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Research

Contrasting effects of host identity, plant community, and local species pool on the composition and colonization levels of arbuscular mycorrhizal fungal community in a temperate grassland

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Summary

- Arbuscular mycorrhizal fungi (AMFs) are important plant symbionts, but we know little about the effects of plant taxonomic identity or functional group on the AMF community composition. To examine the effects of the surrounding plant community, of the host, and of the AMF pool on the AMF community in plant roots, we manipulated plant community composition in a long-term field experiment.
- Within four types of manipulated grassland plots, seedlings of eight grassland plant species were planted for 12 wk, and AMFs in their roots were quantified. Additionally, we characterized the AMF community of individual plots (as their AMF pool) and quantified plot abiotic conditions.
- The largest determinant of AMF community composition was the pool of available AMFs, varying at metre scale due to changing soil conditions. The second strongest predictor was the host functional group. The differences between grasses and dicotyledonous forbs in AMF community variation and diversity were much larger than the differences among species within those groups. High cover of forbs in the surrounding plant community had a strong positive effect on AMF colonization intensity in grass hosts.
- Using a manipulative field experiment enabled us to demonstrate direct causal effects of plant host and surrounding vegetation.

Introduction

Arbuscular mycorrhizal fungi (AMFs) represent an important component of many types of ecosystems across the world, connecting plant roots with the surrounding soil by their mycelia. AMFs are obligate symbionts of a large proportion of vascular plant species (Van der Heijden *et al.*, 2018) and provide multiple important services to primary producers within many terrestrial ecosystems. AMFs trade acquired soil phosphorus (P) and nitrogen (N) with their hosts for photosynthetically fixed carbon (C) (Bücking & Kafle, 2015; Konvalinková *et al.*, 2017). Additionally, AMFs affect water transport to plants (Mariotte *et al.*, 2015), competition between plants (Weremijewicz *et al.*, 2018), and their resistance to herbivores (Middleton *et al.*, 2015).

Despite most AMF species being able to enter symbiosis with most mycorrhizal hosts in a plant community, there seem to be important differences for both partners in the profitability of particular fungus–plant combinations (Bever *et al.*, 1996; Klironomos, 2003). Published studies have explored the differences in AMF communities that can be explained by the functional traits of plant hosts (Chagnon *et al.*, 2013; López-García *et al.*, 2017; Neuenkamp *et al.*, 2018). Trait differences are often summarized by the classification of hosts into functional groups (Dassen *et al.*, 2017; Gui *et al.*, 2018). Some studies identify differences of AMF communities between dicotyledonous herbs (which we henceforth call forbs) and grasses (Albarracín Orio *et al.*, 2016; Gui *et al.*, 2018), but are mostly based on ad hoc sampling of species present across a range of environmental conditions.

Although it seems obvious that besides the functional properties of host plant species we also need to know functional properties of fungal symbionts, their traits have been found difficult to study (Van der Heijden & Scheublin, 2007; Chagnon *et al.*, 2013). As a proxy for the functional differentiation of AMFs, their phylogeny (Maherali & Klironomos, 2012) or even just their taxonomic position at the family level (Chagnon *et al.*, 2013; López-García *et al.*, 2017) have been used.

Besides the mutual effects of the AMFs and their plant hosts, the occurrence and abundance of AMFs are known to be affected by multiple soil parameters, with soil pH playing a notable role (Fitzsimons *et al.*, 2008; Davison *et al.*, 2015; Bouffaud *et al.*, 2016; Oehl *et al.*, 2017; Van Geel *et al.*, 2018), particularly at smaller spatial scales (Rasmussen *et al.*, 2018), as well as the availability of N (Camenzind *et al.*, 2014; Fitzsimons *et al.*, 2008; Van Geel *et al.*, 2018) and P (Gosling *et al.*, 2013; Camenzind *et al.*, 2014).

Lekberg & Helgasson (2018) suggested that, although functions and services provided by AMFs are known from the results of glasshouse and laboratory experiments, it is often not known whether such processes are widely occurring under field conditions, so there is an urgent need to 'move mycorrhizal research into the field'. This concerns also the study of relationships between AMFs and their hosts or between the communities of those two organismal groups and the environment they inhabit, and particularly so using experimental approaches. Although field studies often provide data for describing the strength and directions of such relations (Fitzsimons *et al.*, 2008; Hiiesalu *et al.*, 2014), their conclusions are usually just correlative in their nature, given the exploratory character of the underlying sampling strategy. Yet, to test hypotheses concerning causal relationships between plant and AMF communities, manipulative experiments under realistic field conditions are needed. This particularly applies to the driver and passenger hypotheses of Hart *et al.* (2001), further advanced by Zobel & Öpik (2014). The driver and passenger hypotheses state, respectively, that AMF community variation induces variation in the plant community, and that variation in a plant community affects the AMF community.

This study focuses on causal effects of the plant community on the diversity and composition of AMF communities in the roots of plant seedlings that were established in a temperate grassland. In our experimental design, the effects of functional and taxonomic identity of seedlings were crossed with effects of the surrounding plant community. To effectively maximize variation in plant community composition without introducing too large heterogeneity in environmental conditions, we used a long-term (> 15 yr) field experiment with manipulated presence of two functional groups of plants (plots with mycorrhizal forbs, plots with mycorrhizal grasses, plots with a mixture of both plant groups, and nonmanipulated plots). Additionally, our study took into account the important effect of variation in AMFs available in individual plots (the AMF pool).

We addressed the following questions:

- Is the compositional variation of AMF communities in plant roots affected by the host functional type and taxonomic identity, by the plant community composition (four manipulated vegetation types), and by the pool of AMFs available in plots?
- If the effects of more than one of these factors are ascertained, what is their relative importance (strength) in shaping the AMF community composition?
- What effects do those three factors have on the alpha diversity of AMF communities in seedling roots and their phylogenetic diversity, serving as a proxy for functional diversity?
- What effects do the host functional type and plant community have on AMF colonization intensity?

Materials and Methods

Research site and plant community experiment

We collected data from a site close to the Zvíkov village, Czech Republic (48°59′20″N, 14°36′28″E), *c.* 500 m above sea level. The Cambisol soil has a low availability of macronutrients (DW per 1 kg of soil, A horizon): 2.2 mg ammonium (NH_4^+), 0.6 mg nitrate (NO_3^-), and 3.8 mg of extractable phosphates (Mehlich, 1984). The site is located on a shallow valley slope, and the vegetation represents an oligotrophic, traditionally managed meadow (mown in June, without any fertilization in the past 28 yr). The plant community is species rich (*c.* 85 species), with the following six species being most abundant, accounting for about half of the aboveground biomass at hay-cut time: *Alopecurus pratensis*, *Anthoxanthum odoratum*, *Holcus lanatus*, *Plantago lanceolata*, *Poa pratensis*, and *Sanguisorba officinalis*. The nomenclature of the plant species follows Kubát *et al.* (2002).

The field experiment described in this study was embedded in a long-term experiment, which focused on the role that mycorrhizal grasses and forbs play in a grassland ecosystem and therefore distinguished three groups of vascular plants: mycorrhizal grasses, mycorrhizal forbs, and nonmycorrhizal plant species (four sedge and six forb species). Nonmycorrhizal plant species (contributing *c.* 6–10% of the total aboveground biomass in nonmanipulated vegetation) were removed from three out of the total four plot types. Our initial definition of the set of nonmycorrhizal species was based on Harley & Harley (1987) and was later confirmed from samples collected at our site (P. Šmilauer, unpublished results).

The design of the long-term experiment used 10 randomized blocks arranged in two rows and five columns, running in parallel to local soil moisture and nutrient availability gradients. We modified the original vegetation by removing plants by hand, including part of their belowground organs. This weeding approach has many advantages, utilizing a species pool filtered by the local biotic and abiotic conditions and keeping a natural extent of genetic variation within the present species (Díaz *et al.*, 2003). Each replicate block contained all four designed vegetation types, each represented by a single $1 \times 1\ m^2$ plot: a reference plot (Ref plot) with intact vegetation (i.e. with no removal); a mixture plot (F+G plot) with both mycorrhizal forbs and grasses retained and nonmycorrhizal species removed; a forb plot (F plot) and a grass plot (G plot), both retaining only one functional group. The Ref plots allowed us to separate the potential effect of weeding disturbances by comparing them with weeded F+G plots. The removal of nonmycorrhizal species from the F+G plots ensured that the absence of one functional group in the F or G plots was not confounded by a disturbance effect. The extent of the soil and vegetation disturbance was considerably greater in the F plots and G plots during the initial phases of experiment (> 10 yr ago), but the subsequent yearly maintenance imposed similar disturbance across all three manipulated plot types.

We started weeding between 2001 and 2003 and since then continued with yearly maintenance. All plots (except Ref plots) were weeded three to four times each year, keeping soil disturbance to a minimum, and the soil in each weeded plot was cut at its borders to a depth of 15 cm every spring and autumn, to sever rhizomes or roots growing into plots. Transient changes in the retained components of the vegetation were observed only for the first few years across the weeded plots (Šmilauer & Šmilauerová, 2013).

Soil moisture was measured using an HH2 Moisture Meter with an SM200 sensor (DeltaT Devices Ltd, Cambridge, UK) at five locations inside the central 0.5 × 0.5 m^2 area in each plot on a spring day (April) and on a summer day (July) after a period of hot, dry weather. The availability of soil nutrients was quantified by taking soil samples to 0–10 cm soil depth in early June, measuring N-NH_4 and N-NO_3 concentrations (potassium chloride extraction and flow injection analysis), orthophosphate P concentrations (according to Mehlich, 1984) and the total N content and C : N ratio (using an NC 2100 Soil Analyzer; Carlo Erba, Milan, Italy).

AMF pool estimation and seedling AMF experiment

In mid April 2017, we collected four soil cores (diameter 2.4 cm, depth 10 cm) from each plot (see Fig. 1). The corers were washed with tap water, sterilized using Eli Gene Lab Cleaner A (Elisabeth Pharmacon, Brno, Czech Republic), and rinsed with sterilized deionized water between samples. Each soil sample was sieved using a sterilized sieve with a 2 mm mesh size to separate gross and intermediate roots from soil particles, and any stones or rhizomes detected were removed. We then used both the roots dried at 60°C and the sieved soil for separate DNA extractions, analysing each of the 160 core samples independently.

In early April 2017, we planted seedlings of eight grassland plant species originating from our research site (ninth species was non-mycorrhizal and hence ignored in this study): four grasses (*A. pratensis*, *A. odoratum*, *Agrostis capillaris*, and *H. lanatus*) and four forbs (*Betonica officinalis*, *Centaurea jacea*, *Knautia arvensis*, and *P. lanceolata*) into each experimental plot. We planted one replicate of each species into each of the six planting groups present in each plot (Fig. 1). Seedlings from seeds (surface-sterilized by sodium hypochlorite) were grown on sterilized sand for 1–2 wk before planting.

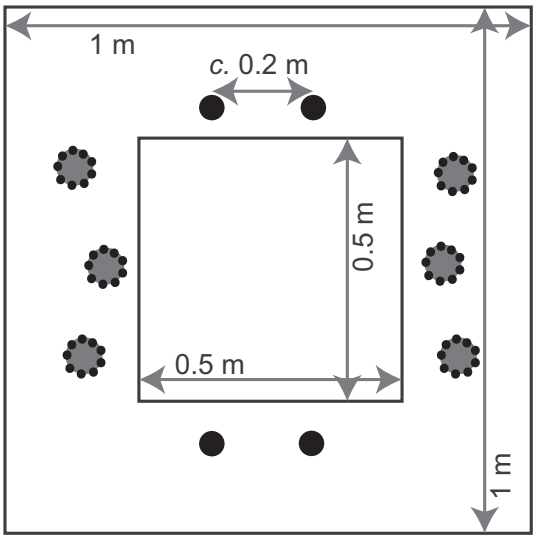

Fig. 1 Schematic outline of one of the 40 experimental plots. The central 0.5 × 0.5 m^2 square was excluded from destructive sampling; the four black-filled circles show the positions of four collected soil cores, and the six grey-filled circles with nine small black dots at their periphery suggest approximate positions of the nine species of seedlings planted. Distance between neighbouring plots and between neighbouring blocks is *c.* 0.5 m.

In early July 2017 (after 12 wk in the field), each of the 240 planting groups (40 plots × 6 groups) was harvested as a soil block, and the root systems of surviving seedlings were isolated. Roots of individual seedlings of a plant species from the same plot were pooled for molecular analyses (described later). Roots were dried at 60°C and frozen before DNA extraction. If there was a sufficient amount of roots available, AMF colonization level was quantified in several lateral roots using light microscopy.

DNA sequencing and bioinformatic analysis

DNA extraction Samples of soil and roots obtained from cores were processed using a modified protocol based on the powdered activated C method (Devi *et al.*, 2015) followed by sodium dodecyl sulphate precipitation (Verbylaitė *et al.*, 2010). Seedling roots were processed using a cetrimonium bromide protocol (Doyle & Doyle, 1987). All DNA extracts were purified using a PowerClean Pro DNA Clean-Up Kit (Qiagen, Venlo, the Netherlands).

PCR amplification and Illumina sequencing A 550 bp fragment of small subunit (SSU) ribosomal DNA was amplified by semi-nested PCR according to Dumbrell *et al.* (2011) except that AML2 (Lee *et al.*, 2008) was used as an AMF-specific primer. The Phusion High-Fidelity DNA Polymerase (New England Biolabs, Ipswich, MA, USA) was used to reduce amplification errors. The second PCR involved a Wanda primer (Integrated DNA Technologies, Coralville, IA, USA) fused with a sample-specific barcode sequence, allowing identification of individual samples. The resulting products were pooled and subjected to sequencing using a MiSeq and Reagent Kit v.3 (Illumina, San Diego, CA, USA) for paired end sequencing of 2 × 300 bp. A more detailed description of the procedures can be found in Supporting Information Methods S1.

Raw data were processed using software tools implemented in SEED v.2.0 (Větrovský *et al.*, 2018), MOTHUR v.1.39.5 (Schloss *et al.*, 2009) and PIPECRAFT v.1.0 (Anslan *et al.*, 2017). Raw reads were assembled and subjected to quality filtering and demultiplexing. Potentially chimeric sequences were removed and sequences were BLAST-identified against an extended version of the MaarjAM database (Öpik *et al.*, 2010) using the SSU pipeline (Vasar *et al.*, 2017). The following criteria were required for a match: sequence similarity of 97%, alignment length not differing from the length of the shorter of the query and subject sequences by > 5%, and a BLAST *e*-value of $< 1 \times 10^{-50}$. We considered sequences with similarities to the closest available AMF sequence exceeding 90% but below 97% as putative novel AMF taxa. These sequences were clustered using a 97% similarity threshold and resulting clusters were evaluated considering monophyly, statistical support and a minimal frequency threshold. We detected no novel AMF taxa using these criteria. Finally, up to 400 AMF sequences were randomly selected from each sample, yielding 321 802 sequences. Samples with < 90

sequences and AMF taxa with read frequency < 0.5% per sample were removed from statistical analysis.

To establish the phylogeny of AMF taxa, the most abundant sequence was selected for each virtual taxon (VTX) detected in our dataset and aligned with type sequences of other taxa from the MaarjAM database. Phylogenetic relationship was assessed under a general time-reversible model (Rodríguez *et al.*, 1990) with a discrete gamma distribution using maximum likelihood as implemented in PhyML v.3.0 (Guindon *et al.*, 2010). Additional details on the bioinformatic analysis can be found in Methods S1.

Light microscopy

Seedling roots selected for microscopy were stained using a modified protocol of Vierheilig *et al.* (2005). The colonization level was quantified as total colonization by AMF, colonization with arbuscules, colonization with vesicles, and the colonization by fine endophyte morphotypes at ×40 magnification. A more detailed description of the procedure can be found in Methods S1.

Statistical analyses

A more detailed description can be found in Methods S2.

Response data (read counts of individual VTX in individual samples from seedling roots) were $\log_e(x+1)$-transformed and standardized by Hellinger transformation (Legendre & Legendre, 2012, p. 331) before normal or partial redundancy analysis (RDA; Legendre & Legendre, 2012, p. 629). In variation-partitioning analyses, the samples of AMF community data were aggregated at the level of host plant functional group (grasses vs forbs), but differences among individual host species were also examined.

We first examined the question whether Ref and F+G plot types differed in AMF community composition. As we found no significant difference, they were pooled in the variation partitioning.

The effects of three predictors – host plant functional type, plot type, and the AMF pool (represented by three co-correspondence analysis (CoCA) axes; see next paragraph) – were compared using variation partitioning (Šmilauer & Lepš, 2014, p. 88) with an RDA. An alternative variation partitioning was also performed with experimental blocks as a covariate.

Case scores on the first three (significant) axes of a predictive CoCA (Šmilauer & Lepš, 2014) were used to describe the explanatory effects of the AMF pool of experimental plots (using combined soil and root data from the cores) on the composition of AMF community in seedling roots.

Variation partitioning was also performed with AMF community data aggregated at the level of AMF families using their definition in the MaarjAM database (Öpik *et al.*, 2010), with the same specification as for VTX data, but with the effect of AMF pool (predictive CoCA scores) based on relative proportions of AMF families.

All multivariate analyses were performed with the Canoco v.5.11 software (ter Braak & Šmilauer, 2018) except predictive CoCA, which was performed with the cocorresp package (Simpson, 2009) in the R software (R Core Team, 2018).

The taxonomic diversity of AMF in seedling roots was compared using two indices: the number of VTXs and Hill's N_2 diversity measure (Legendre & Legendre, 2012, section 6.5.1). Phylogenetic diversity was also used as a proxy of functional diversity, namely the Rao metric (Swenson, 2014), transforming the metric's value into an 'effective number of species' scale, and the mean nearest taxon distance statistic (Swenson, 2014). Both statistics were calculated with the picante package (Kembel *et al.*, 2010) in R.

To examine the effects of host plant functional group (or species identity), vegetation type, and the taxonomic richness of AMF pool, generalized linear mixed-effect models (GLMMs) were fitted with the lme4 package (Bates *et al.*, 2015) in R, assuming a Poisson distribution for VTX richness and gamma distributions for the other three diversity measures. We also examined the conditional effect of host species identity by evaluating the addition of species identifier as a fixed effect into a model already containing the effect of the host plant functional group.

GLMMs with an assumed gamma distribution for random variation and a log link function were used to model the differences of estimated colonization levels (total, arbuscular, vesicular colonization, and the colonization by the fine endophyte) among vegetation types and among functional groups and species, with the lme4 package (Bates *et al.*, 2015).

Spatial variation in the AMF pool composition was related to soil moisture and chemistry, using the AMF community data obtained from both soil and roots (pooled per plot). Partial RDA was used with the vegetation type (Ref and F+G types pooled) as a covariate, to exclude the indirect effects of vegetation manipulation via the AMF pool changes. Stepwise selection of predictors was employed (Blanchet *et al.*, 2008), and selected predictors were visualized in geographical space. To demonstrate the relationship of the effects of soil characteristics and the effects of the AMF pool, we also compared the results of an RDA using seedling AMF community as response data and selected soil parameters as predictors, with another RDA based on the same response and predictor data, but with the AMF pool descriptors (CoCA axes) as additional covariates. Calculations were performed with the Canoco v.5.11 software (ter Braak & Šmilauer, 2018).

Data availability statement

DNA sequences: GenBank accession nos. MK611457–MK611550.

Quality-controlled results of next-generation sequencing, environmental and design-related data are deposited on Dryad platform (www.datadryad.org) with a 1 yr embargo (https://doi.org/10.5061/dryad.5fv6p3p).

Results

Spectrum of AMF taxa

In the dataset representing AMF samples from seedling roots and collected soil cores, we identified 94 VTXs from together eight families of the MaarjAM database (Tables 1, S1 for GenBank

sequences). Sixty-three VTXs were shared among seedling roots, core roots, and core soil. There were eight VTXs unique to seedling roots, seven to core roots, and two to soil cores. Among partially shared taxa, the largest overlap (eight VTXs) was between core soils and seedling roots. Most VTXs were detected in both forbs and grasses (Fig. S1).

AMF community variation: effects of AMF pool composition, host plant, and plant community

We pooled the reference and mixture plot types for subsequent analyses, because we found that they do not differ significantly in the AMF community composition in seedling roots ($R^2_{adj} = 1.6\%$, pseudo-$F = 1.5$, ns).

Variation partitioning (Fig. 2a) identified the composition of AMF communities in the plots (the AMF pool) as the most effective predictor of the community composition in seedling roots (65.8% of the variation explained by all three predictor groups), followed by the effect of seedling functional group (Plant functional group, 21.2%), and the effect of vegetation type (Plot type, 18.0%). The overlap in explanation between AMF pool and Plot type was relatively small (5.0%).

When we removed the compositional variation that could be explained by the differences among the experimental blocks (Fig. 2b), the overall explanatory power of the three factors decreased (from 19.8% to 13.5%), primarily due to the reduced importance of the AMF pool predictor. However, the explained variation shared between AMF Pool and Plot Type predictors substantially increased. All the effects presented in Fig. 2 significantly contributed to the explained variation in AMF community composition of seedling roots (Table 2).

We also tested the differences in AMF community composition in plant roots in response to host species within each host plant functional group. These differences represented a small ($R^2_{adj} = 1.8\%$) but significant (pseudo-$F = 1.8$, $P < 0.001$) part of the total compositional variation (see Fig. 3, where the functional group differences are not excluded). The spread of plant species symbols in Fig. 3 indicates a similar extent of AMF community variation among grass and forb species and also that all the best-fitted VTXs had higher relative abundances in forb roots.

Table 1 Relative dominance of detected arbuscular mycorrhizal fungus (AMF) families, based on number of virtual taxa (VTXs; full dataset) and the count of reads (in seedling root samples).

AMF family[a]	No. of VTXs	Proportion of reads in seedling samples (%)
Glomeraceae	61	58.2
Acaulosporaceae	9	6.2
Claroideoglomeraceae	7	27.8
Diversisporaceae	6	0.3
Archaeosporaceae	4	1.1
Gigasporaceae	3	1.7
Paraglomeraceae	3	4.7
Ambisporaceae	1	< 0.1

[a]The Archaeosporaceae and Ambisporaceae families were pooled in the statistical analyses into the Archaeosporales order.

AMF community variation: effects at family level

In the variation partitioning predicting the relative frequencies of AMF families with the same three predictor groups as previously (and not using experimental block as a covariate), 12.9% of the total variation was explained. The AMF pool was responsible for the largest share of variation explained (50.3%, pseudo-$F = 3.0$, $P = 0.003$ for its unique effect), followed by the host plant functional group (35.4%, pseudo-$F = 5.0$, $P = 0.001$). Unlike the results of the analysis at VTX level, the effect of vegetation (plot) type was much smaller than the other two effects (14.1% of the total explained variation), and it was not significant whether judged as an independent effect (i.e. judging each predictor group independently of the others, pseudo-$F = 1.6$, $P = 0.240$) or as a unique effect (excluding the explained variation shared with the other predictors, pseudo-$F = 1.9$, $P = 0.116$).

When comparing the relative importance of AMF families between grass and forb hosts (Fig. 4), the AMF taxa from the Claroideoglomeraceae family occurred with a higher frequency in the roots of grass seedlings and AMF taxa from the Glomeraceae, Diversisporaceae, and Gigasporaceae families had higher frequency in forb roots, whereas there was no notable preference of the fungi from the other three AMF families.

Diversity of AMF community: effects of AMF pool richness, vegetation type, and host identity

When examining the effects of the three predictor groups (same as in preceding two sections, except that the AMF pool was represented by its richness) on taxonomic diversity of AMF fungi, we found that the only important effect was the host plant functional group (Table 3). Forb seedlings had, on average, a 43% higher richness of AMF VTX (95% confidence interval < 37.3%, 48.5-%>) than did grass seedlings. Similarly, the N_2 diversity was also, on average, 45.5% higher for forbs than for grasses.

The test for VTX richness differences among individual host species within their respective functional groups revealed (marginally) insignificant differences ($\chi^2_6 = 12.2$, $P = 0.057$; see also Fig. 5). For N_2 diversity, the conclusions were similar ($\chi^2_6 = 9.9$, ns).

When examining phylogenetic diversity, we found that only the host species functional group had a significant, strong effect (Table 4), with the AMF community in forb roots being more phylogenetically diverse (Fig. 6a), but with lower mean nearest taxon phylogenetic distance (MNTD, Fig. 6b). There were, however, no significant differences among the plant species within their respective functional groups ($\chi^2_6 = 8.9$, ns, for phylogeny-based effective number of VTXs, i.e. N_2 (VTX phylogeny), and $\chi^2_6 = 7.7$, ns, for MNTD). The differences between grasses and forbs in phylogeny-based N_2 (Fig. 6a) were smaller than for the taxonomy-based N_2 measure (Fig. 5b).

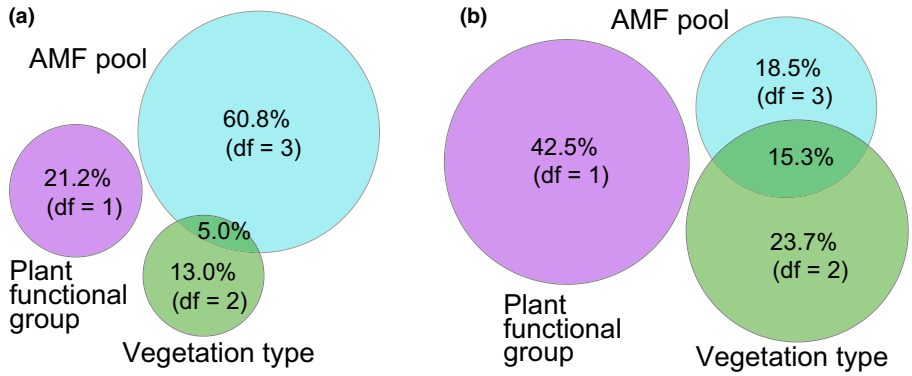

Fig. 2 Explanatory power of three predictors: the plant functional group (violet) factor classifies plant hosts into grasses and forbs; arbuscular mycorrhizal fungi (AMF) pool (cyan) represents the explanatory ability of the AMF community present in soil and roots collected in the plots; plot type (green) characterizes the vegetation type (mycorrhizal forbs, mycorrhizal grasses, or their mixture). Values shown in fractions represent their relative contributions to total explained variation. (a) Variation partitioning without considering the effect of experimental blocks; total variation explained by all three predictors $R^2_{adj} = 19.8\%$. (b) Variation partitioning after removing the differences among experimental blocks; total variation explained by all three predictors (after removing block effects) $R^2_{adj} = 13.5\%$.

Table 2 Results of multivariate Monte Carlo permutation tests of significance of the unique and independent effects of the three predictors, performed without and with the removal of the among-blocks variation.

		All AMF variation		Block effects removed	
Predictor[a]	Effect	Pseudo-F[b]	P[c]	Pseudo-F	P
Plant functional group	Unique	5.1	< 0.001	5.5	< 0.001
	Independent	4.3	< 0.001	5.1	< 0.001
AMF pool	Unique	4.9	< 0.001	1.7	0.014
	Independent	4.9	< 0.001	2.1	< 0.001
Plot type	Unique	2.2	0.003	2.3	0.006
	Independent	2.4	0.007	2.9	< 0.001

[a]Plant functional group classifies the plant hosts into grasses vs forbs; AMF pool represents the explanatory of the AMF community present in soil and roots collected in the plots, summarized as axes of co-correspondence analysis; plot type distinguishes the three vegetation types resulting from the weeding manipulation (mycorrhizal forbs, mycorrhizal grasses, or their mixture).
[b]Pseudo-F is the value of the test statistic.
[c]P is the type I error probability estimate.

Environmental correlates of spatial variation in the AMF pool

To understand the explanatory power of the AMF pool predictors for the composition of AMF community in seedling roots, we visualized the pattern of the three predictors (i.e. three CoCA axes) and of selected soil characteristics in geographical space (Fig. 7) and also used partial RDA to predict AMF community composition in core samples and in seedling roots using soil characteristics measured for individual plots.

Stepwise selection of abiotic descriptors identified the soil NO_3^- content and soil moisture as important predictors of AMF pool composition, explaining together $R^2_{adj} = 9.1\%$ of the total variation. The spatial pattern of soil NO_3^- (in Fig. 8b) most closely matched a combination of the spatial patterns of the first two CoCA axes (Fig. 7a,b), whereas the April moisture pattern (Fig. 8a) matched the pattern of the second CoCA axis (Fig. 7b) and the July moisture pattern (Fig. 8a) matched the pattern of the first CoCA axis (Fig. 7a).

When using selected soil parameters as predictors for AMF community composition in seedling roots, they explained $R^2_{adj} = 10.0\%$ of the total variation (pseudo-$F = 3.9$, $P < 0.001$). After adding the predictors representing AMF pool composition as covariates, the explanatory power of soil parameters dropped to $R^2_{adj} = 2.7\%$ of the total variation (pseudo-$F = 1.9$, $P = 0.003$).

AMF colonization levels

We examined the effects of host plant functional group and vegetation type on the estimated parameters of mycorrhizal colonization (Fig. 9, Table 5). For all the parameters examined there was no significant difference between F+G- and Ref-type of plots, so they were merged in subsequent analyses and for visual presentations.

Both the total and arbuscular mycorrhizal colonizations showed rather similar patterns (Fig. 9a,b), with no or negligible difference in forb root colonization across the three vegetation types (and with generally higher colonization levels than seen in grasses). However, there were significantly increasing colonization levels in the roots of grass seedlings on a gradient from G plots, across the two vegetation types with both functional groups present, to highest levels in the F plots. A similar pattern was seen in vesicular colonization, but with less pronounced differences.

The extent of colonization by fine endophytes was not distinctly different between forb and grass roots in the plots where grasses were present; but in the plots where mycorrhizal forbs grew without grasses (F plots), the frequency of fine endophytes was substantially higher in grass seedlings than in forb seedlings. We also examined the relation of fine endophyte colonization to total AMF colonization in

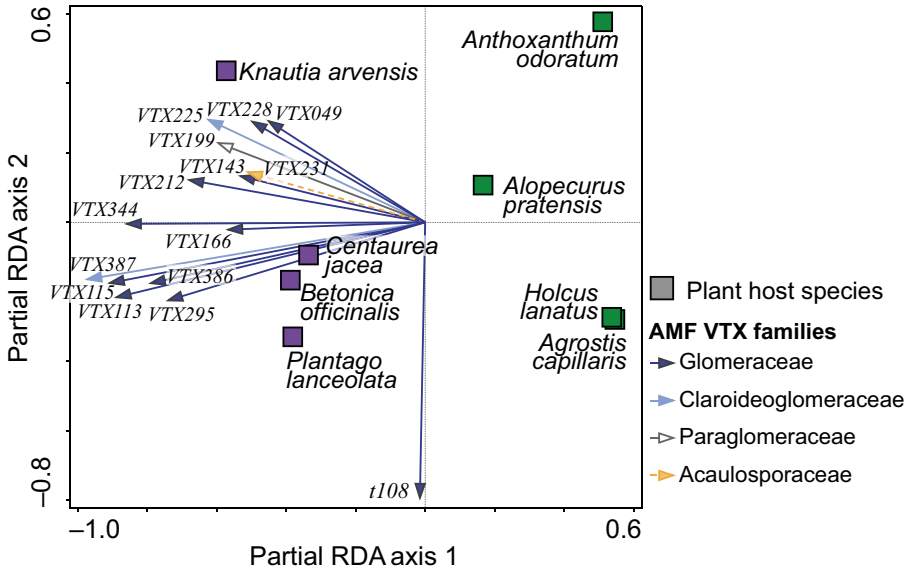

Fig. 3 Biplot diagram with the first two axes of partial redundancy analysis (RDA), in which the arbuscular mycorrhizal fungus (AMF) community in seedling roots is explained by host species identity (plot identity served as a covariate). Host identity explains overall $R^2_{adj} = 6.7\%$ of the community variation using seven constrained axes; the first two axes shown in the diagram summarize 80% of that variation. The arrows display 15 AMF virtual taxa (VTXs) best predicted by the host identity. The membership of a VTX in a particular AMF family is given in the key. Average read frequencies of VTXs found in the roots of individual plant species could be predicted by a perpendicular projection of a plant species symbol (violet for forbs, green for grasses) onto a straight line overlaying a VTX arrow.

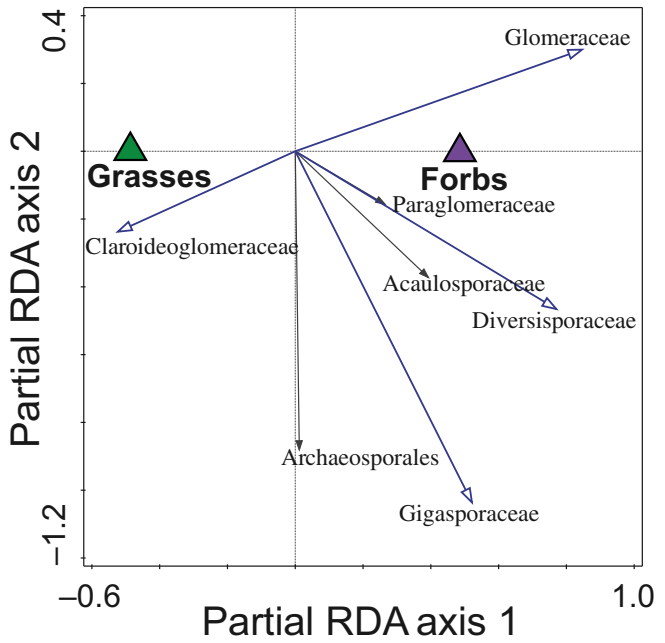

Fig. 4 Biplot diagram of a partial redundancy analysis (RDA) with host functional group (forbs vs grasses) explaining the relative proportions of arbuscular mycorrhizal fungus (AMF) families in molecular data of seedling roots. Vegetation (plot) type and three AMF pool predictors (co-correspondence analysis axes) were used as covariates. Note that only the horizontal (first) axis is constrained (explaining 6.5% of the partial variance), whereas the vertical axis already represents an unexplained variation. The relative preferences of individual families can therefore be judged by a perpendicular projection of arrow tips onto the horizontal axis. Based on a *t*-value biplot (not shown), all families represented by blue arrows with empty heads are suggested to differ significantly between forbs and grasses in their relative frequencies.

Table 3 Likelihood-ratio tests of fixed effects of four focal predictors on the taxonomic richness and Hill's N_2 diversity of arbuscular mycorrhizal fungus (AMF) community in the seedling roots.

	Richness			N_2 diversity		
Model term[a]	χ^2 statistic	df	P[b]	χ^2 statistic	df	P
Plant functional group	111.8	1	<0.001	76.0	1	<0.001
Plot type	4.2	3	ns	4.4	3	ns
Soil AMF richness	3.9	1	0.048	0.03	1	ns
Root AMF richness	0.3	1	ns	0.04	1	ns

df, degrees of freedom; ns, not significant.
[a]Functional group of host plant, manipulated vegetation type and log-transformed counts of AMF taxa found in cores' soil or plant roots.
[b]P is the type I error probability estimate.

combination with the effect of host functional groups. We found a positive effect of increasing AMF colonization on the fine endophyte colonization levels ($\chi^2_1 = 12.31$, $P < 0.001$), and the samples from the roots of grass species seemed to be more responsible for this positive relationship, although the interaction term between the host functional group and AMF colonization level was not significant ($\chi^2_1 = 3.37$, $P = 0.066$).

The total AMF colonization differed significantly among the host species ($\chi^2_7 = 163.0$, $P < 0.001$) and generally followed the pattern of colonization level lower in grasses than in forbs (see Fig. S2).

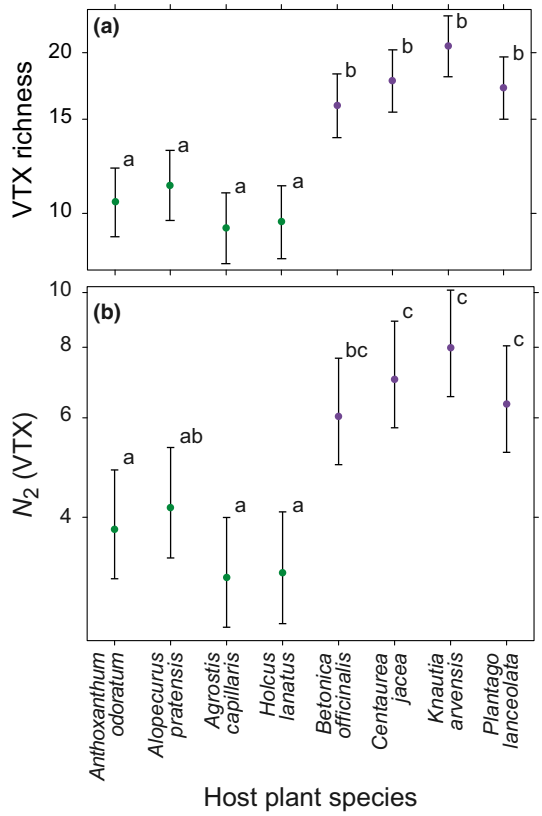

Fig. 5 (a) Average counts of virtual taxa (VTXs) and (b) Hill's N_2 diversity of arbuscular mycorrhizal fungi in the roots of eight plant species (the first four in green are grasses; the other four in violet are forbs). The predicted averages are shown together with 95% confidence intervals (implied by the fitted model, assuming homoscedasticity). Letters identify groups of species without significant differences of means in multiple comparisons.

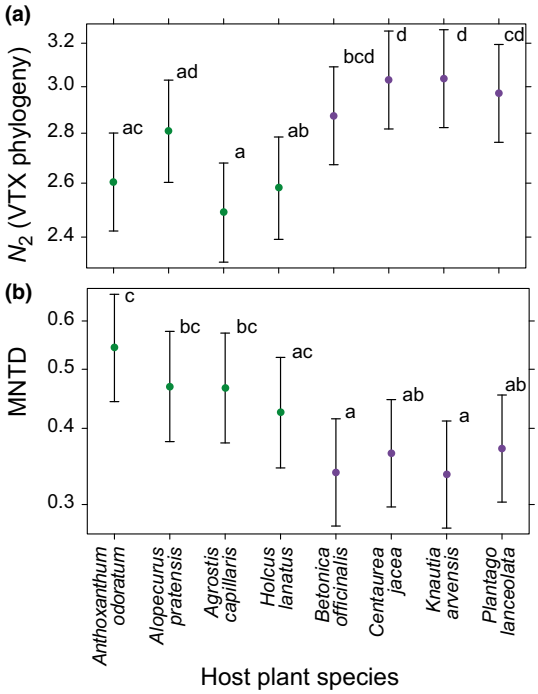

Fig. 6 (a) Effective number of species (Hill's N_2) based on Rao's index of phylogenetic diversity and (b) the mean nearest taxon phylogenetic distance (MNTD) measure of phylogenetic diversity of arbuscular mycorrhizal fungi in the seedling roots of eight plant species (the first four in green are grasses; the other four in violet are forbs). The predicted averages are shown together with 95% confidence intervals (implied by the fitted model, assuming homoscedasticity). Letters identify groups of species without significant differences of means in multiple comparisons.

Table 4 Likelihood-ratio tests of fixed effect of four focal predictors on the phylogenetic diversity measures (phylogeny-based Hill's N_2 diversity and mean nearest-taxon phylogenetic distance, MNTD) of arbuscular mycorrhizal fungus (AMF) community in the seedling roots.

	N_2 (phylogeny)			MNTD		
Model term[a]	χ^2 statistic	df	P[b]	χ^2 statistic	df	P
Plant functional group	29.7	1	< 0.001	35.3	1	< 0.001
Plot type	1.7	3	ns	4.2	3	ns
Soil AMF richness	0.4	1	ns	< 0.1	1	ns
Root AMF richness	0.1	1	ns	< 0.1	1	ns

df, degrees of freedom; ns, not significant.
[a]Functional group of host plant, manipulated vegetation type, and log-transformed counts of AMF virtual taxa found in cores' soil or plant roots.
[b]P is the type I error probability estimate.

Discussion

AMF community composition: effect of species pool and plant community

The composition of AMF communities in seedling roots was by far most affected by the relative abundances of AMF taxa in the plots (the AMF pool). The explanatory power of this AMF pool was strongly spatially structured, as evidenced by a drop in its importance when the effects of experimental blocks were suppressed and also by the visualized spatial covariability in the AMF pool predictors; that is, soil moisture and soil NO_3^-.

In this way, our findings agree to a large extent with those of Horn *et al.* (2017), who found that the AMF community is strongly affected by spatial gradients, which are in turn correlated with the variation in soil properties. Their study was on a larger spatial scale (up to 500 m), which might explain the larger percentage of variation explained by space. Additionally, their study quantified AMF communities in roots and rhizosphere soil of a single host species; namely, the *Festuca brevipila* grass.

Soil N availability has often been reported as a driving factor of AMF community composition (Egerton-Warburton *et al.*, 2007; Fitzsimons *et al.*, 2008; Antoninka *et al.*, 2011; Camenzind *et al.*, 2014), and the variation in soil moisture can similarly select AMF taxa more supportive to plants under drought conditions (Yang *et al.*, 2017). The dominating impact of soil properties on the identity and abundance of AMFs has been widely reported in literature (Bouffaud *et al.*, 2016; Dassen *et al.*, 2017; Van Geel *et al.*, 2018). However, it is surprising to find it at such a small spatial scale of metres.

Even after removing a large part of the spatial variation by excluding the among-block community differences, the overlap between the AMF pool and plant community effects in

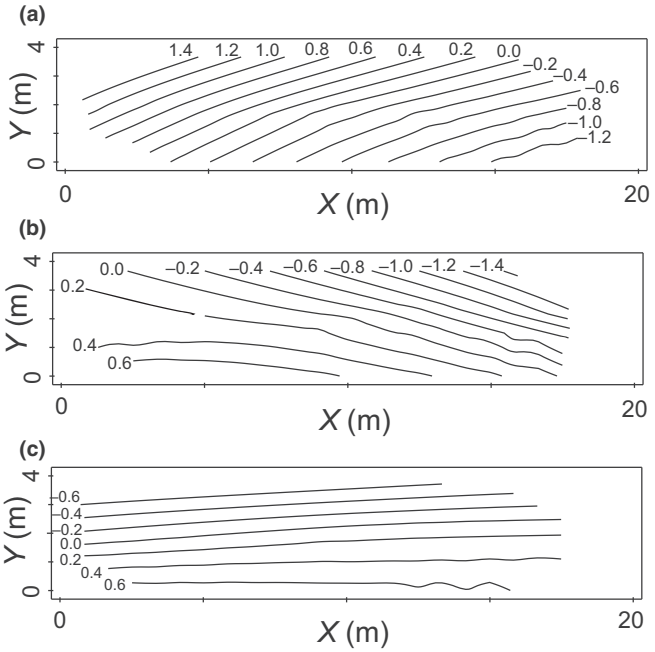

Fig. 7 Spatial variation of arbuscular mycorrhizal fungus (AMF) pool predictors, representing the first, second, and third axis of predictive co-correspondence analysis (CoCA; using the AMF community in seedling roots as the response data and the AMF community in collected soil cores as the explanatory dataset). Plot scores on CoCA axes are shown here across the geographical space of experimental site as isolines connecting places with the same value of axis scores as predicted by the loess model. Displayed area represents a mildly inclined grassland with the slope running down from top to bottom in the plots. The plant community also changes from left to right, with forest edge close to the left margin. Percentages of variation in the AMF pool predictors explained by the displayed loess models are 54.8%, 50.1%, and 9.2% for (a), (b), and (c), respectively.

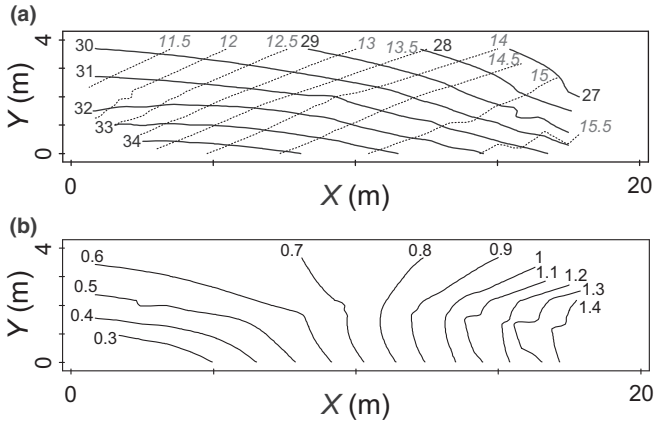

Fig. 8 Variation of values of soil characteristics (chosen in a stepwise selection to explain arbuscular mycorrhizal fungus community composition in soil cores) across the geographical space of the experimental site as isolines connecting places with the same value of soil characteristic as predicted by the loess model. (a) The variation of two moisture readings: percentage of soil moisture on an April afternoon (solid lines, $R^2 = 26.0\%$; as a predictor, it explains 7.7%, pseudo-$F = 3.0$, $P_{Adj} = 0.002$) and on a July morning (dotted lines, $R^2 = 14.4\%$; as a predictor, it explains 5.0%, pseudo-$F = 2.0$, $P_{Adj} = 0.025$). (b) The variation in soil nitrates (mg kg^{-1} soil DW, $R^2 = 43.1\%$; as a predictor, it explains 7.6%, pseudo-$F = 2.9$, $P_{Adj} = 0.004$).

explaining the AMF community in seedling roots remained below 40% of the overall effect of plant community type. This focused our attention on the significant and much larger unique (i.e. not shared with the AMF pool) effect of plant community type. This important fraction of explained variation provides a direct support for the passenger hypothesis (Zobel & Öpik, 2014), because we manipulated the plant community on a long-term temporal scale in a way that was independent of a major part of spatial variation in abiotic conditions.

The exact mechanisms how the plant hosts affect the AMF community must remain a subject of speculation, but besides the expected differences in fitness and compatibility between individual AMF taxa and their plant hosts, the removal of plant functional groups could introduce changes in the plot environment, perhaps affecting the host plant performance. Indeed, in another study (Šmilauerová & Šmilauer, 2016) we have found that changes induced by vegetation manipulation (in light availability and quality, soil surface temperature, and soil moisture) may affect seedling survival and growth.

The lack of significant differences in AMF community composition between regularly weeded F+G plots and the Ref plots with no soil disturbances validates the manipulative approach of our study. The lack of differences was undoubtedly due to the long period of time that our experimental treatment had been imposed on the plots studied. The similarity of F+G and Ref plots also demonstrates that the presence of nonmycorrhizal plant species (at least in the limited extent of our research site) did not affect the composition of AMF communities.

AMF community composition: the effect of host species

The role of plant host identity in determining the set of colonizing AMFs and their quantity has been a frequent topic in current research (Eom *et al.*, 2000; Lekberg & Waller, 2016; Van Geel *et al.*, 2018). When examined with data collected at wider spatial scales, and often based on database records, plant identity has rarely played an important role (Lekberg & Waller, 2016; Van Geel *et al.*, 2018; but see Davison *et al.*, 2015), probably due to a larger variation in environmental conditions and to a nonrandom choice of hosts investigated in studies using database data. Our research, however, belongs to studies performed at a more local level, across one or a few ecosystem types (Eom *et al.*, 2000; De Deyn *et al.*, 2011).

We found a reasonably strong effect of host species on the compositional variation of AMF communities of seedling roots, and this effect was, to a large extent, attributable to a difference between two functional groups (grasses vs dicotyledonous forbs), although the difference among plant species within functional groups was still significant. Our study was not the first in pointing out the effect of host plant functional groups on the AMFs harboured (Eom *et al.*, 2000; König *et al.*, 2010), but here, importantly, this difference is demonstrated for multiple plant species co-occurring in the same community, where they compete

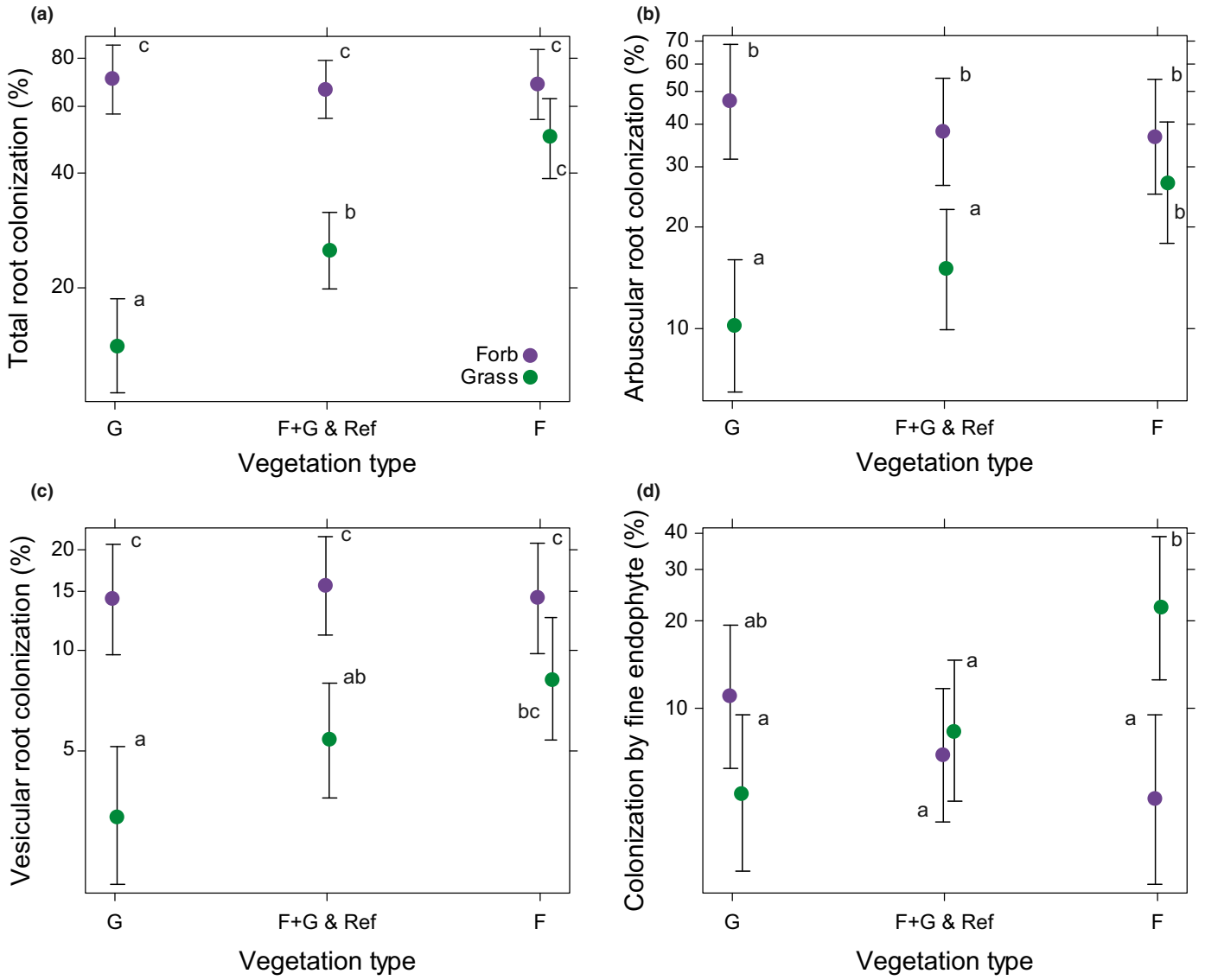

Fig. 9 Mean values of mycorrhizal colonization levels and their 95% confidence intervals for four estimated parameters and three vegetation types (along horizontal axes) and for the roots of forb (violet circles) and grass (green circles) host plants: (a) total colonization level, (b) arbuscular colonization level, (c) vesicular colonization level, and (d) colonization level for the fine endophyte morphotype. The mean values and their confidence intervals are estimated from fitted generalized linear mixed-effect models. The lower-case letters portray the results of multiple comparisons among the six groups shown in each plot: group means sharing the same letter are not significantly different at $\alpha = 0.05$.

for nutrients and light. The observed importance of host identity was revealed by a lack of host effect covariation with environmental (or other spatial) heterogeneity, thanks to the orthogonal experimental design. The significant conditional effect of host species on AMF community, on the top of the effect of plant functional group, provides another piece of evidence for the passenger hypothesis, in addition to the conditional (unique) effect of plot manipulation.

Further, the effect of host functional group remained important when we switched to examining the AMF community data aggregated into fungal families. There are multiple cases of evidence in the literature that the higher taxonomic levels of the Glomeromycota phylum possess signals of varying functionality: efficiency in providing soil P (Yang *et al.*, 2017), relative investment into extramatrical mycelium compared with biomass within host roots (Maherali & Klironomos, 2012), and sensitivity to disturbances (König *et al.*, 2010) or to heavy metals (He *et al.*, 2014). In the grassland studied here, the dicotyledonous hosts supported a larger diversity of AMF families, with only taxa from the Claroideoglomeraceae family being more frequent in grass roots. This matches well the previously described higher reliance of grassland forbs on the retrieval of soil nutrients via the absorptive surfaces of AMF hyphae (Unger *et al.*, 2016), and the higher reported investment of C resources by forbs into AMF symbionts (Gui *et al.*, 2018). We found a similar pattern for AMF taxonomic and phylogenetic diversity, as discussed in the following section.

Table 5 Summary of fitted linear mixed-effect model (lower indices of χ^2 statistics represent degrees of freedom) describing the effects of host functional group and vegetation type, and of their interaction on four parameters of mycorrhizal colonization (first three concerning arbuscular mycorrhizal fungi (AMF), fourth concerning the 'fine endophyte' morphotype).

	Vegetation type		Host functional group		Vegetation type : host functional group	
Colonization	χ^2_2	P^a	χ^2_1	P	χ^2_2	P
Total	13.0	0.002	63.6	< 0.001	55.4	< 0.001
Arbuscular	3.3	ns	11.7	< 0.001	35.0	< 0.001
Vesicular	3.6	ns	20.1	< 0.001	16.9	< 0.001
Fine endophyte	19.4	< 0.001	0.15	ns	57.9	< 0.001

ns, not significant.
[a]P is the type I error probability estimate.

AMF richness and phylogenetic diversity in seedling roots

Only the host functional group (and to some extent also the actual species identity) had a strong (and significant) effect on the richness and phylogenetic diversity of AMF VTXs, with the forb species harbouring substantially more diverse AMF communities, similar to the results of König *et al.* (2010).

The larger phylogenetic distance between nearest pairs of AMF taxa, observed in grass roots, probably follows from a similar spread of a smaller number of VTXs across the AMF phylogeny. Caution should be used when interpreting those patterns in terms of the functional diversification of AMFs, as the relation between phylogeny and functional traits is uncertain.

Root colonization levels

In contrast to the effects of host plant functional group on AMF community composition, the intensity of root colonization by AMFs was more affected by the plot type (vegetation). In the case of total and vesicle-based colonization, the main effect of the host functional group and its interaction with the plot type were of similar size, but the changes in arbuscular colonization frequency in grass seedling roots across plot types were actually more important than the overall differences between forbs and grasses. In this grassland community, the presence of forbs led to higher mycorrhization levels in grass roots. This can partly explain our earlier findings that grass seedlings survived best in forb plots (and vice versa; Šmilauerová & Šmilauer, 2016).

Another intriguing pattern was the high colonization level by the fine endophyte fungi in grass seedling roots in the forb-only vegetation, followed by the second highest average level for forb seedlings in grass-only vegetation. The affinity of fine endophytes to roots of various grasses is already known (see Orchard *et al.*, 2017). Possible interactions among forbs and grasses in supporting fine endophyte populations are difficult to interpret, because we know so little about the ecology of these fungal symbionts. We found a positive correlation between the colonization levels of fine endophyte and AMFs, particularly for the grass seedlings, and vegetation with frequent forbs might support such an interaction.

Pros and cons of experimental manipulation

Manipulative experiments have an advantage of allowing us to distinguish correlative and causal effects due to the randomization of experimental units (Quinn & Keough, 2002), but they also have some disadvantages, mostly related to the side effects of experimental treatments or the unrealistic nature of imposed manipulations, even under field conditions. Overall, we consider the manipulation of plant community and host plant identity in our study to be its essential feature, allowing us to identify both considered effects as indisputable causes of AMF community variation, thereby corroborating the correlative results of earlier exploratory studies.

In the experimental design adopted, the effect of host plant identity could be evaluated independently of the vegetation manipulation and we could observe a full set of considered species of both functional groups within each experimental plot. Given the time constraints and required initial sterility of plant roots, using plants at seedling stage was an obvious choice. Our findings might, therefore, be biased in comparison with the AMF communities of more heterogeneous root systems of adult plants.

Conclusions

Our experimental approach demonstrated an important causal effect of host plant identity on the composition and diversity of the AMF community in a semi-natural grassland. Dicotyledonous forbs hosted more diverse AMF communities than grasses did, and their roots harboured phylogenetically more related taxa. The balance between grasses and forbs in the surrounding vegetation was important for the intensity of root colonization of establishing seedlings. The manipulative experimental approach embedded within a long-term experiment manipulating the community of plant hosts enabled us to identify causal effects of plant host and community on the composition and diversity of AMF community. Our findings highlight the importance of maintaining dicotyledonous forbs in managed agricultural grasslands, as they may provide a rich pool of symbiotic fungi for the dominant grasses.

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Author contributions

PS and MS designed the experiment and data collection, JK and MK optimized and performed molecular and bioinformatic

analyses, PS analysed data, PS and JK wrote the paper, and all authors contributed to its further improvement.

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Supporting Information

Additional Supporting Information may be found online in the Supporting Information section at the end of the article.

Fig. S1 Overlap in AMF virtual taxa between grass and forb seedling roots.

Fig. S2 Total AMF colonisation levels for individual host plant species.

Methods S1 Details for the methods of molecular analysis and light microscopy.

Methods S2 Details for the methods of statistical analysis.

Table S1 GenBank sequences for detected virtual taxa of AMF.

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