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Soil addition improves multifunctionality of degraded grasslands through increasing fungal richness and network complexity

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ABSTRACT

Soil addition is now widely used in the restoration of degraded ecosystems, but how soil addition influences multiple ecological functions of degraded grasslands, and whether these effects depend on the amount and type of soil inoculum, are still not clear. We performed two parallel experiments to examine how two different donor soil types and two amounts of donor soil addition affect the restoration of degraded grassland. In a field experiment at a degraded grassland site where the top layer of the soil was removed (5 cm), we assessed the effect of addition of soil collected from two different ecosystems (upland meadow and meadow steppe) and addition of different amounts of soil (0 cm, 1 cm and 3 cm) on ecosystem multifunctionality. In a microcosm experiment, we examined the effects of soil biotic and abiotic factors on ecosystem functions by inoculating sterilized and non-sterilized soil. Soil addition promoted the restoration of degraded grassland, particularly when higher amounts of soil were added. Both biotic and abiotic factors increased ecosystem multifunctionality. Biotic factors, especially fungal richness and network complexity, had the strongest positive effects on ecosystem multifunctionality. Our study reveals the importance of fungal communities in soil for improving ecosystem multifunctionality in restoration of degraded grassland. Future studies should explore the effects of joint addition of arbuscular mycorrhizal fungi and saprophytic fungi on the ecosystem functions of degraded grasslands.

1. Introduction

Grassland degradation caused by human activities such as climate change or overgrazing leads to a reduction in plant and soil diversity (Li et al., 2020; Lyu et al., 2020) and in the destruction of many ecosystem functions (Zhang et al., 2021), including declining plant production, loss of soil nutrients (Dong et al., 2020) and loss of soil organic carbon (Han et al., 2020). Various measures (replanting, artificial reconstruction and reduction of grazing with enclosures) have been applied to restore degraded grasslands (Huang et al., 2020; Zhang et al., 2020; Gao et al., 2021b; Liu et al., 2022). These studies mainly focus on the changes in plant or soil community diversity or on single ecosystem function, i.e. primary production (Wubs et al., 2019b; Grman et al., 2020; Wolfsdorf et al., 2021). In this study we focus on how soil addition influences multiple ecosystem functions in a degraded grassland restoration experiment. Clarifying the mechanisms and influences of soil addition that promote the restoration of ecosystem functions is of great significance for improving the restoration of degraded grasslands.

Addition of soil from another ecosystem, e.g. the target ecosystem can accelerate the restoration of degraded ecosystems by changing soil physicochemical properties, and by changing belowground biodiversity (Clewell & Aronson, 2013). It also can promote colonization and growth of target vegetation by influencing soil biota and nutrients in the recipient ecosystem (Wubs et al., 2016; Emsens et al., 2022). Lower amounts of soil used to inoculate might defer the establishment of vegetation (Piqueray et al., 2020) while higher amounts of soil inoculum can accelerate effects on the vegetation, e.g. on the height of ectomycorrhizal tree species (St-Denis et al., 2017). This may be due to an

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increase in soil nutrient levels or a better colonization of key soil organisms that favor the restoration of ecosystem functions, when higher amounts soil are introduced at the recipient site. Previous studies that examined the effects of soil addition applied a wide range of soil amounts (from 0.016 cm to 40 cm) (Contos et al., 2021; Han et al., 2022). To study the effect of soil amount on the effectiveness of soil addition, in this study we selected two amounts (1 cm and 3 cm) of soil addition. A large-scale soil inoculation study showed that addition of soils from two different ecosystems (heathland and grassland) leads to development of plant and soil communities at the recipient site towards the corresponding ecosystems (Wubs et al., 2016). In this field study, we selected two similar soil types (meadow steppe and upland meadow) to test whether this also leads to soil-specific directional changes in plants, soil communities and ecosystem functions at the recipient site.

Ecosystem multifunctionality is the ability of ecosystems to simultaneously provide multiple functions (Manning et al., 2018) that occur within an ecosystem, such as productivity, soil carbon storage and nutrient provision (Maestre et al., 2012; Garland et al., 2021). Ecosystem functions are influenced by producers (plants), consumers and decomposers (including microbes and nematodes) (Fierer, 2017; Manning et al., 2018; van den Hoogen et al., 2019). Hence, ecosystem multifunctionality should be best predicted by multi-trophic biodiversity measures (Delgado-Baquerizo et al., 2016; Wan et al., 2022). However, changes in ecosystem multifunctionality also depend on the shifts in environmental factors (Isbell et al., 2011). Multifunctionality relationships, for example, can differ between live soil and sterilized soil (Nuske et al., 2021; Duell et al., 2022). Addition of sterilized soil only alters soil abiotic conditions and has limited effects on ecosystem nutrient cycling, due to the absence of soil organisms that play key roles in nutrient cycling (Nuske et al., 2021). However, addition of sterilized soil can promote plant growth e.g. due to increased nutrient availability. Soil biotic effects on ecosystem functions (e.g. plant productivity) also depend on the effects of abiotic factors such as nutrients (Castle et al., 2016). Hence, distinguishing the abiotic and biotic soil effects on ecosystem functions is essential for understanding the restoration effects of soil addition.

In this study, we performed two parallel experiments. We tested the effects of soil addition on ecosystem multifunctionality across two types (meadow steppe and upland meadow) and amounts (1 cm and 3 cm) of donor soil in a 4-year soil addition field experiment. Further, we distinguished the influence of biotic and abiotic effects of soil addition on ecosystem functions in a microcosm experiment. In the field experiment, we test the following hypotheses. First, we expect that addition of upland meadow soil will lead to a higher multifunctionality than addition of meadow steppe. Second, we expect that the multifunctionality of degraded grassland will be higher when a higher amount of soil is added. In the microcosm experiment, we aimed to disentangle the immediate effects of soil biotic factors from the soil abiotic factors. We used sterilized degraded grassland soil and added live or sterilized soil from the experimental plots and then quantified how soil abiotic and biotic factors influence ecosystem functions. We predicted that in the treatment where live soil was added multifunctionality is higher than in the treatment where sterilized soil was added. With our design we aimed to provide insights into how soil addition affects the biodiversity and functions of degraded grassland.

2. Materials and methods

2.1. Site description

The study was conducted at a degraded grassland near the Erguna Forest-Steppe Ecotone Research Station of the Chinese Academy of Sciences (50° 10′ 46.1″ N, 119° 22′ 56.4″ E, 523 m above sea level), which had been used as a horse track leading to overgrazing (Han et al., 2020). In 2018, we selected an area in the degraded grassland (DG) as recipient site and three meadow steppes (DonorS) and three upland meadows (DonorM) as donor sites for the soil addition experiment (Fig. S1). The three replicates of each donor ecosystem are located about 1 km apart. At the experiment site (DG) we created three replicated blocks with a randomized block design. In each block there were 14 plots, each plot was 2 m × 2 m and was separated by 2 m wide paths. For 13 plots in each block the topsoil (0–5 cm) was removed and for one plot no topsoil was removed (NTR). The NTR plot was not included in the analysis and was established to observe the effects of natural vegetation development in the degraded grassland. The soil from the 6 donor sites (2 soil types: 3x meadow steppe (S) and 3x upland meadow (M)) was added at two different amounts (1 cm and 3 cm) so that there were a total of 12 plots with donor soil addition. These 12 experimental plots in each block thus contained 3 replicates of 4 groups, i.e. S1cm, S3cm, M1cm and M3cm. The remaining plot where topsoil was removed without soil addition served as control. In total, 42 plots ([[2 soil types (S and M) × 2 added amounts (1 cm and 3 cm) × 3 replicates] + 1 Control + 1 NTR] × 3 blocks) were established. In each plot, a 1 m × 1 m subplot was randomly selected and fixed for the annual vegetation survey and a 25 cm × 25 cm subplot was selected in a counter clockwise direction around the 1 × 1 m subplot for soil and plant samplings in each year. The current study focuses on data collected in 2021.

2.2. Plant and soil sampling

At the end of August 2021, we conducted a vegetation survey, and recorded species richness and the percentage cover of each plant species. We collected plant shoot samples from each subplot (25 cm × 25 cm) by clipping at 1 cm soil level. Then ten soil cores (0–10 cm and 2.5 cm in diameter) were collected from each subplot and the soil was homogenized. Plant roots were collected from one soil core (8 cm diameter, 15 cm deep). After field sampling, the soil for the root samples was washed over a 1 mm mesh sieve and roots were collected from the sieve. All plant shoot or root samples were oven-dried at 65 °C to constant weight and weighed. All soil samples were passed through a 5 mm sieve to homogenize the soil and to remove large roots and stones. The soil samples were then divided into three parts. One subsample was stored at 4 °C for the analysis of soil enzyme activities, microbial biomass and nematode extraction, one sample was stored at −80 °C for microbial DNA extraction, and the third one was air-dried for soil physicochemical analysis.

2.3. Soil nematode analysis and microbial DNA sequencing

The cotton-wool filter method was used to extract nematodes from 100 g of fresh soil (Townshend, 1963). The nematode suspensions obtained were fixed in formalin solution and counted using a stereo microscope (Leica stereo microscopy MZ 12.5, Germany). From each sample, 100 individuals were randomly selected and identified at the genus level (Bongers, 1994).

Soil microbial DNA was extracted using the Soil FastDNA™ Spin Kit (MP Biomedicals, USA). The bacterial and fungal genes were amplified with primer sets 515F/907R (Biddle et al., 2008) and ITS86F/ITS4R (Op De Beeck et al., 2014), respectively. PCR products were paired-end sequenced using a MiSeq PE300 platform (Illumina, San Diego, America). Amplicon sequencing and bioinformatics analyses are described in the appendix. To account for differences among samples in sequence depth, we rarefied the bacteria and fungi to 31,077 and 45,888 reads, respectively. The raw sequencing data was deposited in the China National Microbiology Data Center (Accession: NMDC10017881).

2.4. Microcosm experiment

In the microcosm experiment we used sterilized degraded grassland soil (bulk) and live or sterilized soil collected from the experimental plots. The pots (d = 13.5 cm, h = 9.5 cm), were filled with 300 g bulk soil that was topped up with 150 g live or 150 g sterilized soil collected from

the plots as live soil addition and sterilized soil addition, respectively. So, for each plot a sterilized and a live soil sample was used to disentangle the biotic and abiotic soil addition effects. In 2021, we randomly selected one block (block 2) and took 18 soil cores (0–10 cm depth) at random locations from each of the 12 plots with donor soil addition in that block. A total of 2 kg of soil of each plot was collected, homogenized and brought back to the laboratory to be used in the microcosm experiment. The experiment had the following four soil treatments: "M1cm" (1 cm upland meadow addition), "M3cm" (3 cm upland meadow addition), "S1cm" (1 cm meadow steppe addition) and "S3cm" (3 cm meadow steppe addition) collected from the field experiment. We sterilized soils using gamma-irradiation (c. 35 kGy; Harbin Radiance Radiation Technology Co., Ltd, China). We also included 18 no inoculum pots (NI) filled with 450 g sterilized degraded grassland (DG) soil. This resulted in a total of 42 microcosms [2 soil types (S and M) × 2 soil amounts (1 cm and 3 cm) × 3 replicate donors] × 2 soil sterilization levels (sterilized/non-sterilized) + 18 NI (no inoculum treatment).

One week after soil addition, 20 surface sterilized seeds (70% ethanol and 10% sodium hypochlorite) of *Leymus chinensis* (false wheatgrass or Chinese rye grass) were planted into each pot. Ten days after germination, we weeded the microcosms so that only 10 seedlings were kept per microcosm. Seedlings were watered every two days and grown for three months. Soil moisture was maintained at 20% using the weighing method and microcosms were randomly placed on racks with lighting in a plant-growth climate room, at a 30 °C: 20 °C temperature regime and under 12 h day: 12 h night conditions. At harvest, the roots were separated from the shoots for each pot. Roots were washed with water to remove soil. Shoots and roots were placed in separate paperbags, oven-dried at 65 °C and weighed. Soil enzyme activities, microbial biomass and physicochemical properties were analyzed as described above.

2.5. Measurement of physicochemical properties and quantification of ecosystem multifunctionality

We selected 11 soil and plant indicators to calculate the ecosystem multifunctionality (MultiFunc) index as the ability of an ecosystem to maintain multiple functions simultaneously. These 11 indicators represent soil carbon cycling (soil organic carbon, microbial biomass carbon, soil respiration and β-1, 4-glucosidase), soil nitrogen cycling (total nitrogen, microbial biomass nitrogen and β-1,4-N-acetylglucosaminidase), soil phosphorus cycling (total phosphorus and acid phosphatase) and primary production (shoot biomass and root biomass) (Table S1). In addition, soil moisture and pH, which are not included in the calculation of multifunctionality, were also measured. The detailed measurements for the indicators are described in the appendix.

Averaging (MultiFunc) and multi-dimensional measure (PCA-MultiFunc) approaches were used to quantify multifunctionality (Wagg et al., 2019). The results of MultiFunc and PCA-MultiFunc were highly correlated (Fig. S2B). In addition, PCA-MultiFunc can avoid potential collinearity issues between each single function (Meyer et al., 2018; Wagg et al., 2019), so the PCA-MultiFunc was used in the main text. We calculated MultiFunc by averaging the normalized 11 ecosystem function indicators using the maximum–minimum method ($f(x) = [x - \min(x)]/[\max(x) - \min(x)]$, ranging from 0 to 1) (Byrnes et al., 2014; Gamfeldt & Roger, 2017). The PCA-MultiFunc was calculated by performing a PCA analysis on the normalized function indicators and calculating the composite score by weighting the eigenvalues (Meyer et al., 2018). Soil carbon, nitrogen and phosphorus cycling and primary productivity indices were calculated in the same way as the PCA-MultiFunc (Maestre et al., 2012).

2.6. Statistical analyses

Data normality and homoscedasticity were assessed before statistical analyses. Logarithmic or square-root transformation was performed on data that did not meet the requirements, depending on the data type. For the analyses of the field experiment, first a linear mixed model was conducted with the "lmer" and "Anova" functions in the LME4 and CAR packages to examine the effects of added soil type (S and M), amount (1 cm and 3 cm) and their interaction, with block and donor sites as random factors. A post-hoc Tukey test was used for multiple comparisons. For this analysis only plots were included where soil was added (not the control plots). In this analysis we examine whether donor soil type, and amount of donor soil influence the response variables. Subsequently, to examine the effects of soil addition, we compared the control with each addition treatment (S1cm, S3cm, M1cm and M3cm). Here there were 5 categories analyzed using one-way ANOVA (with addition treatments as fixed factor, block as random factor), followed by post hoc Dunnett test, where the four addition treatments were each compared to the control. The no-topsoil removal (NTR) plots were not included in the statistical analysis and are only presented as background information. A non-parametric Kruskal-Wallis test was used when normality assumption was violated with the "scheirerRayHare" function in the RCOMPANION package. Unconstrained principle coordinates analysis (PCoA) and permutational analysis of variance (PERMANOVA) were performed based on a Bray-Curtis distance matrix, to assess the effects of added soil type, and amount and their interaction on the composition of the multifunctionality indicators, and plant and soil communities. To compare the control treatment with each added soil treatment (S1cm, S3cm, M1cm and M3cm), data from these 5 groups were analyzed using one-way PERMANOVA (all soil addition treatments as fixed factor). The PCoA and PERMANOVA were performed using the VEGAN package (Dixon, 2003). Mantel tests (Pearson correlation) were used to determine the associations between plants, soil microbes (at OTU level) and nematode community composition (at genus level) and ecosystem multifunctionality through Bray–Curtis distance using the GGCOR package (https://rdrr.io/github/houyunhuang/ggcor). For univariate measures such as soil physicochemical and biological α-diversity, we use Euclidean distances for analysis. Random forest analysis was used to evaluate important predictors of ecosystem multifunctionality among different abiotic and biotic factors significantly correlated with MultiFunc in the mantel test using RFPERMUTE (Significance of each predictor) and A3 (Significance of the model) package (Jiao et al., 2018). As soil C, N and P contents are part of the ecosystem multifunctionality calculation, we did not include these indicators in the model.

Fungal and bacterial co-occurrence networks were calculated with the IGRAPH package (Csardi & Nepusz, 2006) based on Spearman's correlation matrices. Only OTUs that were present in at least 1/3 of the samples were retained for network analysis (Zhou et al., 2011; Banerjee et al., 2019; Yuan et al., 2021). We focus on OTUs that strongly co-occurred in the network with a FDR-adjusted cutoff of $P < 0.05$ (Qiao et al., 2021) and $r \geq 0.65$ (Chen et al., 2022; Jiao et al., 2022). The networks were visualized with Gephi (version 0.9.2) software. The network of each group and topological properties of the network for each sample were calculated using the "subgraph" function via the IGRAPH package (Ma et al., 2016). The properties included the complexity of the network (node numbers, edge numbers, average degree, cluster coefficient, average path length and betweenness centrality) (de Vries et al., 2018). Typically, a highly complex network has a greater number of nodes, edges, average degree, and cluster coefficient, but lower values of average path length and betweenness centrality (Barberan et al., 2012; Qiu et al., 2021). Network complexity was calculated using the same method as the calculation of PCA-MultiFunc (Manning et al., 2018) after getting the inverse of average path length and betweenness centrality (Jiao et al., 2021). A combination of degree and closeness centrality, was used to statistically identify microbial key OTUs. OTUs with degree ≥ 3 and closeness centrality > 0.3 were selected as potential key taxa (Gao et al., 2021a; Xiong et al., 2021). Each fungal key taxa based on the functional groups was classified using the FUNGuild database at the 'Highly Probable' and 'Probable' confidence rankings. In the microcosm experiment, the analysis of ecosystem

functions among different treatments was as described for the field experiment. For addition of soil from the field plots, we assessed the effects of soil type (S and M), amount (1 cm and 3 cm), soil sterilization (sterilized or unsterilized soil) and their interactions, with donor sites as random factor using LMM. A post-hoc Tukey test was used for multiple comparisons. To compare the control (No-addition) treatment with each added soil treatment (sterilized or unsterilized soil from S1cm, S3cm, M1cm and M3cm field plots), data from these 9 groups were analyzed using one-way ANOVA (all soil addition treatments as fixed factor), followed by a post-hoc Dunnett test.

3. Results

3.1. Effects of soil addition on ecosystem multifunctionality in the field experiment

The amount of added soil significantly affected the ecosystem multifunctionality (MultiFunc) of the degraded grassland ($P < 0.001$, Table 1). Addition of different soil types did not lead to differences in the composition of ecosystem functions. However, addition with a higher amount of soil led to the composition of ecosystem functions that more closely resembled the donor ecosystems than addition of a lower amount of soil. Both centroids of S1 and M1 were closely located to the centroid of the control in the multivariate plot (Fig. 1B). The amount of added soil also significantly affected soil nutrient cycling functions of the degraded grassland ($P < 0.001$, Table 1), with the highest soil carbon, nitrogen and phosphorus cycling indices observed in the treatments where the higher amount of soil was added (Fig. 1). Soil addition had no significant effects on the primary production of plants (Fig. 1F).

3.2. Effects of soil addition on biotic and abiotic parameters in the field experiment

Soil addition significantly affected the soil microbiomes and plant communities in the degraded grassland (Table 2; Table S2). Richness of fungi was significantly affected by soil addition (Fig. 2F). Increasing the amount of soil had a positive effect on the richness of nematodes ($P < 0.05$) and fungi ($P < 0.01$). There were no significant effects on the Shannon diversity of plants, bacteria, fungi and nematodes (Table S2; Fig. S3).

Soil pH was significantly affected by the type of added soil ($P < 0.05$), with higher pH observed in plots where meadow steppe soil was added than in plots added with upland meadow soil (Table S4). Increasing the amount of soil had a positive effect on soil moisture ($P < 0.01$) but a negative effect on soil pH ($P < 0.05$).

The results of Mantel tests indicated that soil moisture ($P < 0.001$), fungal richness ($P < 0.001$), nematode richness ($P < 0.05$) and plant Shannon diversity ($P < 0.05$) had positive effects on ecosystem multifunctionality, while microbial qCO_2 had a negative effect ($P < 0.05$) on MultiFunc (Fig. 3A). The random forest model showed that fungal richness ($P < 0.01$) and soil moisture ($P < 0.05$) were the main factors influencing ecosystem multifunctionality (Fig. 3B).

3.3. Soil biological network complexity in the field experiment

Soil bacterial (Fig. S7A) and fungal (Fig. 4A) community networks showed different symbiotic patterns among different treatments. There was no significant difference in the complexity of bacterial networks among different treatments four years after initiating the experiment. There was no obvious relationship between the complexity of bacterial networks and ecosystem multifunctionality (Fig. S7C). However, the type and amount of soil addition significantly affected fungal network complexity ($P < 0.001$), with higher complexity found in plots where upland meadow soil was added than in plots where meadow steppe soil was added. Increasing the amount of inoculum also had a positive effect on fungal network complexity (Fig. 4B). These results show that soil addition affected fungal associations, and increased the complexity of soil fungal community networks. The changes in network complexity were significantly correlated with ecosystem multifunctionality (Fig. 4C).

Saprotroph fungi, arbuscular mycorrhizal fungi and soil pathogens were identified as key functional groups (key guilds) for the restoration of degraded grassland (Fig. 5A and B). There was a higher proportion of arbuscular mycorrhizal fungi in the control plots where no soil was added and in the plots where a low amount of soil was added. The proportion of saprotrophs was higher in plots where 3 cm soil was added, than in plots where 1 cm was added. The proportion of pathogens was lower in plots where upland meadow soil was added than in plots where meadow steppe soil was added. Moreover, the plot with a higher amount of soil addition had lower pathogens than the other treatments.

The added soil type significantly affected the proportion of *Achroiostachys, Cercophora, Clonostachys, Myrmecridium, Paraphaeosphaeria* and Diversisporaceae (Table S7). The amount of soil added significantly affected the proportion of Nectriaceae ($P < 0.05$). Among them, *Achroiostachys* and *Cercophora* only existed in the plots where meadow steppe soil was added. The proportion of *Clonostachys*, *Myrmecridium* and Diversisporaceae in the soil of plots where meadow steppe was added was higher than in plots where upland meadow soil was added. The proportion of *Paraphaeosphaeria* in the plots with upland meadow soil was higher than in plots with meadow steppe soil. The proportion of Nectriaceae was higher in the soil where 3 cm soil was added than in plots where 1 cm soil was added (Fig. 5C). The proportions of *Achroiostachys* and Nectriaceae, both saprophytic fungi, were positively correlated ($P < 0.05$) with ecosystem multifunctionality (Fig. S9).

3.4. Effects of biotic and abiotic factors on multifunctionality in the microcosm experiment

The type ($P < 0.001$) and amount ($P < 0.01$) of added soil and whether the inoculated soil was sterilized or not ($P < 0.05$) significantly affected ecosystem multifunctionality in the microcosm experiment (Table 3). Live soil addition resulted in higher ecosystem multifunctionality than sterilized soil addition. Compared to the control plots, addition of live and sterilized soil from plots increased ecosystem multifunctionality, but not for soil collected from S1cm plots (Fig. 6A).

Table 1
One-way ANOVA results for effects of soil addition (Control, S1cm, S3cm, M1cm and M3cm) and two-way ANOVA results for effects of soil type (S and M) and soil amount (1 cm and 3 cm) on the ecosystem functions of degraded grassland.

	One-way ANOVA			Two-way ANOVA								
Treatment	Soil addition			Soil type (S)			Soil amount (A)			S * A		
	df	F	*P*	df	F	*P*	df	F	*P*	df	F	*P*
Ave-MultiFunc	4, 32	15.96	<0.001	1, 4	0.28	0.62	1, 26	72.33	<0.001	1, 26	1.19	0.29
PCA-MultiFunc	4, 32	16.38	<0.001	1, 4	0.25	0.64	1, 26	75.14	<0.001	1, 26	0.96	0.34
Soil C cycle	4, 32	9.10	<0.001	1, 4	0.11	0.76	1, 26	46.52	<0.001	1, 26	0.00	0.98
Soil N cycle	4, 32	8.70	<0.001	1, 4	0.03	0.87	1, 26	42.22	<0.001	1, 26	2.63	0.12
Soil P cycle	4, 32	10.42	<0.001	1, 4	0.02	0.89	1, 26	46.13	<0.001	1, 26	1.03	0.32
Primary production	4, 32	1.68	0.18	1, 4	3.06	0.16	1, 26	0.08	0.78	1, 26	0.01	0.92

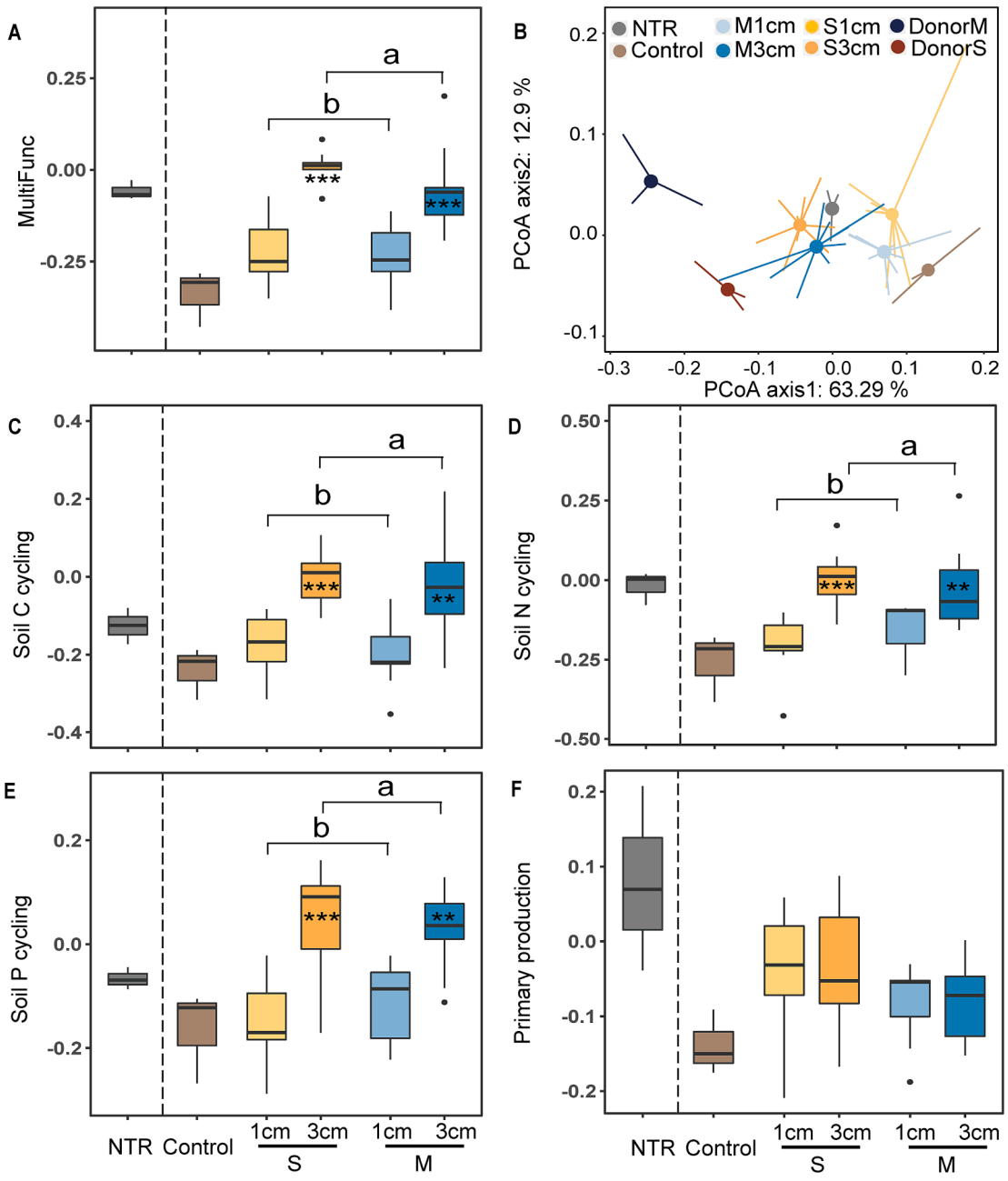

Fig. 1. Effect of soil addition on ecosystem functions of degraded grassland. Ecosystem multifunctionality (A), composition of ecosystem functions (B), soil carbon cycling index (C), soil nitrogen cycling index (D), soil phosphorus cycling index (E) and primary production (F). In (B) the percentage given on each axis refers to the variation explained by this axis as part of the total variation. Soil (S): the origin of added soil; Amount (A): the amount of added soil. The colors depict soil addition treatments: Control, plot with topsoil removal; NTR, plot without topsoil removal; S1cm, meadow steppe soil 1 cm added; S3cm, meadow steppe soil 3 cm added; M1cm, upland meadow soil 1 cm added; M3cm, upland meadow soil 3 cm added; DonorS, donor meadow steppe; DonorM, donor upland meadow. The endpoint of each line represents the replicated sample points in each treatment. Different lowercase letters indicate significant differences in ecosystem functions comparing two different amounts of added soil based on post hoc Tukey test at $P < 0.05$ after a 2-way ANOVA. Asterisks within each bar denote significant differences from the Control treatment based on a Dunnett's test: ** $P < 0.01$; *** $P < 0.001$.

Overall, higher ecosystem multifunctionality was found in the treatments where upland meadow soil (M1cm and M3cm) was added than in plots where meadow steppe soil (S1cm and S3cm) was added. Ecosystem multifunctionality in the microcosms where soil was added that originated from field plots of higher amount of soil additions was higher than soil from plots where a lower amount of soil was added (Fig. 6A).

N cycling of the soil, overall, was significantly higher in microcosms where live soil was added than in ones with sterilized soil from the field plots ($P < 0.05$). Addition of live or sterilized soil did not result in significant differences ($P > 0.05$) for soil carbon and phosphorus cycling and for primary productivity.

4. Discussion

We assessed the effects of soil addition on ecosystem multifunctionality in a degraded grassland and show that soil addition can promote the restoration of degraded grassland, particularly when higher amounts of soil are added. Both soil biota and abiotic conditions influenced ecosystem multifunctionality, but biotic factors, especially fungal diversity, had the strongest effect on ecosystem multifunctionality.

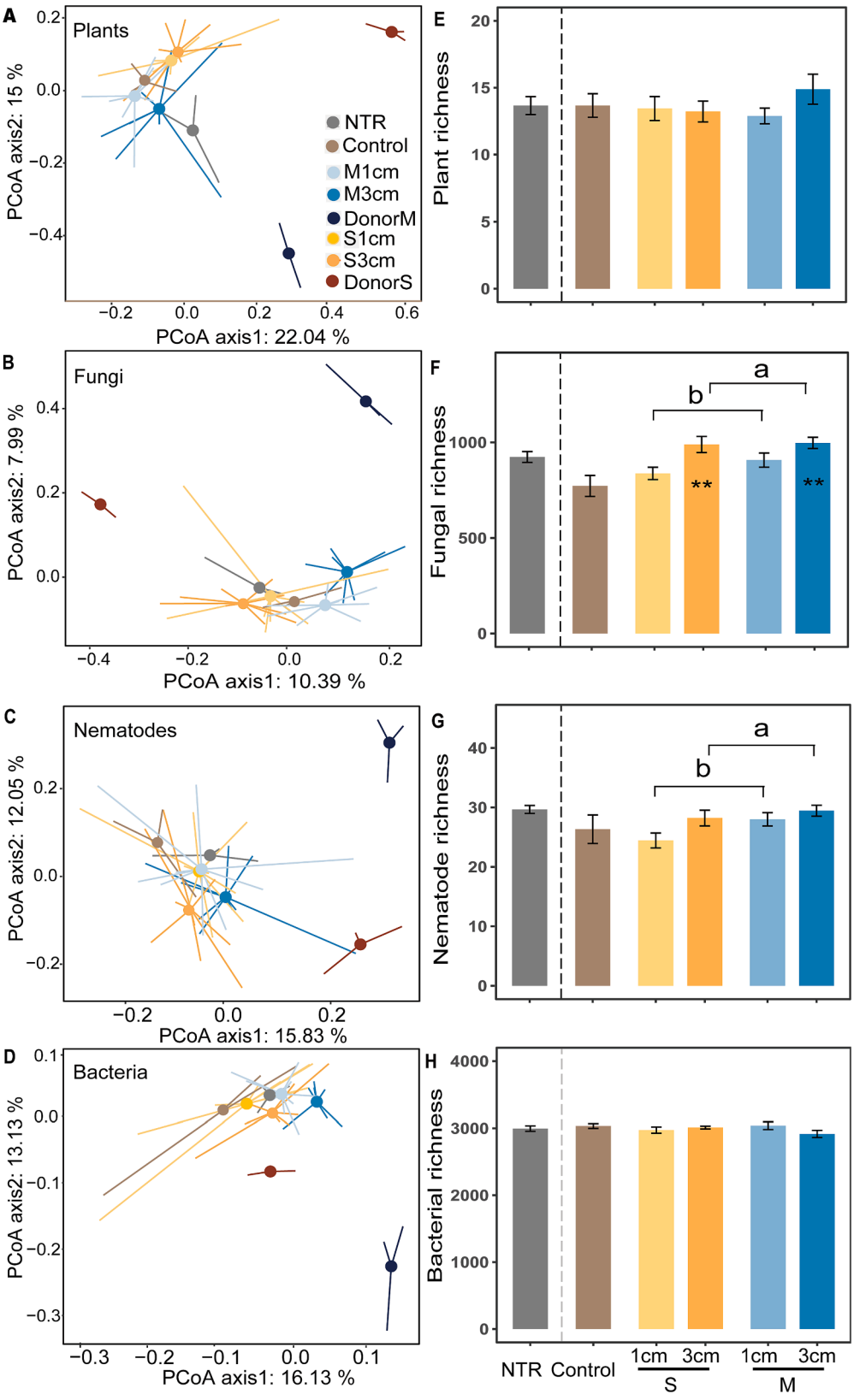

Fig. 2. Effects of soil addition on the community composition and richness of plants (A, E), fungi (B, F), nematodes (C, G) and bacteria (D, H). The percentage given next to each axis refers to the variation explained by this axis as part of the total variation. Soil (S): the origin of the added soil; Amount (A): the amount of added soil. The colors depict soil addition treatments: Control, plots with topsoil removal; NTR, plots without topsoil removal; S1cm, meadow steppe soil 1 cm added; S3cm, meadow steppe soil 3 cm added; M1cm, upland meadow soil 1 cm added; M3cm, upland meadow soil 3 cm added; DonorS, donor meadow steppe; DonorM, donor upland meadow. Different lowercase letters indicate significant differences in the richness of fungi and nematodes among the two different amounts of added soil based on a post hoc Tukey test at $P < 0.05$. Asterisks within each bar denote significant differences from the Control treatment based on a Dunnett's test: ** $P < 0.01$.

4.1. Addition with different soil types changed complexity of fungal networks

Higher biodiversity can accelerate ecosystem processes and functions, such as nutrient cycling, carbon sequestration and plant productivity (Wardle et al., 2004; van der Heijden et al., 2008). In this study, we found that addition of different soil types had no significant effect on richness of the microbial community and plant species richness, but that it affected nematode richness. Addition of the two different soil types resulted in a divergence in the composition of plant,

Table 2
One-way PERMANOVA results for effects of soil addition (Control, S1cm, S3cm, M1cm and M3cm) and two-way PERMANOVA results for effects of soil type (S and M) and soil amount (1 cm and 3 cm) on the composition structure for bacteria, fungi, nematodes, plants and ecosystem multifunctionality.

	Treatment	df	Bacteria		Fungi		Nematodes		Plants		MultiFunc	
			F	*P*	F	*P*	F	*P*	F	*P*	F	*P*
One-way	Soil addition	4, 34	1.49	<0.01	1.75	<0.01	1.09	0.26	1.99	<0.01	4.26	<0.01
Two-way	Soil type (S)	1, 32	1.97	<0.01	2.65	<0.01	1.07	0.35	3.49	<0.01	1.10	0.33
	Soil amount (A)	1, 32	1.51	<0.01	1.65	<0.01	0.99	0.48	1.85	0.04	11.40	<0.01
	S * A	1, 32	1.04	0.31	1.17	0.10	0.89	0.60	0.97	0.48	0.26	0.94

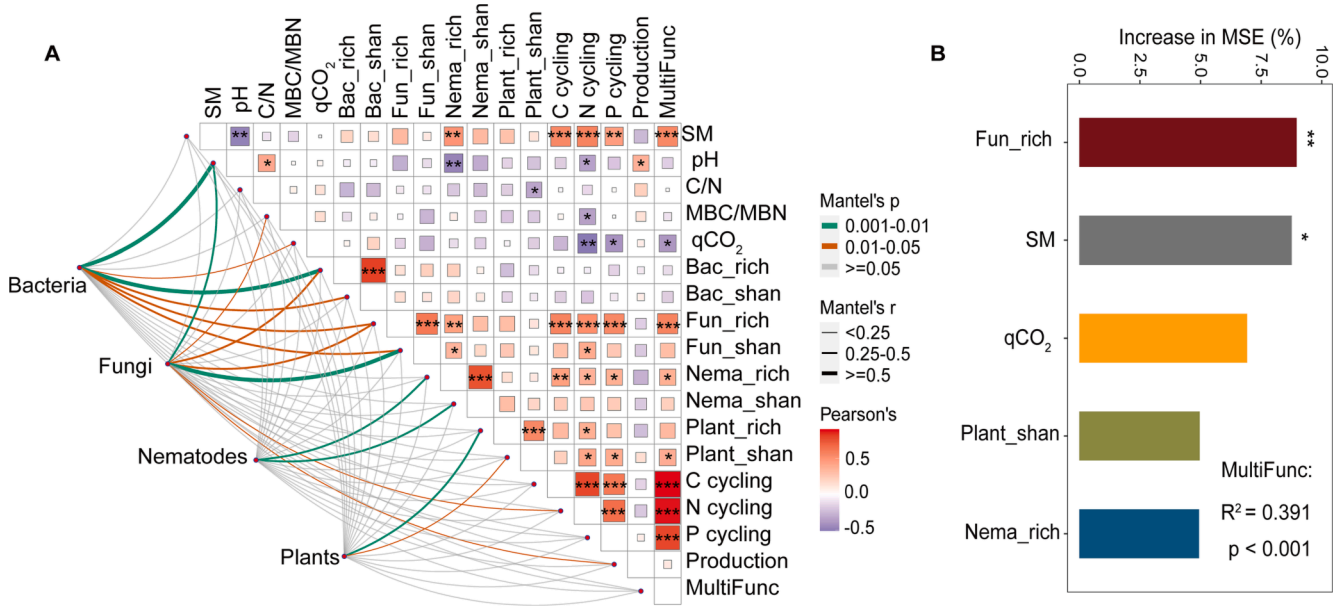

Fig. 3. Main biotic and abiotic factors influencing ecosystem multifunctionality of degraded grassland based on a Mantel test (Pearson's correlations) (A) and random forest model (B). In A, community data is calculated based on Bray-Curtis distance, and the single environmental and biological indicators are calculated based on Euclidean distance. SM: soil moisture; pH: soil pH; C/N: soil Total C/Total N; MBC/MBN: soil microbial biomass C/microbial biomass N; qCO_2: soil microbial qCO_2; Bac_rich: bacterial richness; Bac_shan: bacterial Shannon diversity; Fun_rich: fungal richness; Fun_shan: fungal Shannon diversity; Nema_rich: nematodes richness; Nema_shan: nemaodes Shannon diversity; Plant_rich: plant richness; Plant_shan: plant Shannon diversity; C cycling: soil carbon cycling; N cycling: soil nitrogen cycling; P cycling: soil phosphorus cycling; Production: primary productivity. Bacterial and fungal communities are analyzed at OTU level. Nematode communities are analyzed at genus level. Plant communities are analyzed at species level. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

fungal and bacterial communities towards the corresponding ecosystems. However, ecosystem multifunctionality or single functions (soil C, N, P cycling and primary production) did not differ in the degraded grassland when comparing the addition of upland meadow and meadow steppe soil. This is inconsistent with our first hypothesis. A correlation analysis revealed that particular fungal key taxa such as OTU5651 and OTU7873 that were found in both donor systems correlated with multifunctionality (Fig. S8). Our results therefore suggest key taxa that were present in both donor systems maybe essential for maintaining plant growth and the biogeochemical cycles of the ecosystem (Toju et al., 2018).

Interestingly, we found that the complexity of the fungal networks was higher after addition of upland meadow soil than after addition of meadow steppe soil. Moreover, although the key species guilds were the same among the soil addition types, the plots where upland meadow soil was added had higher relative abundance of saprophytic fungi and lower abundance of potential pathogens. As both the richness and the composition of biota are important to support ecosystem functions, it is possible that over time we will observe a divergence in ecosystem functions (Mori et al., 2018). Future research should examine how additions with different soil microbiomes change network connections and key species of plant and soil organisms over longer time periods, and assess the links between these changes and ecosystem functions.

4.2. *Higher amounts of added donor soil improve ecosystem multifunctionality*

The successful restoration of terrestrial ecosystems depends on the presence and activity of soil biota (Wubs et al., 2019a). Hence, improving the conditions that are favorable to increase the richness and activity of soil organisms has been proposed as a close-to-nature method for ecosystem restoration (Coban et al., 2022). We observed higher species richness of fungi and nematodes, and higher ecosystem multifunctionality in the treatments where a higher amount of soil was added. These results support our second hypothesis that addition of a higher amount of soil will improve ecosystem multifunctionality of degraded grasslands more than addition of a lower amount. It suggests that more fungi and nematode species can survive in the soil after higher amounts of donor soil are added. Interestingly, the Mantel test and random forest model provided evidence that fungal richness plays a more obvious role than bacterial or nematode richness in the recovery process of grassland functions after addition of donor soil. This emphasizes the important links between fungal richness and ecosystem multifunctionality. In addition, higher fungal richness can lead to higher association complexity within fungal communities, and to greater association among individuals and this can support functions provided by the fungi (Wagg et al., 2019). This is in line with the result that there was a positive connection between ecosystem multifunctionality and the

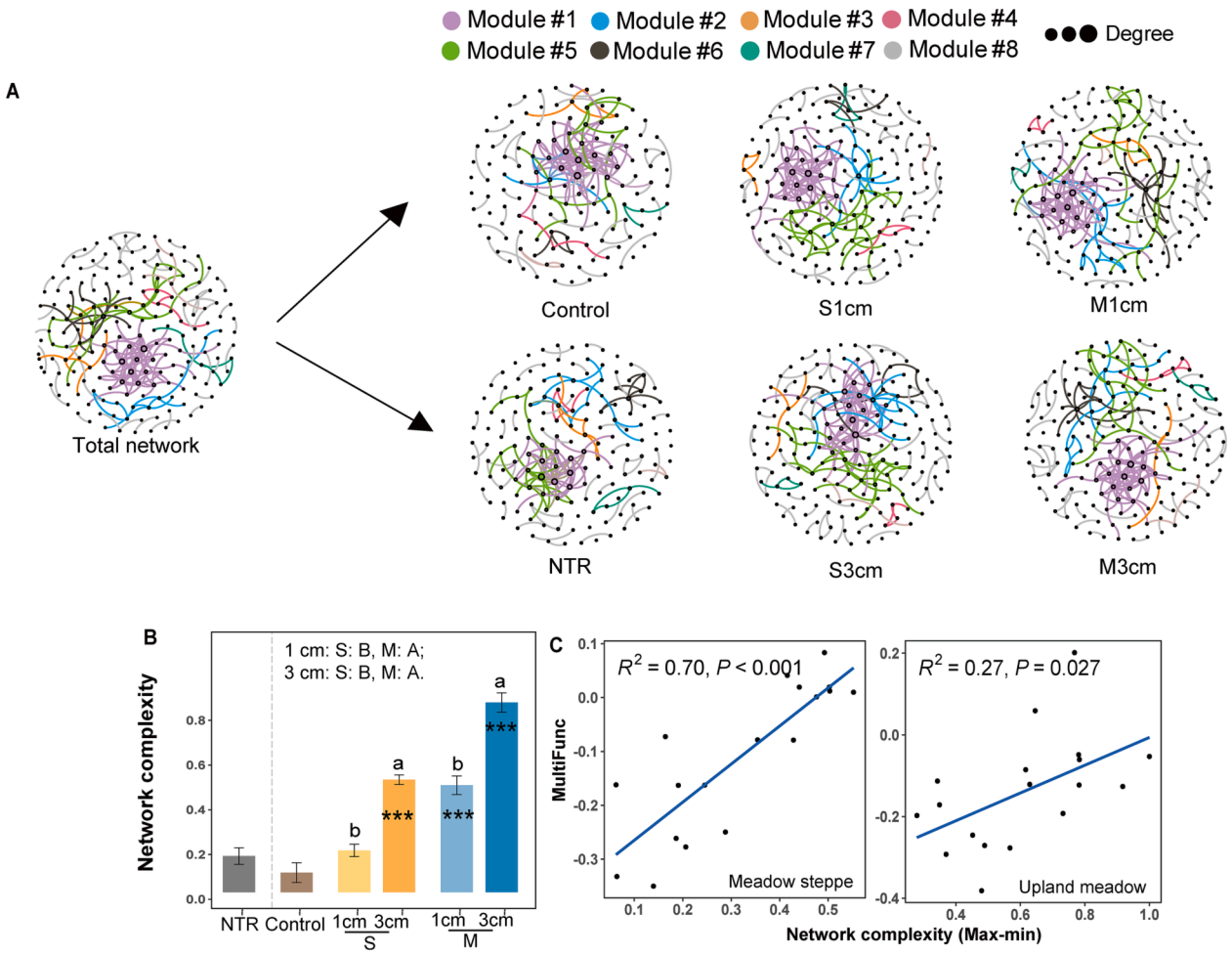

Fig. 4. Ecological networks of soil fungi at OTU level (A), a composite index used to estimate the complexity (B), relationship between network complexity and ecosystem multifunctionality of degraded grassland (C). The nodes are colored according to fungal models, and node size indicates the degree of connection. Soil (S): the origin of soil for addition; Amount (A): the amount of soil for addition. The colors depict soil addition treatments: Control, plots with topsoil removal; NTR, plots with no topsoil removal; S1cm, meadow steppe soil 1 cm added; S3cm, meadow steppe soil 3 cm added; M1cm, upland meadow soil 1 cm added; M3cm, upland meadow soil 3 cm added. Different lowercase letters indicate significant differences in network complexity between soil amounts in each soil type based on a post hoc Tukey test at $P < 0.05$. Different uppercase letters indicate significant differences in network complexity between soil types in each soil amount based on a post hoc Tukey test at P < 0.05. Asterisks within each bar denote significant differences from the Control treatment based on Dunnett's test: *** $P < 0.001$. R^2 and P are also presented.

number of links per fungal node in the association network (network complexity). Previous studies have found that resource availability increased when fungal networks become more complex (Banerjee et al., 2019; Guo et al., 2020), which may further benefit plant growth.

Key taxa are highly connected in biological networks (Banerjee et al., 2018). They may be deployed to stimulate favorable soil biota for the restoration of ecosystem functions. In our results, saprophytic fungi, arbuscular mycorrhizal fungi (AMF) and pathogenic fungi were identified as key functional guilds (Chen et al., 2021). A higher proportion of AMF and a lower proportion of saprophytic fungi was observed in the topsoil removal treatment and in the 1 cm soil addition treatment than in the 3 cm soil addition treatment. This may be related to the nutrient levels in the plots of the different treatments. Plants tend to have a higher dependency on AMF in soils with lower nutrient availability (Kleikamp & Joergensen, 2006), as AMF can positively affect plant nutrient acquisition and accelerate organic material decomposition (Leigh et al., 2009). However, AMF cannot directly decompose organic matter, as they do not have saprophytic capacity. Thus saprotrophs are also needed to decompose organic matter in soils with lower concentrations of available nutrients (e.g. Control, S1cm and M1cm). The relationship between AMF and saprotrophs is not always positive. When nutrient availability is higher, plants tend to allocate less carbon to AMF, and the latter could act as parasites in such situation (Cao et al., 2022). This could explain the higher soil nutrient cycling functions but lower plant production functions in the treatments with the higher amount of added soil compared to the control plots without top soil removal, as the treatment with higher amount of added soil had a higher proportion of saprotrophic fungi and a lower proportion of AMF.

In this study, we identified two saprophytic fungi that were positively related to ecosystem multifunctionality, namely *Achroiostachys* and Nectriaceae. These fungi can improve soil carbon and phosphorus cycling (Silva et al., 2021), which in turn can affect biogeochemical cycling and plant growth in degraded grasslands. Furthermore, we detected two groups of AMF species, *Glomus* (Glomerales) and Diversisporaceae (Diversiporales) which were related to the acquisition of nitrogen and phosphorus. Glomerales is a well-known order and a dominant commercial bio-inoculant (Basiru et al., 2021). Our study highlights that Diversiporales may also be important in degraded grasslands. Previous studies have found that Glomerales may not always be a good biological inoculant, and that its use has limitations (Salomon et al., 2022). Thus the combination of *Glomus* and Diversisporaceae might improve the success rate of AMF addition. However, our study does not provide direct evidence for effects of fungal richness and the addition of keystone species on ecosystem functions. This needs to be

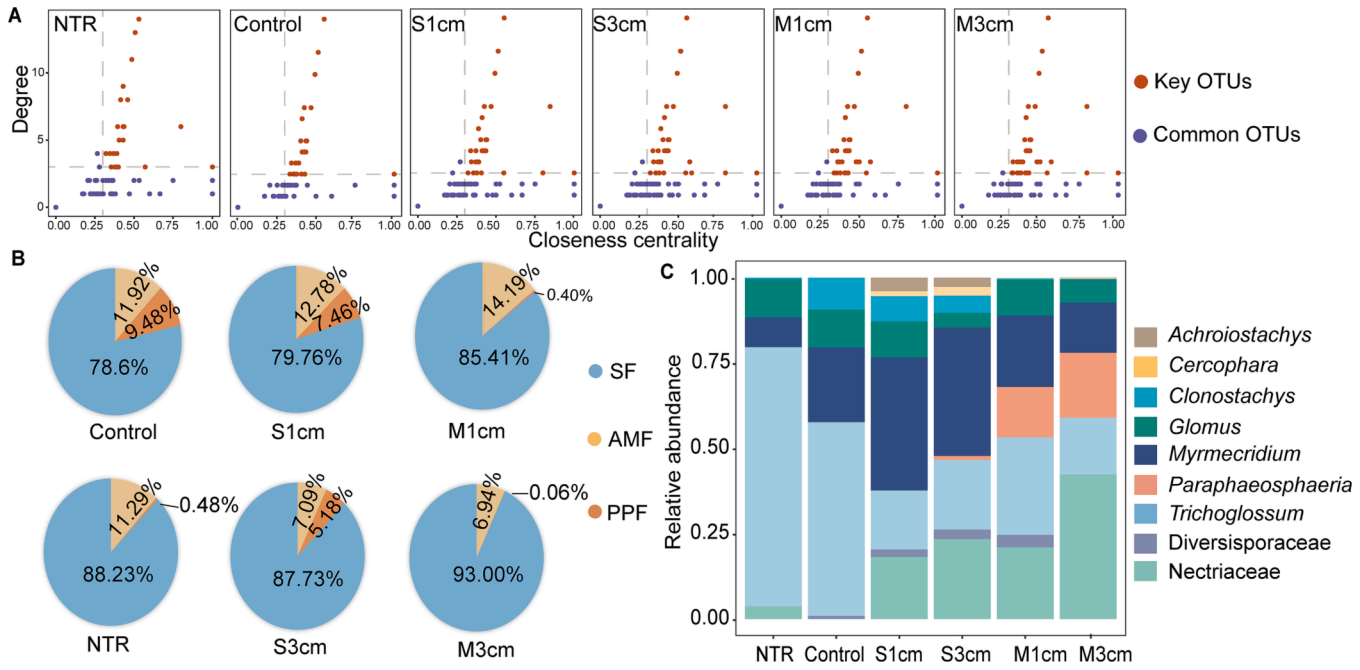

Fig. 5. Scatterplot of OTUs according to degree and closeness centrality in the field experiment. Orange dots represent fungal key OTUs, purple dots represent common fungal OTUs (A). The proportion of different fungal guilds classified by key OTUs, SF: saprophytic fungi; AMF: Arbuscular mycorrhizal fungi; PPF: plant pathogen fungi (B), and fungal taxa classified by key OTUs (C) of the networks. The colors depict soil addition treatments: Control, plot with topsoil removal; NTR, plot without topsoil removal; S1cm, meadow steppe soil 1 cm added; S3cm, meadow steppe soil 3 cm added; M1cm, upland meadow soil 1 cm added; M3cm, upland meadow soil 3 cm added.

Table 3
Results from a one-way ANOVA comparing the effects of control (no-addition) and soil addition treatments (8 group of live soil and sterilized soil addition); and a three-way ANOVA comparing the effects of soil type (S and M), soil amount (1 cm and 3 cm) and levels of soil sterilization (live soil and sterilized soil) on the ecosystem functions of degraded grassland in the microcosm experiment.

	Treatment	df	MultiFunc		Soil C cycle		Soil N cycle		Soil P cycle		Primary production	
			F	*P*	F	*P*	F	*P*	F	*P*	F	*P*
One-way ANOVA	Soil addition	8, 18	8.14	<0.01	7.44	<0.01	3.93	0.01	0.62	0.75	3.69	<0.01
Three-way ANOVA	Soil type (S)	1, 14	9.10	<0.01	2.83	0.10	8.89	<0.01	0.05	0.83	1.08	0.30
	Soil amount (A)	1, 14	12.84	<0.01	13.91	<0.01	9.38	<0.01	0.82	0.37	2.26	0.14
	Sterilization (ST)	1, 14	12.66	<0.01	6.11	<0.01	3.17	0.02	4.12	<0.01	18.33	<0.01
	S * A	1, 14	13.07	<0.01	9.98	<0.01	1.99	0.16	1.57	0.22	0.28	0.60
	S * ST	1, 14	5.74	<0.01	2.03	0.09	1.38	0.25	0.68	0.64	4.39	<0.01
	A * ST	1, 14	4.90	<0.01	4.95	<0.01	3.06	0.02	0.40	0.85	0.31	0.91
	S * A * ST	1, 14	2.07	0.09	2.24	0.07	1.47	0.22	0.30	0.91	3.44	0.01

tested in further experiments that include different nutrient gradients to disentangle the effects of fungi and carbon and nutrient stocks. It is important to mention that soil carbon, nitrogen and phosphorus sequestration are all part of ecosystem multifunctionality. Hence, in our experiment, the addition of soils with higher nutrient levels could also directly increase the multifunctionality of the degraded grassland. This is revealed from an additional random forest model (Fig. S6), where we included total soil carbon, and nutrient stocks, although we emphasize nutrient sequestration is also included in the multifunctionality calculation. Interestingly, fungal richness is still one of the significant predictors of ecosystem multifunctionality when STC and STP are included as explaining variables in the model, which highlights the importance of fungal diversity for the restoration of ecosystem function in degraded grasslands. Future experiments are also needed to quantitatively assess the relationship between AMF, saprophytic fungi and ecosystem multifunctionality. These studies should focus on absolute abundances of these key fungal guilds and examine how inoculating these key genera in different proportions will influence ecosystem functions in degraded grasslands.

4.3. Biotic and abiotic factors are both important components that influence ecosystem multifunctionality

In the microcosm experiment with soil collected from the field plots, we found that ecosystem multifunctionality tended to respond directionally similar for the abiotic and biotic components of the soil, independent of the origin of the soil. Soil collected from plots where 1 cm steppe soil was added supported the lowest ecosystem multifunctionality. Further, adding live soil collected from plots, on average, supported higher ecosystem multifunctionality than adding sterilized soil. Moreover, adding soil collected from plots where a higher amount of soil was added originally, supported higher ecosystem multifunctionality than soil from plots where lower amount of soil was added. This may be because more soil organisms were introduced when more soil was added, and this can be important for the functions the soil organisms support, such as organic matter decomposition and soil nutrients cycling (Bardgett & van der Putten, 2014; Garland et al., 2021). The result that higher ecosystem multifunctionality was observed in the treatments with live soil collected from plots where high amounts of soil were added stresses the key role of biotic factors in improving ecosystem

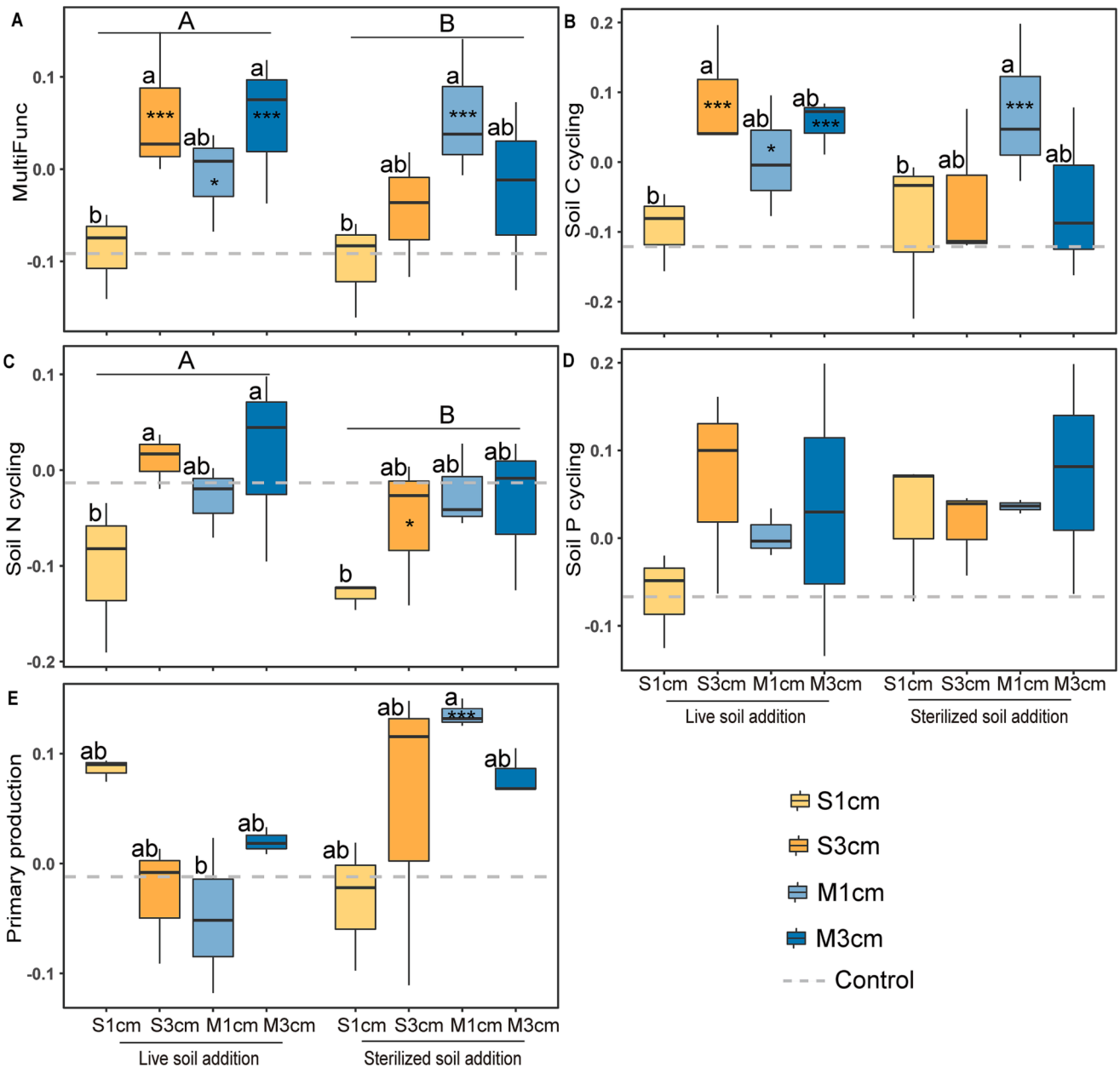

Fig. 6. Multifunctionality in the microcosm experiment. Biotic and abiotic effects of soil addition on ecosystem multifunctionality with soils collected from experimental plots (A). Biotic and abiotic effects of soil addition on soil carbon cycling index (B), soil nitrogen cycling index (C), soil phosphorus cycling index (D) and primary production (E). The gray dotted line represents "No-inoculum". Soil (S): the origin of soil that was added; Amount (A): the amount of soil added; Sterilization (ST): whether sterilized soil or non-sterilized soil was added. NI, not inoculated, only contains sterilized degraded grassland soil; S1cm, soil from field plots where 1 cm of meadow steppe soil was added; S3cm, soil from field plots where 3 cm of meadow steppe soil was added; M1cm, soil from field plots where 1 cm of upland meadow soil was added; M3cm, soil from field plots where 3 cm of upland meadow soil was added. Different lowercase letters indicate significant differences at $P < 0.05$ based on a post-hoc Tukey test between 8 soil addition treatments (adding live or sterilized soil from "S1cm", "S3cm", "M1cm" and "M3cm" field plots). Different uppercase letters indicate significant differences at $P < 0.05$ based on post-hoc Tukey test between 2 levels of soil sterilization treatments (live or sterilized soil), regardless of the type and amount of added soil originally. Stars within each bar denote significant differences from the Control (No-inoculum) treatment based on Dunnett's test: * $P < 0.05$; *** $P < 0.001$.

functions. However, our study also shows that abiotic factors are important. Addition of live soil from experimental plots resulted in higher soil nitrogen cycling than addition of sterilized soil, indicating that soil organisms that contribute to soil nitrogen cycling are adequately present in the soils. This result is consistent with our field experiment, where we observed that the soil biological community mainly promoted the function of soil nitrogen cycling. A previous study also found that the availability of soil nitrogen is the main limiting factor for the restoration of degraded grassland (LeBauer & Treseder, 2008). Our result suggests that soil biota related to soil nitrogen cycling may be an important group of soil organisms to focus on in degraded grassland restoration. Future studies should employ metagenomic techniques to systematically analyze the relationship between soil microorganisms and the soil nitrogen cycle. This can further clarify the mechanisms by which soil inoculation can improve restoration of degraded grasslands.

Notably, in the microcosm experiment where we used soil from the field plots, we detected significant differences between the two donor soils in multifuntionality while we did not detect such effect in the field plots itself. This is interesting because it suggests that even though it was not visible in the field plots, the donor soils differentially affected soil

functioning. In the microcosm experiment, we introduced field soil into sterilized grassland soil and this can result in high survival rates and diversity of introduced soil organisms, as there is no competition (e.g. for nutrients and space) with native soil organisms. As the inocula originated from the field plots, four years after the start of the experiment, it suggests that in the field the additions of different donor soils led to different communities and that these communities persisted in the field. Taken together, the findings of the microcosm experiment demonstrate that biotic and abiotic soil effects are both important components that can influence ecosystem functions of degraded grassland, but also that soil biotic effects can guide the development of functional ecosystem characteristics.

5. Conclusion

This study investigated how addition of soil from different origins and the amount of soil added affects ecosystem functions of a degraded grassland. Our findings indicate that soil addition increased ecosystem multifunctionality of the degraded grassland and that this was linked to fungal richness and network complexity. Key fungal guilds (AMF, saprophytic fungi and potential pathogens) differed among treatments after soil addition, and these have unique bio-interactions with plants and soil nutrients. As the relationship between AMF and saprophytic fungi is not always positive, our results suggest that in grassland restoration, more emphasis should be placed on co-addition of AMF and saprophytic fungi to improve the ecosystem functions of degraded grassland, including plant productivity and soil nutrients. Although high amounts of soil addition are less applicable to the restoration of large-scale degraded grasslands, our results highlight the importance of soil organisms and nutrients (i.e. soil nitrogen) in the restoration of degraded grasslands. Our study also provides some new ideas for the restoration of degraded grasslands. For example, simultaneous application of soil nutrients and organisms (i.e. AMF and saprophytic fungi) by spraying and such approaches need to be tested in future trials.

CRediT authorship contribution statement

Yuhui Li: Data curation, Formal analysis, Writing – original draft. **Xu Han:** Data curation, Formal analysis, Writing – review & editing. **Bing Li:** Data curation, Formal analysis. **Yingbin Li:** Data curation, Writing – review & editing. **Xiaofang Du:** Data curation, Formal analysis. **Yixin Sun:** Data curation. **Qi Li:** Conceptualization, Writing – review & editing. **T. Martijn Bezemer:** Conceptualization, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data that support the findings of this study are available from the corresponding author upon request. Raw sequencing data is available from the China National Microbiology Data Center (Accession: NMDC10017881).

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.geoderma.2023.116607.

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