49	0.34	0.39	0.19	0.10	0.12	0.01	15.50	15.5
REF-B		Donor	51.33940	5.00120	3.17	0.048	0.009	9.93	0.86	33.60	0.15	10.12	0.83	0.21	0.29	0.12	0.04	0.06	0.01	11.66	12.3
REF-C		Donor	51.33940	5.00120	3.19	0.092	0.012	12.38	0.89	32.20	0.22	11.07	1.51	0.28	0.32	0.15	0.04	0.06	0.05	13.47	16.8

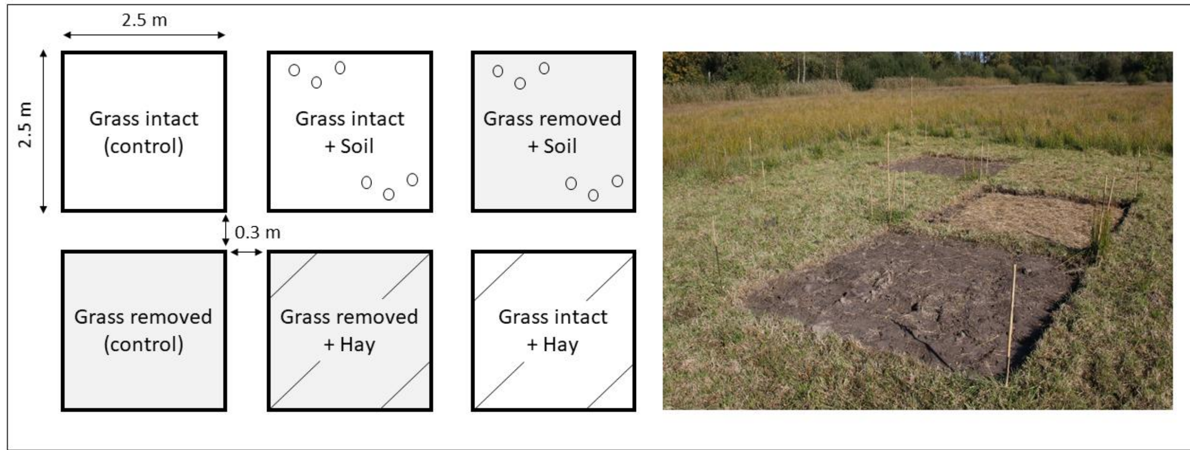

Fig. S1 Example of the experimental setup at one grassland site, including six plots with different treatment combinations. The order of the treatment combinations was randomized at each of the 16 sites.

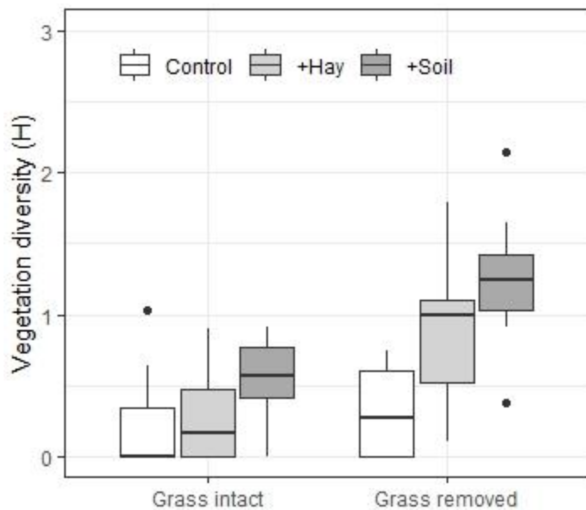

Fig. S2 Effects after three growing seasons of different treatments on vegetation diversity in 16 post-agricultural grasslands, only including target species that occurred in the donor grassland. Experimental treatments included initial removal of the grass layer (yes or no), and nested within were addition treatments of nothing (control), soil inoculation or hay transfer (both collected in the donor site). Diversity was calculated as the Shannon-Wiener diversity index (H) of the vegetation matrix that included target species only.

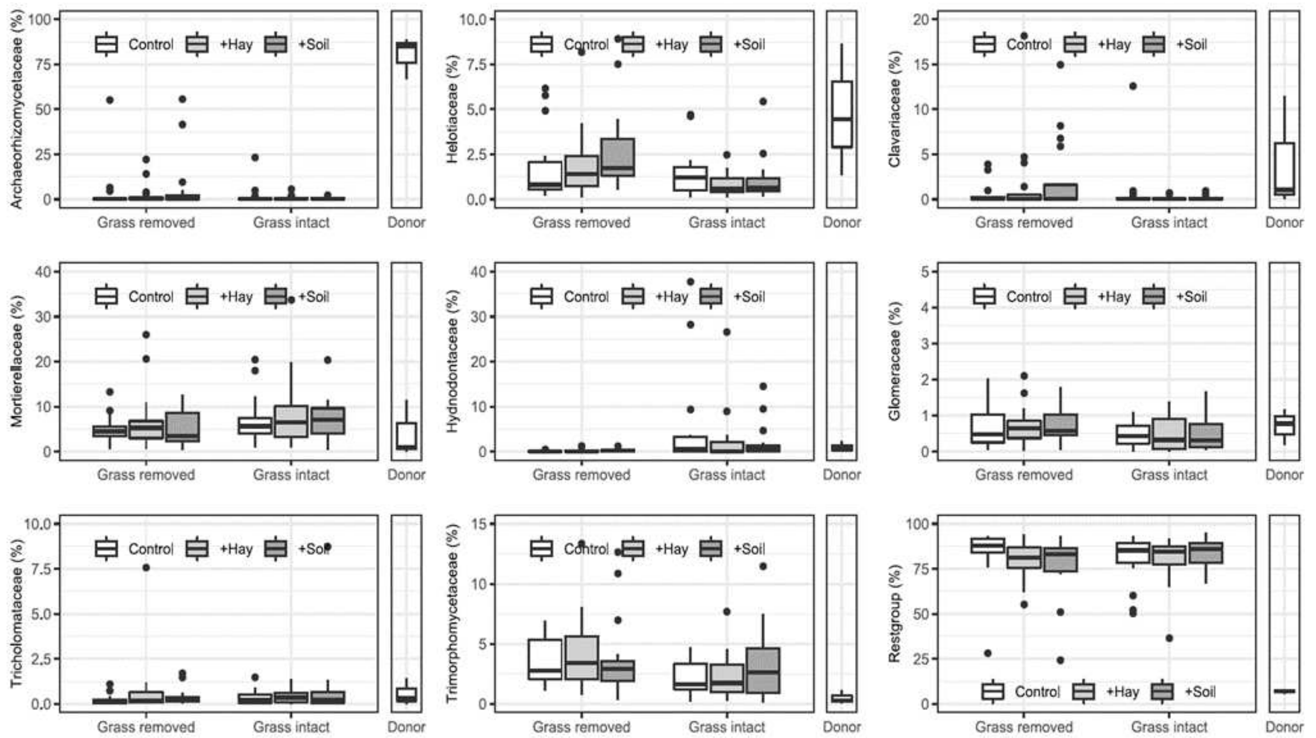

Fig. S3 Effects of different treatments on relative abundances (% reads) of the eight most dominant families in the late-successional donor grassland. The rest group contains a remaining 259 families. Experimental treatments included initial removal of the grass layer (yes or no), and nested within were addition treatments of nothing (control), soil inoculation or hay transfer (both collected in a late-successional donor site). Families are ranked from highest (top left) to lower (bottom right) abundance in the donor grassland.

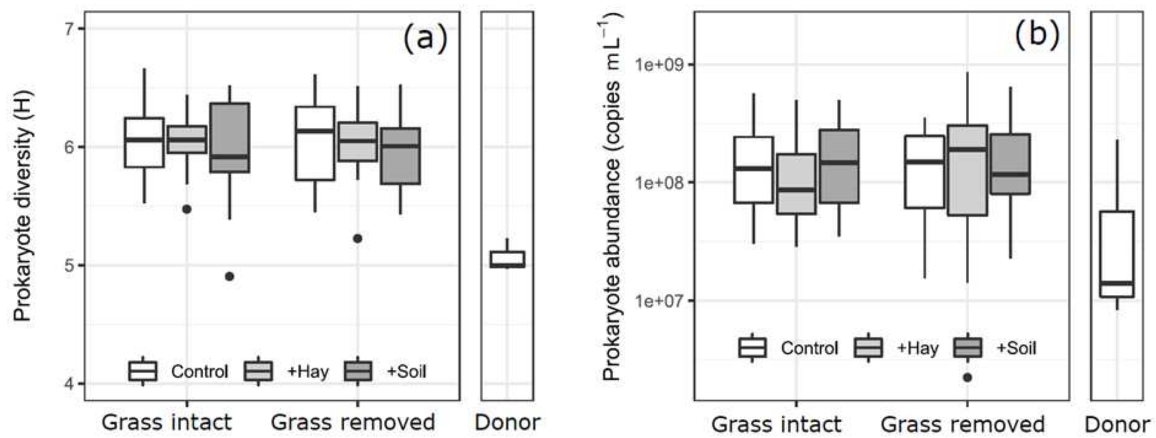

Fig. S4 Effects after three growing seasons of different treatments on (a) prokaryote diversity and (b) prokaryote abundance (log10-scale) in the topsoil of 16 post-agricultural grasslands, with the late-successional donor site as comparison. Experimental treatments included initial removal of the grass layer (yes or no), and nested within were addition treatments of nothing (control), soil inoculation or hay transfer (both collected in the donor site). Diversity was calculated as the Shannon-Wiener diversity index (H). Prokaryote abundance was calculated as 16S rRNA gene copy numbers per milliliter of soil.

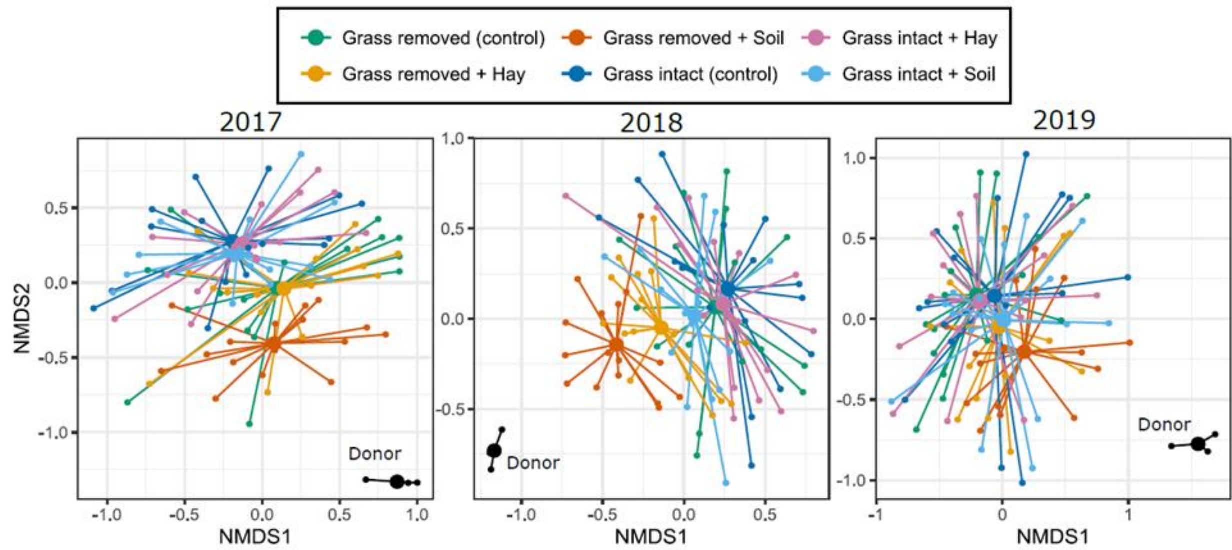

Fig. S5 Effects during three growing seasons of different treatments on vegetation community composition (stress = 0.24, 0.19 and 0.18) in 96 experimental plots distributed over 16 post-agricultural grasslands and one late-successional donor grassland, using nonmetric multidimensional scaling (NMDS). The main (colored) panels show plot grouping by experimental treatment, the embedded (black) panels show the same ordination but with plot grouping by grassland site. The analyses are based on Bray-Curtis dissimilarity indices of the sample*species matrix. Groups are joined by a central "circle" representing group centroids, whereas only small points represent true individual sample scores.

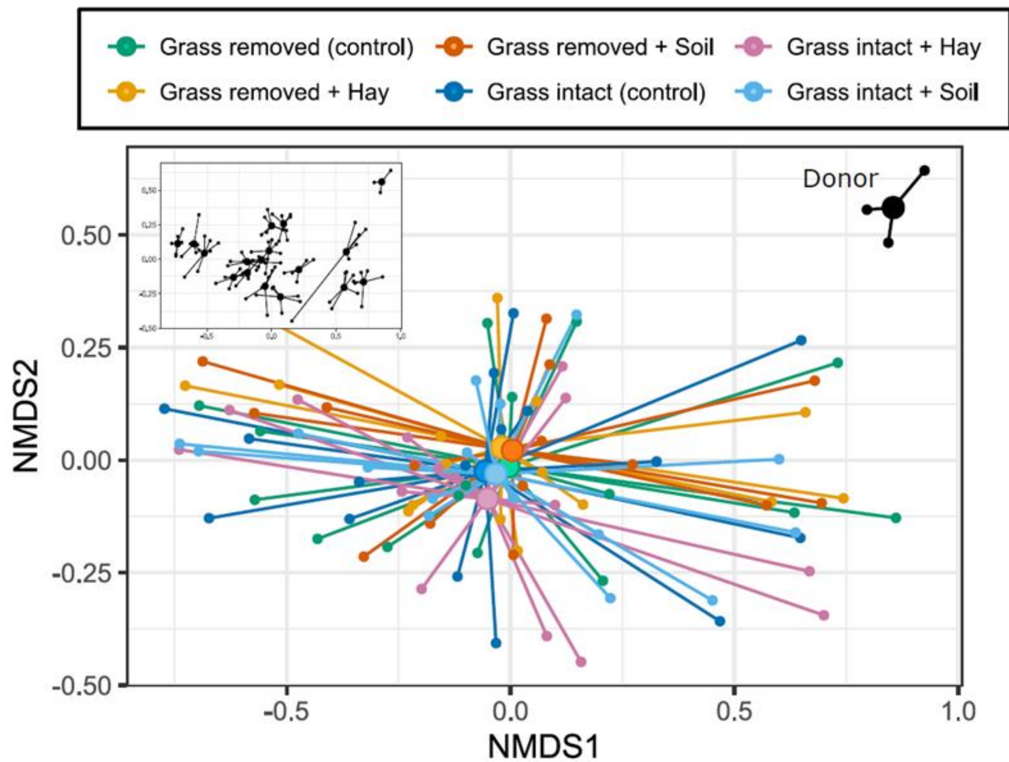

Fig. S6 Effects after three growing seasons of different treatments on prokaryote (stress = 0.09) community composition in 96 experimental plots distributed over 16 post-agricultural grasslands and one late-successional donor grassland, using nonmetric multidimensional scaling (NMDS, k = 2). The main (colored) panels show plot grouping by experimental treatment, the embedded (black) panels show the same ordination but with plot grouping by grassland site. The analyses are based on Bray-Curtis dissimilarity indices of the sample*species and sample*OTU matrices. Groups are joined by a central "circle" representing group centroids, whereas the small dots represent the individual sample scores.

Fig. S7 The number of fungal OTUs, grouped per fungal family, that were identified as indicators for the different treatments. Families are ranked from highest (read) abundance in the late-successional donor grassland (left on x-axis) to lowest abundance (right on x-axis). Blue bars represent the cumulative number of indicator OTUs from the corresponding family that were also simultaneously found in the donor grassland, while red bars represent the number of indicator OTUs from that family that were not found in the donor grassland. OTUs that could not be attributed to a corresponding family were excluded from the graph. The larger the number and height of the blue bars, the more likely it is that taxa within the corresponded treatment originated from the donor site.

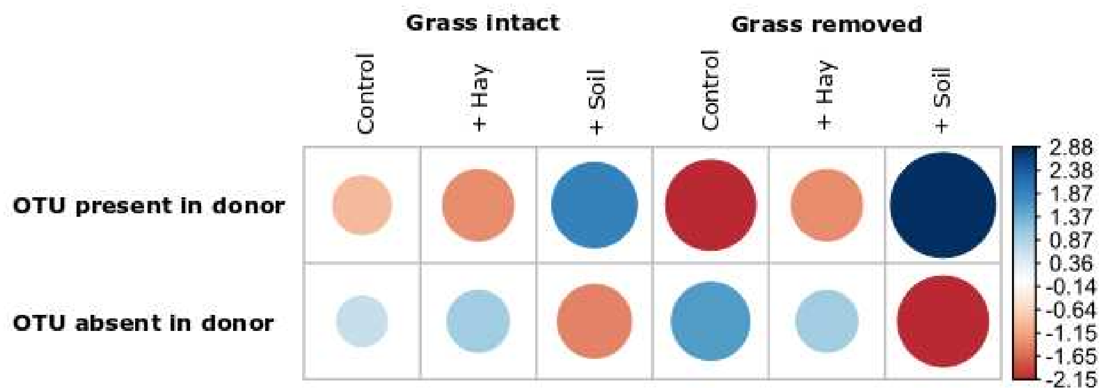

Fig. S8 Distribution of the number of fungal indicator OTUs of the different treatments that were also found in the late-successional donor grassland (top row) or that were absent in the donor grassland (bottom row). The size of the circles is indicative for the number of indicator OTUs within the corresponding group, and the analysis includes all indicator OTUs (also of unknown families). Positive standardized pearson residuals (in blue) indicate a positive association between the corresponding row (presence or absence) and column (treatment) variables, while negative residuals (in red) indicate a negative association between the corresponding row and column variables. For example, the soil inoculation treatments ("+ Soil") contained a relatively large proportion of indicator OTUs that also occurred in the donor grassland (blue circles in top row), and relatively few OTUs that did not occur in the donor grassland (red circles in bottom row).