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The structure of root-associated fungal communities is related to the long-term effects of plant diversity on productivity

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Abstract

Root-associated fungi could play a role in determining both the positive relationship between plant diversity and productivity in experimental grasslands, and its strengthening over time. This hypothesis assumes that specialized pathogenic and mutualistic fungal communities gradually assemble over time, enhancing plant growth more in species-rich than in species-poor plots. To test this hypothesis, we used high-throughput amplicon sequencing to characterize root-associated fungal communities in experimental grasslands of 1 and 15 years of age with varying levels of plant species richness. Specifically, we tested whether the relationship between fungal communities and plant richness and productivity becomes stronger with the age of the experimental plots. Our results showed that fungal diversity increased with plant diversity, but this relationship weakened rather than strengthened over the two time points. Contrastingly, fungal community composition showed increasing associations with plant diversity over time, suggesting a gradual build-up of specific fungal assemblages. Analyses of different fungal guilds showed that these changes were particularly marked in pathogenic fungi, whose shifts in relative abundance are consistent with the pathogen dilution hypothesis in diverse plant communities. Our results suggest that root-associated fungal pathogens play more specific roles in determining the diversity–productivity relationship than other root-associated plant symbionts.

Jose G. Maciá-Vicente and Davide Francioli contributed equally to this work.

KEYWORDS
fungi, grasslands, mutualists, pathogens, plant diversity–productivity relationship, roots

1 | INTRODUCTION

Biodiversity is consistently and positively associated with ecosystem functioning, affecting processes such as net primary productivity or carbon sequestration (Cardinale et al., 2012; Weisser et al., 2017). This relationship has been particularly well studied in temperate grasslands (Adler et al., 2011), where numerous experiments that manipulate plant diversity have repeatedly shown that species-rich plant communities are more productive than species-poor ones (Isbell et al., 2015; Tilman et al., 2001; Van Ruijven & Berendse, 2009). Despite the generality of the positive plant diversity–productivity relationship across biodiversity experiments, its underlying mechanisms remain debated (Barry et al., 2019; Eisenhauer, Schielzeth, et al., 2019; van Ruijven et al., 2020). The relationship was initially interpreted to arise from plant-centred processes such as spatial resource partitioning (Barry et al., 2019; Tilman et al., 2014), but mounting evidence has attributed soil microbiota a major role (Hendriks et al., 2013; Schnitzer et al., 2011; van Ruijven et al., 2020). This is partly supported by observations that the increase in productivity with plant diversity becomes stronger with grassland age (Cardinale et al., 2007; Guerrero-Ramírez et al., 2017; Meyer et al., 2016; Reich et al., 2012; Van Ruijven & Berendse, 2009), suggesting that plant communities gradually build up changes in soil microbial communities that increasingly feedback on plant performance over time (Eisenhauer et al., 2012; Eisenhauer, Bonkowski, et al., 2019).

Eisenhauer et al. (2012) proposed that the strengthening of plant diversity–productivity relationships with time results from the lapse it takes plant communities to accumulate specific soil biota, with increased feedback effects on plants over time. It is hypothesized that species-poor plant communities provide high host densities for specialized plant pathogens, thus promoting a build-up of pathogen populations over time that progressively impairs biomass production (Burdon et al., 2006). Conversely, high diversity in plant communities can decrease pathogen pressure via pathogen dilution (Keesing et al., 2006; Rottstock et al., 2014), and/or provides a greater variety of niches for other microorganisms performing functions that sustain overall productivity (Wagg et al., 2014), such as mutualists and decomposers (Eisenhauer et al., 2011; Latz et al., 2012). Under these assumptions, microbial diversity increases with plant diversity and leads to effects on plant performance ranging from detrimental in plant communities with low richness, to positive in rich plant communities. However, reaching the full influence of plant richness on soil microbial communities and functions may take years following the establishment of grassland communities (Eisenhauer et al., 2010, 2011), and thus potential feedback effects on plant growth may be equally delayed. As a result, the long-term build-up of plant community-specific microbial communities is expected to strengthen the relationship between plant diversity and productivity with ecosystem age (Eisenhauer, Bonkowski, et al., 2019).

Fungi may play a particularly important role as determinants of the plant diversity–productivity relationship because they encompass major groups (guilds) of pathogens, mycorrhizal mutualists and soil and litter saprotrophs that directly or indirectly affect plant growth and competition (Bever et al., 2015; Francioli, van Ruijven, et al., 2020; Maron et al., 2011; Schnitzer et al., 2011; Tedersoo, Bahram, & Zobel, 2020). They also form communities with higher specificity towards standing vegetation and habitat conditions than those formed by bacteria (Coleman-Derr et al., 2016; Dassen et al., 2017; Lauber et al., 2008; Millard & Singh, 2010; Thiergart et al., 2020), which makes their contribution to the plant diversity–productivity relationship theoretically stronger (Semchenko et al., 2022). However, studies in natural and experimental systems have yielded mixed support for a link between plant and fungal diversity (Dassen et al., 2017; Francioli, van Rijssel, et al., 2020; Mommer et al., 2018; Shen et al., 2021; Tedersoo et al., 2014; Tedersoo, Anslan, et al., 2020; Waldrop et al., 2006), probably stemming from differences in methodology, scale and plausibly, also from confounding effects of ecosystem age (Vogel et al., 2019). To our knowledge, no study has explicitly tested the hypothesis formulated by Eisenhauer et al. (2012) about how temporal changes in microbiota may strengthen feedback effects on plant productivity. In addition, studies addressing the relationship between plant-associated microorganisms and plant diversity and/or productivity most often target microbial communities in soil (Dassen et al., 2017; Leff et al., 2018; Schmid et al., 2019, 2021), but neglect those tightly associated with roots (but see Ampt et al., 2022; Francioli, van Ruijven, et al., 2020) that show stronger variation of fungal communities across different plant species, and likely have more direct effects on plant performance (Coleman-Derr et al., 2016; Maciá-Vicente et al., 2020, 2022).

We assessed the contribution of root-associated fungal communities to the plant diversity–productivity relationship by formally testing the model proposed by Eisenhauer et al. (2012). Using a long-term biodiversity experiment encompassing 1 and 15-year-old grassland plots varying in plant richness and composition, we studied whether and how the relationship between fungal and plant communities is affected by ecosystem age. Previous results from this experiment confirmed a strengthening of the plant diversity–productivity relationship over time which was associated with an increase in total soil microbial biomass (Lange et al., 2015; Vogel et al., 2019; Weisser et al., 2017), but how these effects relate to changes in fungal diversity and fungal community composition in roots remains unknown. Here, we hypothesized (1) a strengthening of the fungal diversity relationships with plant richness and productivity with plant community age (i.e., increased slope over time), as a result of an increased availability of niches sustaining fungal growth

in older, species-rich plant communities. Moreover, we expected that (2) grassland age would also strengthen the plant diversity effects on the fungal community composition, reflecting the long-term build-up of plant community-specialized fungal communities and (3) that this specialization would lead to a long-term accumulation of fungal antagonists (e.g. soil-borne pathogens) in species-poor plant communities, and of fungal guilds that sustain plant growth (e.g. arbuscular mycorrhizal fungi and soil saprotrophs) in species-rich plant communities.

2 | MATERIALS AND METHODS

2.1 | Study site and experimental design

This study was done within the ΔBEF Experiment (DELTa-BEF; short for DEterminants of Long-Term Biodiversity Effects on Ecosystem Functioning Experiment; Vogel et al., 2019), which consists of established plots from the Jena Biodiversity Experiment (Main Experiment; Roscher et al., 2004; Weisser et al., 2017) and newly created plots (Vogel et al., 2019). The ΔBEF Experiment is aimed at testing how ecosystem age alters the relationship between biodiversity and ecosystem functioning. Both the ΔBEF and the Jena Experiment have been described in detail elsewhere (Roscher et al., 2004; Vogel et al., 2019), and hence we only summarize the main experimental design here.

The Main Experiment was established in 2002 in Jena (Germany; 50.95 N, 11.62 E, 130 m a.s.l.) and comprises a pool of 60 plant species typical of semi-natural mesophilic grasslands (Figure S1) planted in experimental plant communities (20 × 20 m plots) varying in species richness (1, 2, 4, 8, 16 and 60) and functional group richness (1–4 plant functional groups [PFGs], including legumes, grasses, small forbs and tall forbs). The plots were randomly assembled in four blocks across a gradient in edaphic conditions (Figure S1) and sown at a density of 1000 viable seeds per m^2 with equal representation of all target species. The field site is managed according to a typical hay meadow in this region, with two yearly mowing events in June and September respectively. To maintain the plant diversity gradient, plots are weeded three times per year to remove spontaneous species different from those planted. For more details, see Roscher et al. (2004).

The ΔBEF Experiment was established in May 2016 and consists of 80 plots from the Main Experiment with three treatments each. Each treatment was performed on 1.5 × 3 m-sized subplots randomly distributed across five fixed locations at the margin of the main plots (Figure S1), and differ in their soil and plant community history. This study focuses on the two of the three treatments varying only in plant history, referred to as ΔBEF2 and ΔBEF3. The ΔBEF3 subplots represent the original treatments from the Main Experiment with undisturbed soil and plant communities sharing soil and plant history since 2002. Thus, hereafter we refer to these treatment subplots as 'old' plant communities. The ΔBEF2 subplots represent shared soil history with the Main Experiment but no plant history, and hence are referred to in the following as 'young' plant communities. In the ΔBEF2 subplots, the plant sod and large roots were removed while keeping the original soil, which was carefully homogenized up to a depth of 30 cm. Whereas such homogenization may disrupt soil fungal communities forming web-like mycelium in topsoil layers (e.g. arbuscular mycorrhizal fungi; Säle et al., 2015) more than other fungal guilds, the treatment was necessary to minimize spatially uneven legacies of previous vegetation within subplots. After homogenization of the soil, new seed mixtures matching the original plant species composition in the respective plot of the Main Experiment were sown at the same density of 1000 viable seeds per m^2, with the aim to establish similar plant community compositions in the ΔBEF2 and ΔBEF3 subplots. This experimental treatment allowed us to evaluate the effects of plant community age on fungal root colonization irrespective of changes in soil mycobiota. Differences in fungal colonization can be the result of different paces of establishment in roots across fungal species in soil, as well as to physiological and/or structural changes in roots that may affect fungal colonization. The ΔBEF2 subplots were separated from adjacent treatments by 30-cm-deep plastic barriers to prevent soil mixture.

2.2 | Collection of root samples and measurements

We sampled roots from both experimental treatments in the ΔBEF Experiment in June 2017, that is, 13 months and 15 years after the establishment of the young and old plant communities respectively. We collected roots from each subplot by taking three soil cores (diameter 4 cm) to a depth of 5 cm and pooling them. These composite samples were stored at 4°C before washing them gently to remove soil particles and debris. The root samples were well mixed before taking a representative subsample of approximately 100 mg fresh weight, which was stored at −20°C until use for molecular analysis. The remaining roots were dried at 70°C for 48 h and weighed. Seven samples had very low amounts of roots, so in these cases, all roots were used for molecular analyses and the dry biomass was scored as zero.

We retrieved additional data from the JExIS database (https://jexis.idiv.de/) on aboveground plant biomass, soil microbial biomass carbon, soil mineral nitrogen and soil organic carbon in the ΔBEF Experiment, measured around the same time as our collection of root samples (Vogel et al., 2019). Aboveground biomass was harvested in May/June 2017 and in August 2017 by clipping 3 cm above soil surface in two randomly placed, 20 × 50 cm^2 frames per subplot. Mass from individual target species and weeds were separately dried and weighted, and dry biomass of target species was summed to community level values for each subplot. We used the summed biomass values of the two harvests, extrapolated on a m^2, as an estimate of annual biomass production per subplot. Soil microbial-biomass carbon was measured using an O_2-microcompensation apparatus from soils sampled in June 2017, collected by pooling six soil cores (4 cm diam. × 5 cm depth) per subplot. The values are expressed as μg C g^{-1} soil dry weight and

represent the biomass of all active bacteria and fungi in the soil. Soil mineral nitrogen (sum of NH_4^+-N and NO_3^--N) and organic carbon were determined in the same soil samples, respectively, using a colorimetric continuous flow analysis and combustion in an elemental analyser (Vogel et al., 2019).

2.3 | Fungal amplicon sequencing and analysis

Total DNA extraction from the washed roots and the targeted amplification and sequencing of the fungal rDNA internal transcribed spacer 1 (ITS1) were done following the procedures described in Francioli, van Ruijven, et al. (2020). In short, we extracted DNA using the DNeasy Plant Mini kit (Qiagen, Hilden, Germany) and used it to amplify the ITS1 region using primers ITS1F and ITS2 (Gardes & Bruns, 1993; White et al., 1990). The ITS1 amplicons were paired-sequenced on an Illumina MiSeq platform at the Wageningen University & Research Shared Research Facilities (Wageningen, the Netherlands).

Sequence reads were assembled, quality-filtered and clustered into amplicon sequence variants (ASVs) using the DADA2 pipeline (Callahan et al., 2017) running on R v4.1.3 (R Core Team, 2022). We discarded non-fungal ASVs by comparing all sequences against a local copy of NCBI GenBank using BLASTN v2.2.31+, and taxonomically annotated the ASVs retained by comparisons with the UNITE database v8.3 (https://doi.org/10.15156/BIO/1265786) of fungal ITS sequences (Kõljalg et al., 2005), using the Naïve Bayesian Classifier tool of MOTHUR v1.39.5 (Schloss et al., 2009; Wang et al., 2007). We assigned ASVs to putative functional guilds by collating their taxonomic annotations against the FungalTraits database (Põlme et al., 2020), that compiles expert-curated, lifestyle-related traits on fungi at the genus level. We annotated each ASV with the 'primary lifestyle' field of FungalTraits.

Of the initial 160 samples (80 plots × 2 treatments), we discarded 12 after quality-filtering of reads and removal of samples with low sequencing depth (<4000 reads). We did not rarefy the data set to a common number of reads per sample because rarefaction curves showed adequate coverage of ASV richness across samples (Figure S2).

2.4 | Statistical analyses

All statistical analyses were done in R v4.1.3. We calculated fungal ASV richness and Shannon diversity (H') per sample using package VEGAN v2.6-2 (Oksanen et al., 2019). We selected Shannon diversity (expressed as effective number of 'species'; ES = $e^{H'}$; Jost, 2006) over ASV richness as the main descriptor of fungal diversity in downstream analyses because of its lesser sensitivity to the presence of rare ASVs (Chao et al., 2014), and because richness was positively correlated with read abundances (Pearson's $r = .59$, $p < .001$) whereas diversity was not. To analyse fungal community composition, we first standardized ASV abundances using the Hellinger transformation (Legendre & Gallagher, 2001) and then calculated pairwise Bray–Curtis dissimilarities among samples.

To estimate the effects of experimental treatments on fungal diversity and community composition, we used plant history (PH; with young and old plant communities included as a dummy variable), the number of plant functional groups (PFGs) and the number of plant species (plant richness) of each subplot as explanatory variables. Because plant richness and PFG were collinear ($r = .5$, $p < .001$), downstream analyses using either variable yielded similar results, and thus we only show results for models including plant richness. Similarly, we did not include the variables soil mineral nitrogen and organic carbon as additional variables, because they have previously been shown to covary with the experimental manipulation of plant history and plant richness (Vogel et al., 2019), thus their feedback effects on plant growth cannot be disentangled (i.e. they can be regarded as plant history and richness effects). In addition, both variables were strongly correlated with one another ($r = .98$, $p < .001$) and also with soil microbial-biomass carbon ($r = .78–.79$, $p < .001$), which was included in some of these models as a descriptor of total fungal abundance. To meet normality assumptions in downstream analyses, we used the $log(x)$ transformation of plant richness and soil microbial-biomass carbon, the square-root of aboveground plant biomass and the $log(x + 0.1)$ of root biomass after visually inspecting the data distributions.

We built two linear mixed-effects models using package LME4 v1.1-29 (Bates et al., 2014) to investigate the relative contribution of plant richness and plant history in predicting fungal diversity and soil microbial-biomass carbon (hypothesis 1). We included the interaction of both predictors as fixed term to assess if plant diversity effects on the two response variables varied between young and old plant communities. To account for the spatial structure of the experimental design, we included block, and plot nested in block, as random intercept terms. We tested for significance in the fixed term effects ($\alpha = .05$) with Type III ANOVAs using the Satterthwaite's approximation for degrees of freedom (Luke, 2017), as implemented by package LMERTEST v3.1-3 (Kuznetsova et al., 2017). Since these are based on partial sums of squares, they enabled us to evaluate the relationship between the variables of interest after accounting for the effects of all other variables in the model.

A second set of models was aimed at assessing the effects of fungal diversity and total microbial biomass on plant productivity across young and old treatments (hypothesis 1). We built two linear mixed-effects models using aboveground plant biomass and root biomass as response variables, and plant history, fungal diversity, soil microbial biomass and the interactions of plant history with fungal diversity and microbial biomass as fixed terms. We included the same structure for random effects described above.

We tested for the effects of plant richness, above- and belowground plant productivity and plant history on fungal community composition (hypothesis 2) using permutational multivariate analysis of variance (PERMANOVA; McArdle & Anderson, 2001) as implemented in function *adonis2()* of VEGAN. In this case, we included soil mineral nitrogen and organic carbon as last terms in the sequential sums of squares test, to assess their explanatory power on fungal community composition after the effect of other factors had been accounted for. As in previous

models, we assessed the interaction between all predictor variables and plant history. The analyses were run with 999 permutations, but restricting permutations across blocks and plots to account for spatial structures in the experimental design. In addition, we used the function *betadisper()* of VEGAN to determine differences in multivariate dispersion between samples from young and old treatments, by calculating the median distance of data points to the group centroid and subjecting them to ANOVA. To visualize differences in fungal community composition across samples, we used 3-dimensional non-metric multidimensional scaling (NMDS) ordinations built with VEGAN's function *metaMDS()*. In this case, we also explored how fungal community composition varied across grassland plots hosting only one PFGs, since PFG has been shown to be a significant driver (surpassing plant species identity) of community composition in root-associated fungi (Francioli, van Ruijven, et al., 2020; Mommer et al., 2018; Sweeney et al., 2020). We used this to detect potential links between particular plant and fungal groups that could help interpret the patterns observed in the full data set. We obtained taxonomic summaries of fungal community composition at the order level using relative proportions of abundance or richness, and used bar plots to visualize them. Relative abundances of the main fungal orders across treatments were compared with linear mixed-effects models, using PFG and plant history as fixed effects terms, with adjustment of *p*-values after multiple comparisons by the Benjamini–Hochberg method.

A last series of models were aimed at identifying patterns of variation in diversity and relative abundances of particular fungal guilds across plant history and plant richness (hypothesis 3). We focused on four guilds ('primary lifestyle' as defined by Põlme et al., 2020) that we deemed relevant as likely determinants of plant community productivity in grasslands: arbuscular mycorrhizal fungi, soil saprotrophs and litter saprotrophs as potential facilitators of plant growth; and plant pathogens as antagonists. For each of them, we separately modelled Shannon diversity and total relative abundance (i.e. the sum of all ASV abundances) as a function of plant richness and plant history, using linear mixed-effects models with the same structure for fixed and random effects used previously. In addition, we investigated the individual patterns of occurrence of the 10 most abundant fungal genera (in relative abundance) within each guild, using again the same model structure as above but adjusting the resulting *p*-values with Benjamini–Hochberg's method to account for multiple testing. We used bar plots to visualize the overall patterns of variation in relative abundance for each fungal genus.

3 | RESULTS

3.1 | Description of the fungal sequencing data set

The quality-filtered data set resulting from the ITS amplicon sequencing of root-associated fungi comprised 3,015,859 sequence reads grouped into 3916 ASVs, with median values of 19,486 reads (range 4542–46,223) and 154 ASVs (67–278) per sample (Figure S2). The fungal ASVs were assigned to 558 species-level taxa and 313 genera distributed across 80 orders (including *incertae sedis* taxa). At the phylum level, most sequence reads belonged to ASVs in the Ascomycota (62.7% of total reads), distantly followed by the Basidiomycota (22.7%), Mortierellomycota (7.3%), Glomeromycota (4.2%) and Olpidiomycota (1.5%), with other phyla being represented by less than 1% of total reads. At the order level, the 10 most represented taxa were the Helotiales (19.7%), Pleosporales (11.8%), Mortierellales (7.3%), Hypocreales (6.1%), Agaricales (5.8%), Sordariales (5.4%), Pezizales (4.3%), Glomerales (3.6%), Cantharellales (3.5%) and Sebacinales (3.4%). We classified ASVs in 20 functional guilds by collating the taxonomic ASV annotations with the FungalTraits database (Põlme et al., 2020), but we only focused on four guilds that we considered relevant for grassland root-associated fungi: arbuscular mycorrhizal fungi (13.3% of total number of ASVs), soil and litter saprotrophs (9.3% and 9.5%) and plant pathogens (9.8%). Of all ASVs, 1659 (42.4%), representing 30.2% of all reads, could not be assigned a guild due to incomplete identification or undefined guild status in FungalTraits.

3.2 | Effects of plant history on fungal diversity

Total soil microbial biomass (encompassing both fungi and other soil microorganisms) exhibited a marked increase in old versus young plant communities (1.3 ± 1.04 [SE] μg microbial C g^{-1} of dry soil, $F_{1/75} = 46.9$, $p < .001$), and a positive relationship with plant richness (1.14 ± 1.03 μg C g^{-1}, $F_{1/75} = 19.0$, $p < .001$) that became more pronounced with plant history (interaction term: 1.05 ± 1.02 μg C g^{-1}, $F_{1/76} = 5.0$, $p = .028$; Figure 1a). In contrast, fungal diversity was significantly associated with plant species richness (2.8 ± 1.22 ES) but not with plant history (Table 1; Figure 1b). Contrary to what we expected, the slope of the relationship between fungal diversity and plant richness decreased with grassland age, so that a positive plant–fungal diversity relationship as identified in young communities disappeared in older ones (interaction term: -2.7 ± 1.19 ES; Figure 1b).

We did not find any significant relationship of fungal diversity with above- and belowground plant biomass after accounting for the effects of plant richness and soil microbial biomass (Table 1; Figure 1c,d). In contrast, both aboveground and root plant biomass were positively associated with soil microbial biomass (aboveground: 4.9 ± 1.07 g m^2; belowground: 1.7 ± 1.13 g) and plant richness (aboveground: 8.1 ± 0.19 g m^2; belowground: 1.2 ± 1.04 g; Table 1). The effect of plant richness on both plant productivity measures became stronger in older plant communities (interaction terms, aboveground: 1 ± 0.12 g m^2; belowground: 1.1 ± 1.04 g), as evidenced by significant interactions with plant history (Table 1).

3.3 | Relationships with fungal community composition

We evaluated whether plant richness, plant history, plant productivity and soil properties were associated with changes in fungal

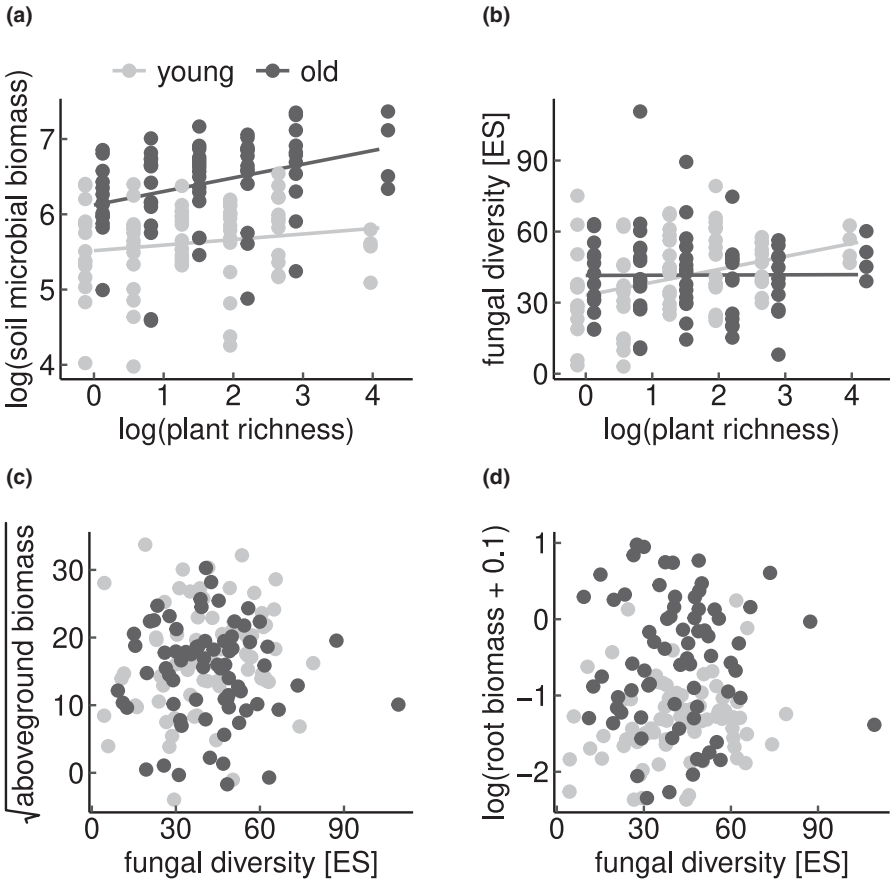

FIGURE 1 Relationships between plant richness and measures of soil microbial biomass (a) and fungal diversity (b), and of primary plant production and fungal diversity (c, d). Shading in point colours indicates measures taken in young or old experimental plant communities (see key). Response variables (*y*-axes) are represented by partial residuals, after the effects of covariates were accounted for in multiple linear mixed-effects regression analyses (see Table 1 for the effects of individual predictors). Regression lines between response and predictor variables are only shown for significant relationships ($p \leq .05$). Fungal diversity was calculated according to the Shannon diversity index and is represented as numbers of effective ASVs (ES). The variables plant richness, aboveground biomass and root biomass were square-root or log-transformed, as indicated in the respective axis labels, to comply with the assumption of residual distribution in linear mixed-effects models.

community composition. All predictors tested had a significant relationship with fungal community composition ($p \leq .05$; Table 1), although they explained relatively small proportions of fungal community variance. Plant history explained the most variance ($R^2 = .036$) followed by plant richness ($R^2 = .022$), whereas the other variables explained less than 2% of the total variance each ($R^2 < .015$). Fungal community composition was strongly affected by plant richness, plot age and their interaction (Table 1).

Visualization of changes in fungal community composition using NMDS ordinations showed a weak clustering of samples by experimental factors (Figure 2a,b). We first explored community changes in plant communities containing only one PFG (3D stress = 0.17; Figure 2a; Figure S3), which showed a significant separation of fungal communities across PFGs ($R^2 = .117$, $p < .001$; Figure 2a) that suggests that different PFGs associate with specific fungal assemblages. However, this pattern was no longer visible when assessing all samples, including plots with mixtures of PFGs (3D stress = 0.18; Figure 2b; Figure S3), which brought together a core set of fungal ASVs. Despite the variation in fungal communities across PFGs, all PFGs hosted a similar suite of the 10 main fungal orders in their roots (Figure 2c) and only affected significantly the relative abundance of one order (Hypocreales, with highest abundance in legumes; $F_{1/58} = 4.2$, $p_{adj} = .049$). In both analyses, including plots with only one PFGs or with all PFG mixtures, plant history was a main predictor of fungal community composition ($R^2 = .032$, $F_{1,62} = 2.2$, $p < .001$ for unique PFGs; $R^2 = .036$, $F_{1,147} = 5.6$, $p < .001$ for all plots; Table 1; Figure 2a,b), resulting in a greater dispersion in pairwise dissimilarities between samples of old compared to young grasslands (multivariate dispersion; one PFGs, $F_{1,61} = 5.6$, $p = .02$; all plots, $F_{1,146} = 26.9$, $p < .001$; i.e. breadth of grey areas in Figure 2a,b).

3.4 | Changes in fungal guild associations with plant community age

We studied how diversity and relative abundance of the four selected fungal guilds was associated with plant species richness and plant history. In terms of fungal diversity (Figure 3a), arbuscular mycorrhizal fungi showed greater diversity in old than in young plant communities ($F_{1/77} = 5.8$, $p = .018$), litter saprotrophs had increased diversity with increasing plant richness irrespective of plant community age ($F_{1/75} = 5.0$, $p = .028$) and diversity of plant pathogenic fungi had a relationship with plant richness that shifted from positive to negative in young respect to old grasslands (i.e. a significant interaction term; $F_{1/76} = 6.6$, $p = .012$). In terms of relative abundance, arbuscular mycorrhizal fungi decreased with both plant richness ($F_{1/81} = 5.6$, $p = .02$) and plant community age ($F_{1/81} = 5.6$, $p = .02$; Figure 3b). The abundance of plant pathogenic fungi followed the same pattern as their diversity, increasing with plant richness in young communities and decreasing in old communities ($F_{1/77} = 4.5$, $p = .04$; Figure 3b).

Analyses of individual genera within the focal fungal guilds (Figure 3c) revealed significant positive effects ($p_{adj} \leq .05$) of grassland

TABLE 1 Effects of experimental treatments on fungal community structure and plant productivity.

	Fungal diversity[a]		Aboveground biomass[a]		Root biomass[a]		Fungal community composition[b]		
	F	*p*	*F*	*p*	*F*	*p*	R^2	*F*	*p*
Plant history (PH)	3.6	.060	2.2	.137	0.0	.923	**.036**	**5.6**	**.001**
Plant richness (S)	**5.2**	**.026**	**42.1**	**<.001**	**17.9**	**<.001**	**.022**	**3.4**	**.001**
PH × S	**5.2**	**.026**	**7.8**	**.006**	**7.8**	**.007**	**.008**	**1.3**	**.043**
Fungal diversity	n.d.	n.d.	0.05	.816	0.0	.949	n.d.	n.d.	n.d.
Soil microbial biomass	n.d.	n.d.	**4.5**	**.034**	**17.6**	**<.001**	n.d.	n.d.	n.d
PH × Fungal diversity	n.d.	n.d.	1.2	.277	0.0	.863	n.d.	n.d.	n.d.
PH × Soil microbial biomass	n.d.	n.d.	3.4	.067	0.0	.889	n.d.	n.d.	n.d.
Aboveground biomass (ABM)	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	**.010**	**1.5**	**.006**
Root biomass (RBM)	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	**.013**	**2.1**	**.001**
PH × ABM	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	.007	1.0	.369
PH × RBM	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	.006	0.9	.662
Soil mineral nitrogen (SMN)	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	**.011**	**1.8**	**.004**
Soil organic carbon (SOC)	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	**.012**	**1.9**	**.001**
PH × SMN	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	.006	0.9	.816
PH × SOC	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	.006	0.9	.775

Note: Significant effects ($p \leq .05$) are indicated in bold-face.

Abbreviation: n.d., not determined.

[a]Test statistics from analysis of variance with Satterthwaite's method, based on linear mixed effects models with random intercepts accounting for the spatial structure of the experimental design.

[b]Test statistics from a permutational multivariate analysis of variance using Bray–Curtis distances between Hellinger-transformed read abundances of fungal ASVs.

age on the relative abundances of two genera of arbuscular mycorrhizal fungi (*Rhizoglomus* and *Septoglomus*) and two genera of soil and litter saprotrophs (*Cadophora* and *Agrocybe*). Alternatively, grassland age was negatively related to one genus of arbuscular mycorrhizae (*Funneliformis*), two genera of litter saprotrophs (*Chaetomium* and *Cystofilobasidium*) and one plant pathogen (*Fusarium*; Figure 3c). Only some plant pathogenic genera had relative abundances significantly affected by the interaction between plant richness and plant history, including *Ilyonectria*, whose abundance increased with plant richness only in old communities; and *Boeremia*, *Paraphoma* and *Gibberella*, which showed associations with plant richness that decreased over time (Figure 3c).

4 | DISCUSSION

Here, we show that plant community age affects the relationship between plant species richness and the diversity and structure of fungal communities in roots. However, in contrast to what we anticipated, the relationship between fungal diversity and plant richness weakened rather than strengthened with time (Figure 1b). Further, we did not find any significant response of primary productivity to fungal diversity after accounting for other factors, such as plant richness or soil microbial biomass, that may influence such a link (Figure 1c,d). Importantly, the changes in fungal community composition with plant history were consistent with a gradual recruitment of plant community-specific fungal assemblages in roots (Figure 2a,b; Table 1), as proposed by Eisenhauer et al. (2012). These changes were particularly evident for fungal pathogens, whose diversity and relative abundance decreased with plant richness in older grasslands (Figure 3a,b), potentially relieving plants from detrimental growth effects in richer plant communities. This would suggest that fungal pathogens may play a more important role in determining long-term effects of plant diversity on productivity than mutualistic and saprotrophic fungi.

4.1 | The relationship between fungal and plant diversity does not increase with time

Our results reject our first hypothesis that fungal diversity and plant richness have a positive relationship that increases with plant history. Indeed, the results show a weakening of a plant–fungal diversity relationship with time (Figure 1b). Several previous experiments have tested the hypothesis that plant diversity begets belowground fungal diversity owing to an increased availability of niches, but reached conflicting conclusions. For example, Dassen et al. (2017) found a significant association between plant richness and total fungal diversity in bulk soil of the Jena Experiment. They did not observe such a positive relationship for arbuscular mycorrhizal fungi

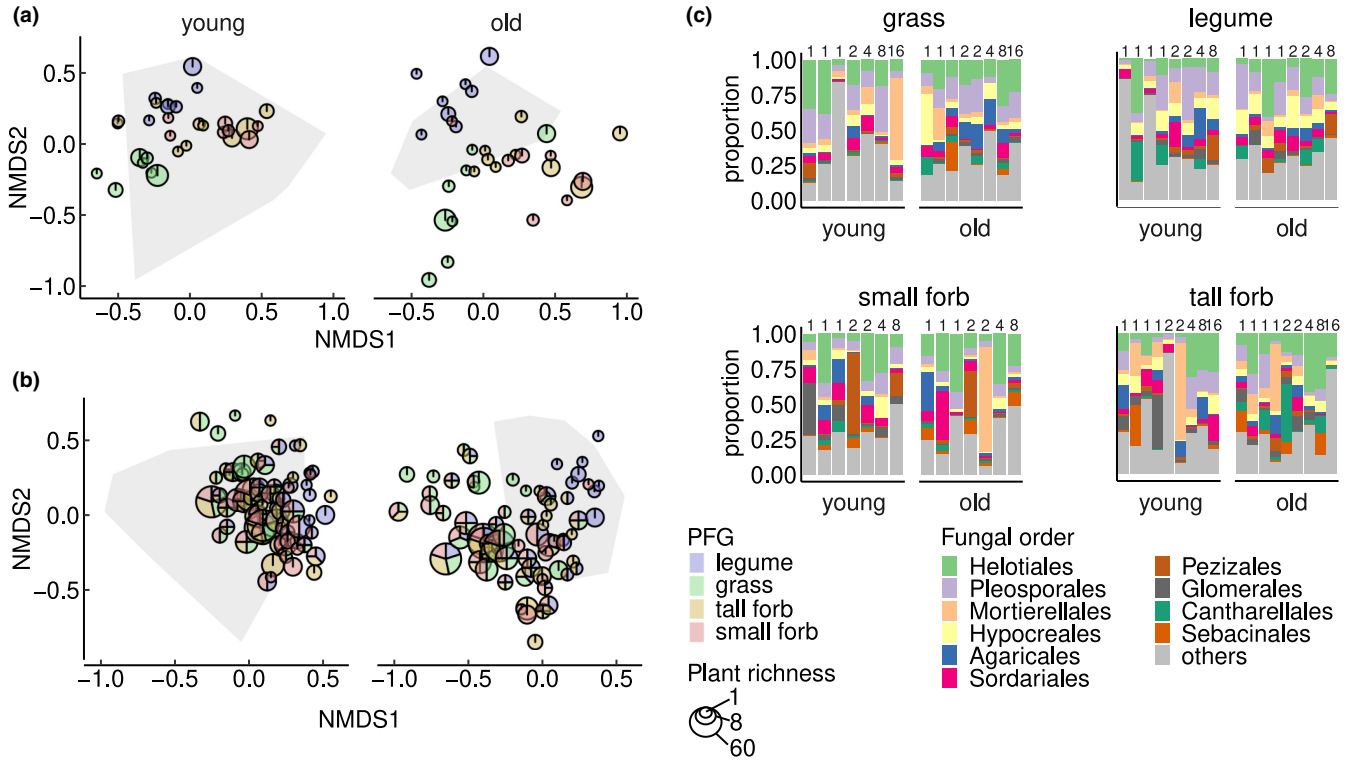

FIGURE 2 Variation in community composition of root-associated fungi across experimental treatments. (a) Non-metric multidimensional scaling (NMDS) of fungal communities in experimental plots hosting unique plant functional groups (PGFs; including legumes, grasses, small forbs and tall forbs). (b) NMDS of fungal communities in all experimental plots, including mixtures of different PFGs. For clarity, samples from young and old plant communities are shown in separate panels, with grey areas within each panel delimiting the distribution of data points in the other panel. Data points are represented by pie charts, with coloured sectors indicating the proportion of PFGs per sample, and pie sizes representing plant richness (logarithmic relationship, with the smallest indicating one plant species, and the largest 60 species). (c) Bar plots showing the proportion of ASV abundances for the 10 most abundant fungal orders in samples from plots with one PFG. Samples from young and old plant communities are shown as separate panels, with each bar representing an individual sample (data points in a). Numbers above bars indicate the plant species richness in each sample.

though, so the authors attributed the effect on fungal diversity to the accumulation of diverse litter materials in soil rather than to plant host specificity. Similarly, in another grassland biodiversity experiment, Francioli, van Rijssel, et al. (2020) found a positive association between plant diversity and richness of fungal decomposers in roots, whereas Mommer et al. (2018) reported no such relationship when examining the total root-associated fungal diversity in the same experiment. The results from these studies agree with our finding that, among all fungal guilds tested, only litter saprotrophs increased in diversity along with plant species richness (Figure 3a). Altogether, this supports the conclusion of Dassen et al. (2017) that the effects of plant richness on fungal diversity may be more related to the accumulation of diverse litter materials in soil than to an actual specificity of fungi towards colonizing roots of particular plant species, which appears to be relatively low across root-associated fungal communities (Coleman-Derr et al., 2016; Glynou et al., 2018; Maciá-Vicente et al., 2020; Maciá-Vicente & Popa, 2022; Põlme et al., 2018; Rotoni et al., 2022; Semchenko et al., 2022; Thiergart et al., 2020). Indeed, our observation of a weakening over time in the relationship between overall fungal diversity and plant richness discounts our hypothesis that old, species-rich grasslands select for many fungal taxa, each specialized towards colonizing one or a few plant species. Instead, our results are in line with the prediction made by Buscot (2015), who hypothesized that older plant communities would favour only a few fungi from the local species pool with generalist habits, that is, able to colonize roots from most plant species in the community.

In line with the weak and decreasing association between fungal and plant diversity over time, we did not find a relationship between fungal diversity and plant productivity (Figure 1c,d; Table 1). This result is consistent with previous results from the Jena experiment showing that manipulation of soil microbial communities had much lower effects on productivity than manipulations of plant history and richness (van Moorsel et al., 2018, 2021). In contrast to fungal diversity, total microbial biomass appeared as a relevant predictor of plant diversity effects on productivity, as previously highlighted by Vogel et al. (2019). However, here we cannot pinpoint whether the increase in soil microbial biomass with plant richness, and in old versus young grasslands, is a consequence or a cause of the increased plant productivity. Enhanced primary production directly caused by increases in plant richness could result in a higher quantity of plant inputs into the

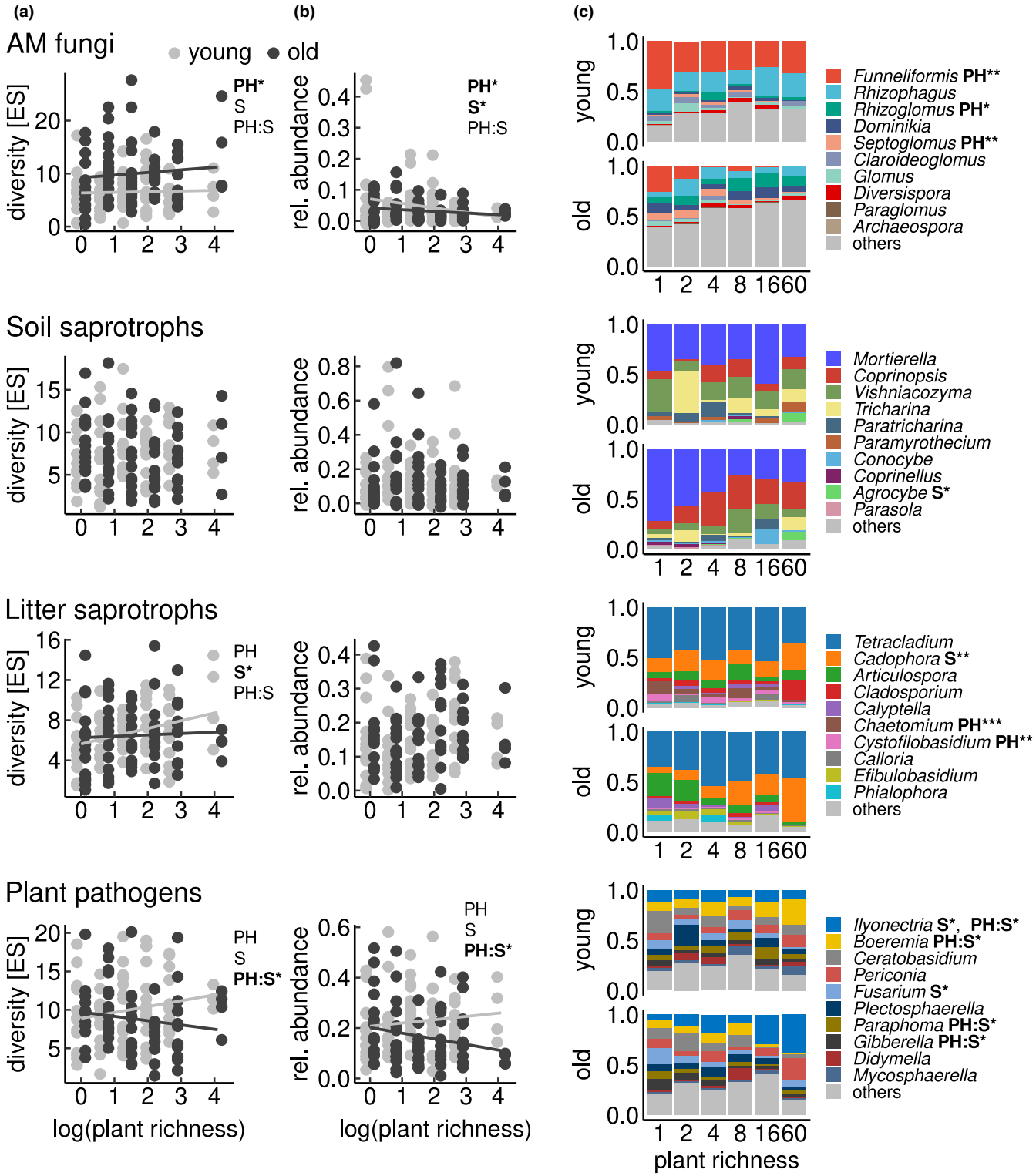

FIGURE 3 Relationships of plant history and plant richness with fungal diversity (a), relative abundance (b) and genus composition (c) of selected functional guilds: arbuscular mycorrhizal (AM) fungi, soil saprotrophs, litter saprotrophs and plant pathogens. In scatterplots (a and b), shading in point colours indicates measures taken in young or old experimental plant communities (see key) and regression lines summarize significant relationships ($p \leq .05$) between response and predictor variables. Bar plots in c show the relative abundances of the 10 most abundant genera within each guild across plant history and plant richness levels. Linear mixed-effects models were used to test significance in the relationships of total fungal diversity and abundance per guild, and of individual genus abundances, with plant history (PH), plant richness (S) and the interaction between them (PH:S). One, two and three asterisks identify significant effects in total or genus-level fungal occurrences at $p \leq .05$, .01 and .001 respectively. Plant richness was log-transformed in the linear mixed-effects models analyses to comply with the assumption of residual distribution.

soil (De Deyn et al., 2011), which could lead to an accumulation of microbial biomass over time (Eisenhauer et al., 2010; Strecker et al., 2016) akin to the one we observed. Alternatively, plant species richness could diversify the soil resource pools (Eisenhauer et al., 2017; Hooper et al., 2000), thereby sustaining a more varied and larger microbial biomass that could in turn feedback positively on plant growth through the provision of stronger and more diverse ecosystem functions (Wagg et al., 2014, 2019).

4.2 | Plant communities build up specialized fungal communities with time

We found a significant influence of plant history on the association between plant diversity and fungal community composition (Table 1). Although this was in line with our expectations (hypothesis 2), the overall effects of plant history and plant diversity on fungal community turnover were small (less than 4% total variation), and so was the interaction between these two variables. Comparatively, PFG was a much stronger predictor of fungal community composition, explaining above 11% of the total variance when experimental plots hosting unique PFGs were compared, which is in line with previous reports (Dassen et al., 2017; Francioli, van Rijssel, et al., 2020; Leff et al., 2018; Schmid et al., 2021; Sweeney et al., 2020). Plant functional groups aggregate sets of traits that have been associated with a differential recruitment of microbial taxa or functions, such as root architecture and chemistry affecting root colonization by arbuscular mycorrhizal fungi (Cortois et al., 2016; Sweeney et al., 2020) and saprotrophic communities (Francioli, van Rijssel, et al., 2020), or leaf nitrogen content affecting colonization by pathogens (Semchenko et al., 2018). Our results show that the specificity of fungal communities towards PFGs increases with plant history, as evidenced by greater dispersion in fungal community dissimilarities among samples from old versus young grasslands. This agrees with the prediction made by Eisenhauer et al. (2012), as well as with previous observations made in bulk soil fungal communities at the Jena experiment (Schmid et al., 2021), and altogether suggest a gradual accumulation of fungal communities more adapted at exploiting the resources and conditions created by certain plant species, and that could be more efficient at carrying out functions likely to affect plant productivity under local conditions.

4.3 | Fungal guilds relate differently with the long-term effects of plant diversity on productivity

Our assessment of the richness and relative abundances of fungal guilds potentially acting as facilitators or antagonists of plant growth provided only partial support for our third hypothesis. We only observed a significant shift in line with what we had anticipated in the case of fungal pathogens, whose overall diversity and relative abundance decreased with plant richness in older grasslands (Figure 3a,b). This pattern is consistent with a dilution of specific pathogens in diverse plant communities due to the presence of non-host species impairing pathogen transmission (Ampt et al., 2018, 2022; Civitello et al., 2015; Keesing & Ostfeld, 2021; Mommer et al., 2018; Rottstock et al., 2014). Ultimately, this would result in a reduced risk of disease in individual plant species, and a consequent increase in overall community productivity.

Conversely, arbuscular mycorrhizal fungi and saprotrophs that are predicted to contribute positively to plant productivity did not exhibit increasing associations with plant richness over time, contrary to what we had expected (Figure 3a,b). The diversity and abundance of arbuscular mycorrhizal fungi varied with plant community age or richness (with trends akin or unlike those found elsewhere; e.g. Dassen et al., 2017; Hiiesalu et al., 2014; König et al., 2010; Lekberg et al., 2013), but not in response to the interaction between these two factors. In particular, mycorrhizal diversity increased significantly in old grasslands, which could result in overall benefits for older plant communities irrespective of their richness (Figure 3a), thus not contributing to strengthening the plant diversity–productivity relationship. Alternatively, the reduced diversity of arbuscular mycorrhizal fungi in young versus old grasslands could be due to the soil homogenization applied during the preparation of the ΔBEF2 ('old') subplots, which is known to break hyphal networks and disrupt mycorrhizal communities (Säle et al., 2015). However, the results for arbuscular mycorrhizal fungi may be affected by known biases in the amplification and sequencing of Glomeromycota taxa resulting from using general primers targeting the fungal ITS region (Stockinger et al., 2010; Tedersoo et al., 2015). Lekberg et al. (2018) showed that general ITS fungal primers disclose similar responses to experimental factors in arbuscular mycorrhizal fungal communities compared to Glomeromycota-specific primers based on the rDNA small subunit, although the latter detect a broader taxonomic richness within the group, and may thus provide a better description of its variation patterns.

Of the saprotroph guilds, none showed significant changes in their relative abundances across treatments, although diversity of litter saprotrophs increased with plant diversity, irrespective of plant community age. This relationship, specific to litter saprotrophs, could result from an actual input upon plant decay of new fungal species that normally inhabit aboveground tissues as endophytes but switch to saprotrophic lifestyles upon tissue senescence (Fanin et al., 2021). These aboveground fungi are thought to exhibit greater host-specificities than fungal groups dwelling belowground, and thus their diversity would be more readily affected by plant richness (Semchenko et al., 2022).

Our findings, therefore, suggest a stronger influence of fungal pathogens in driving plant diversity effects on productivity than other guilds of root-associated fungi. This is in agreement with studies showing that plant biomass increases in species-poor plant communities upon treating the soil with fungicides that decrease pathogen pressure, whereas increasing the soil inoculum of arbuscular mycorrhizal fungi did not affect the plant diversity–productivity relationship (Maron et al., 2011; Mommer et al., 2018; Schnitzer et al., 2011). However, our results also draw a more complex picture

of the roles played by fungi, since the overall patterns disclosed for different fungal guilds are dependent upon finer-grained differences in the occurrence of specific taxa, with individual fungal genera responding differently to the experimental treatments. For example, whereas the relative abundances of several pathogenic genera were related to the interaction between plant richness and plant history, the direction in these changes differed across genera; some of which mirrored the main pattern for the entire guild, while others followed an opposing trend. Similar results were found for other ecological guilds, such as mycorrhizal fungi, whose genera showed opposing responses to plant history. This highlights the need for a better understanding of the trophic interactions, host specificity and plant effects of plant-associated fungi, beyond classification into broad ecological groups that provide little nuance about the lifestyles of individual taxa (Kia et al., 2017; Semchenko et al., 2022).

4.4 | Methodological considerations

There are two potential limitations of our study that may have influenced our conclusions. First, we assessed root-associated fungal communities using (mixed) roots pooled from each experimental subplot rather than from individual plants. This possibly downplayed our measurements of total fungal diversity in species-rich plant communities due to a reduced sampling effort per plant species (irrespective of the proper coverage of sequence reads per pooled sample). In addition, it may have biased our characterization of fungal community composition due to an unequal representation in root biomass per plant species and sample (e.g. see Hiiesalu et al., 2014), limiting our capacity to detect specific associations between plant and fungal taxa. Second, the annotation of fungal ASVs into ecological guilds may be too coarse, due to unreliable species identifications based on single, short DNA barcodes, the incompleteness and low taxonomic resolution of the reference database for guild assignment (Põlme et al., 2020), and the ecological versatility of fungi (Selosse et al., 2018). Therefore, our data only provide a rough inference about the ecological functions played by individual fungi. Despite these caveats, our approach enabled us to assess overall fungal diversity patterns across a wide range of plant communities with enough replication in a single experiment, and to provide a broad overview of the role played by fungal communities in driving the positive plant diversity–productivity relationship. Future work should pay increasing attention to fungal community properties in individual plant species (Ampt et al., 2022), and target fungal taxa with well-known host preferences and plant growth effects (Mommer et al., 2018). Moreover, it will be important to combine information on fungal diversity with fungal biomass data to gain additional insights into the potential strength of fungal effects on plant growth.

5 | CONCLUSIONS

Here, we provide partial support for the model proposed by Eisenhauer et al. (2012) to explain the strengthening relationship between biodiversity and productivity over time as a function of changes in soil biota. Our data suggest that changes in fungal community composition, more than in fungal diversity, may underlie the purported role of root-associated fungi in determining the positive plant diversity–productivity relationship in experimental grasslands. Notably, our results are consistent with previous studies that attribute a major role to fungal pathogens, over other groups of plant symbiotic fungi, in driving the plant diversity–productivity relationship. However, the roles played by specific fungi, and of the overall contribution of fungi to plant diversity effects on productivity with respect to other groups of soil microorganisms, remain questions to be tackled in future work. Such understanding of the effects of biodiversity on ecosystem function will be crucial to predict the consequences of undergoing species losses worldwide, especially under the threats posed by environment change, as well as to manage ecosystems under changing environmental conditions.

AUTHORS' CONTRIBUTIONS

JGMV, DF, AW, NE, JvR and LM designed the research. DF, AE and JvR conducted fieldwork and performed experiments. JGMV and DF analysed the data. JGMV drafted the manuscript. All authors contributed to the interpretation of the results and the writing of the final version of the manuscript. JGMV and DF contributed equally.

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CONFLICT OF INTEREST STATEMENT

The authors declare no competing interests.

DATA AVAILABILITY STATEMENT

The Illumina MiSeq sequence data generated in this study are deposited in the NCBI Sequence Read Archive under BioProject number PRJNA952922. All the data produced in this study have been made available online in the Jena Experiment database (JExIS; https://jexis.idiv.de).

BENEFIT-SHARING STATEMENT

Benefits from this research accrue from the sharing of our data and results on public databases as described above.

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