



Arbuscular mycorrhizal fungi and plant diversity drive restoration of nitrogen-cycling microbial communities

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Abstract

Soil microbial communities, key players of many crucial ecosystem functions, are susceptible to environmental disturbances, which might cause the loss of microbial diversity and functions. However, few ecological concepts and practices have been developed for rescuing stressed soil microbial communities. Here, we manipulated an experiment with or without arbuscular mycorrhizal fungi (AMF) inoculation and at three levels (one, three and six species) of plant diversity to disentangle how the AMF and vegetation rescue soil nitrogen (N) -cycling microbial loop from simulated degraded soil ecosystem. Our results showed that AMF inoculation improved the restoration of soil N-cycling microbial communities. This improved restoration was related to the role of AMF in enhancing interactions within the N-cycling microbial loop. Furthermore, increased plant diversity strengthened the role of AMF in rescuing N-cycling microbial communities. Our findings provide novel insights into the roles of AMF and plant diversity in facilitating the rescue of microbial communities in degraded terrestrial ecosystems.

KEYWORDS

arbuscular mycorrhizal fungi, degradation, nitrogen-cycling microbial community, plant diversity, restoration

1 | INTRODUCTION

Soil microorganisms intimately interact with each other, and with plants, to provide essential ecosystem services by driving nutrient biogeochemical cycling, maintaining carbon sequestration, and regulating climate change (Cavicchioli et al., 2019; Lau & Lennon, 2011). However, soil microbial communities are susceptible to natural and anthropogenic disturbances, which might cause the loss of biodiversity and the functions they provide (Bardgett & Putten, 2014). Given the intrinsic linkages among biotas, developing intentional regulation approaches from the perspective of biological interactions is imperative to rescue microbial communities, and thus facilitate the

maintenance of ecosystem functions and services. However, in contrast to vegetation restoration, few ecological concepts or practices have been developed for rescuing microbial communities and function (Calderón et al., 2017; Kollmann et al., 2016), due to the poor understanding of the processes and factors driving restoration.

Arbuscular mycorrhizal fungi (AMF) are vital connectors between plants, other microorganisms, and soil (Tedersoo et al., 2020). The rhizosphere of the mycorrhizal plant, known as the mycorrhizosphere, provides a critical link between the soil and organisms, both above and belowground, through nutrient cycling and mass exchange (Barea et al., 2002; Rockström et al., 2009). Numerous studies have demonstrated that mycorrhizal inoculation reverses degradation in

plant communities by increasing the richness and structure similarity compared with noninoculation treatment (Kozioł & Bever, 2017; Neuenkamp et al., 2019). Several mechanisms have been advocated to explain the promotion capability of AMF in ecosystem recovery (Asmelash et al., 2016): improving soil aggregation, providing resistance for host plants, improving seedling growth, and increasing soil microbial diversity and abundance. Amongst these mechanisms, the function of AMF regulating aboveground-belowground linkages to assist ecosystem establishment is of increasing global interest (Heijden et al., 2016; Kardol & Wardle, 2010; Lance et al., 2020). Besides AMF, plant diversity also significantly influences microbial growth and functions (Steinauer et al., 2015). Higher plant diversity increases the diversity of soil microbes by increasing the variety of available substrates and supporting more niches (Prober et al., 2015); in contrast, diverse vegetation communities may also cause fierce competition between plants and soil microorganisms (Kuzyakov & Xu, 2013).

Soil nitrogen (N)-cycling processes are model ecosystem functions and important for plant nutrition and human society, and that they are dominated by a microbial N loop (Knops et al., 2002; Stein & Klotz, 2016). Previous studies demonstrated that soil N-cycling microbes could interact with aboveground communities in a positive or negative manner (Berg & Smalla, 2009; Hodge & Fitter, 2013; Moreau et al., 2015). N-cycling microbes can compete with plants for available soil nutrients, exhibiting negative interactions with the plant community (Blagodatskaya et al., 2014; Hodge & Fitter, 2013). However, they can also have mutually beneficial (positive) relationships with plants, for instance, root exudates provide organic matters to N cycling microbes that in turn can fix atmospheric N₂ and transform it into NO₃⁻, a preferred form of N for plants (Singh et al.,

2004; Zhang et al., 2013). AMF can alter plant community composition and influence the composition of microbial community in the rhizosphere and mycorrhizosphere (Rillig, 2004). By changing the physiology of host plant, AMF could modify the rhizodeposits in the rhizosphere and induce the shifts of N-cycling processes, such as nitrification (Veresoglou et al., 2019). Moreover, a greater number of plant species can contribute a more diverse range of potential hosts for symbiotic AMF, which can synergistically affect soil microbes including N-cycling microbial communities (Zhang et al., 2013).

Despite the wide recognition of the ecological associations between aboveground and belowground biota, the lack of knowledge regarding the recovery of microbial communities under the intervention of AMF and aboveground plant diversity represents an important research gap in restoration ecology. A recent study using a removal-by-dilution approach found that although the diversity of some soil microbial compositions were successfully increased after being diluted, soil N-cycling function was not significantly restored (Calderón et al., 2017). Their results imply that N-cycling microbial community might be distinct from other soil communities (e.g., different growth rates) in a degraded soil state. However, it remains unknown whether and to what extent AMF and plant diversity will modulate the restoration of soil N-cycling microbial community.

Hence, we focused on the restoration of soil N-cycling microbial community under the conditions of different AMF inoculation status and plant diversity gradient (Figure 1). Two degraded soil states were simulated with or without AMF inoculation. Three levels of plant diversity (one, three and six species) were planted in experimental pots for 22 weeks. The restoration of six N-cycling microbial genes was investigated. We hypothesized that AMF inoculation would improve

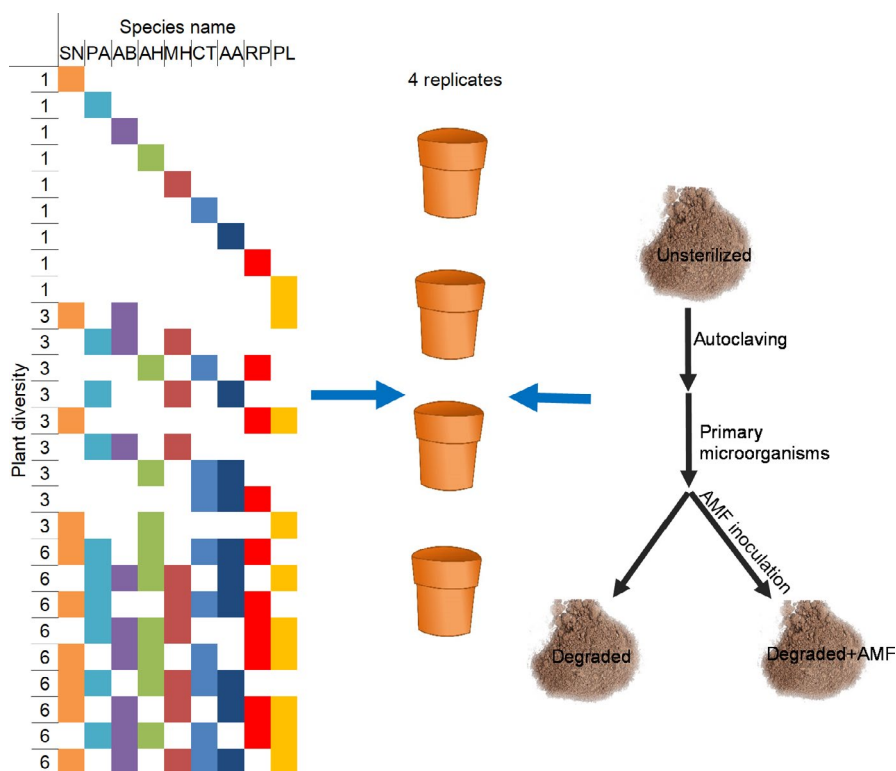


FIGURE 1 Experimental design. Species names: SN, *Solanum nigrum*; PA, *Plantago asiatica*; AB, *Achyranthes bidentata*; AH, *Arthraxon hispidus*; MH, *Mosla hangchowensis*; CT, *Corchoropsis tomentosa*; AA, *Acalypha australis*; RP, *Rostellularia procumbens*; PL, *Polygonum lapathifolium*. AMF, Arbuscular mycorrhizal fungi [Colour figure can be viewed at wileyonlinelibrary.com]

the restoration of soil N-cycling microbes, and higher plant diversity would reinforce this promotion.

2 | MATERIALS AND METHODS

2.1 | Preparing of herbaceous species pool

Pot experiments were conducted in a greenhouse located at Taizhou University, Taizhou, China. A survey of the local herbaceous species in the mountainous areas around Taizhou was carried out (Table S1), and nine native species that occurred frequently were chosen to prepare the aseptic seedlings (Figure 1). *Plantago asiatica* and *Achyranthes bidentate* are perennial forbs, the other seven species are annual grasses (Table S2). Sterilized seeds were sown in plastic boxes (28 × 21 × 8 cm) and placed in a climate greenhouse ($T = 25^{\circ}\text{C}$, $\text{RH} = 70\%$, and 16 h day lengthen at 5000 Lux illumination) at Taizhou University. Germinated seedlings (about 5 cm height) were selected and transplanted to plastic pots (27.5 cm diameter × 31 cm height). Each pot was filled with a soil mixture comprising of native soil, peat substrate (Klasmann-Deilmann GmbH), sand and vermiculite in the proportion of 2:1:1:1 with total N (TN) of 1.600 ± 0.317 g/kg, total phosphorus (TP) of 0.581 ± 0.177 g/kg, and soil organic matter (SOM) of 13.22 ± 0.504 g/kg. The native soils used here were collected from the same multispecies grassland at 0–15 cm depth in Taizhou.

2.2 | Experimental design

To evaluate the effect of restoration of N-cycling microbes, three soil states were produced as described below (Figure 1): (a) without any manipulation (unsterilized), (b) wet autoclaved (121°C , 1 h) and reinoculated with primary microorganisms (degraded), and (c) wet autoclaved and reinoculated with primary microorganisms and native AMF spores (degraded + AMF). The primary microorganisms for degraded and degraded + AMF soils were added within a 10 day period at the beginning of the experiment to each pot with 50 ml of filtrate obtained from original fresh soils. For the reason that the majority of AMF spores are higher than $45 \mu\text{m}$, which are higher than bacterial cells ($0.2\text{--}10 \mu\text{m}$) (Katz et al., 2003) and 1000 times higher than other fungal spores (Aguilar-Trigueros et al., 2019), this filtrate was produced with a soil-to-water ratio of 1:4 and filtering the supernatant through a $45 \mu\text{m}$ filter, which could retain the AMF propagules (Klironomos et al., 1993). The mixed native AMF spores were collected using a wet-sieving technique (Klironomos et al., 1993) from 50 g soil and added along with 50 ml sterilized water to each pot of degraded + AMF at the beginning of the experiment within a 10 day period for three times. Pots of degraded received equivalent amounts of sterilized water without spores.

A total of 324 pots were used to generate 81 combinations, which were three different soil states (unsterilized, degraded, degraded + AMF) and three plant diversity levels (one, three and six species) × nine combinations of plant species, with four replicates

for each combination. Each pot held 5 kg of soil mixture was planted with six seedlings selected from the nine species, and every species had the same occurrence in each plant diversity level. Pots positions were randomized in the greenhouse ($T = 28^{\circ}\text{C}$, $\text{RH} = 70\%$), and no additional artificial light was provided during the experiment. Every pot was watered daily with 500 ml sterile distilled water. Plants were harvested after 22 weeks of transplanting and individual biomass was weighed after oven-drying at 60°C for 48 h.

2.3 | Analysis of soil properties and DNA extraction

After harvesting, soil samples were collected for further analysis of soil properties and DNA extraction. SOM was measured using $\text{K}_2\text{Cr}_2\text{O}_7$ oxidation-reduction titration method. TN was digested by H_2SO_4 , TP was digested by $\text{HClO}_4\text{-H}_2\text{SO}_4$, and both TN and TP were measured using Autoanalyzer 3 (BRAN + LUEBBE, Germany). Soil pH was measured with a soil-to-water ratio of 1:2.5.

Soil DNA was extracted from 0.3 g fresh soil using MoBio PowerSoil DNA Isolation Kits (MoBio Laboratories) according to the manufacturer's instructions. The quantity and quality of extracted DNA were measured using a Nanodrop ND-1000 spectrophotometer (NanoDrop Technologies), and the DNA samples were stored at -80°C for further analysis.

2.4 | Assessing soil N-cycling microbial communities

Six typical genes encoding the key enzymes of the N-cycling microbial loop were investigated in the present study: nitrogenase reductase gene (*nifH*) of nitrogen fixation, ammonia monooxygenase gene (*amoA*) in both ammonia-oxidizing archaea (AOA) and ammonia-oxidizing bacteria (AOB) of ammonification, nitrate reductase gene (*narG*) of nitrate reduction, nitrite reductase gene (*nirK*) of nitrite reduction, and nitrous oxide reductase (*nosZ*) of nitrous oxide reduction.

The abundances of six N-cycling microbial genes were quantified by quantitative PCR (qPCR). Reactions of qPCR were performed using a Roche LightCycler 480 system (Roche Life Science). Each reaction was performed in a 20 μl volume containing 10.0 μl of SYBR Premix Ex Taq (2 \times , Takara, TaKaRa Biotechnology Co.), 8.6 μl dd H_2O , 1 μl DNA ($\sim 10\text{--}30$ ng) and 0.2 μl (10 μM) of each primer. Ten-fold serial dilution of PMD19-T plasmid containing the correctly inserted genes fragments for qPCR as standard curves were developed. All the qPCR efficiencies ranged from 90% to 110%, and the R^2 values were higher than 0.99. The possibility of PCR inhibitors in the DNA samples was determined by serial dilutions, and no severe inhibition was found in any of the extracted DNA. Detailed information about the primers and the PCR protocols for the six genes is provided in Table S3.

Terminal restriction fragment length polymorphism (T-RFLP) was used to analyse the richness and compositions of the six N-cycling genes. The same primers used in qPCR (Table S3) were also used in

the T-RFLP analyses except the forward primers were labelled with 6-Carboxyfluorescein. Amplification was performed in triplicate in a total volume of 50 μ l with 21.8 μ l dd H₂O, 25 μ l Premix Taq (2 \times , Takara), 2 μ l DNA, and 0.6 μ l (10 μ M) of each primer. Controls without DNA templates were conducted to test for contamination. PCR protocol details for the six genes are given in Table S3. After amplification, the triplicate PCR products were pooled and purified using the PCR cleanup Kit (Axygen Biosciences). The purified PCR products of different genes were digested with 10 units of their respective restriction enzymes (Table S3) at 37°C for 6 h and then denatured at 80°C for 10 min. After separating via capillary electrophoresis, the fragment sizes were precisely estimated by ABI 3730xl DNA Analyser (Applied Biosystems). The T-RFLP profiles were generated by GeneMapper 4.0 software (Applied Biosystems) based on the relative abundance of total peak areas (higher than 500 fluorescence units). The terminal restriction fragments (T-RFs) at ± 1 bp with relative abundance lower than 0.1% were excluded from the further analyses using Microsoft Excel and macro treeflap (Rees et al., 2004). The richness was the observed numbers of T-RFs for each sample. To remove the primer dimers and incompletely digested fragments, and considering the PCR products lengths of six genes, fragment sizes ranged from 40–350 bp for *nifH*, 40–550 bp for AOA-*amoA*, 40–450 bp for AOB-*amoA*, 40–450 bp for *nirK*, 40–550 bp for *narG*, 40–550 bp for *nosZ* were included for the further analyses.

Although T-RFLP technique based on the fingerprint method has some disadvantages, T-RFLP method is especially efficient to study functional genes which are low diversity and/or account for a minor fraction of the overall microbial community (Trivedi et al., 2019). Therefore, the richness of each N-cycle gene was calculated based on the observed T-RFs numbers of each replicate pot to estimate the α -diversity.

2.5 | Clone library analysis of N-cycling genes

To identify the T-RFs of the N-cycling microbial T-RFLP profiles, clone libraries were constructed using the same primers as T-RFLP without 6-FAM labelled. Purified PCR products of DNA samples were cloned into the PMD19-T plasmid and introduced into competent cells (*E. coli* Top 10). Fifty clones for each gene were sequenced using ABI PRISM 3730 sequencer (Applied Biosystems), and low quality sequences were removed. The virtual digests with restriction enzyme were carried out on the sequences retrieved from the clone libraries to assign the identity to individual T-RF, results are shown in Table S4. Totally 284 sequences obtained in this study were deposited in GenBank accession numbers MW980963-MW981246.

2.6 | Assessing recovery of N-cycling microbial communities

The individual recovery index (IRI) of abundance or richness for each N-cycling microbe was calculated between both degraded soil states

(degraded and degraded + AMF) and referred against unsterilized soil state. IRI was calculated referring to a previous formula for plant recovery (Ruijven & Berendse, 2010) with some modification concerning the replicates in the present study. Hence, IRI for abundance or richness was calculated as: $IRI = \text{Log}_e$ (abundance or richness value in degraded or degraded + AMF/abundance or richness value in unsterilized). Because the four replicates were set in random, therefore, the value of N-cycling gene abundance in each replicate pot of degraded or degraded +AMF was pairwise divided by the corresponded values of four replicates of unsterilized, and then averaged and Log_e transformed. The pairwise Bray-Curtis similarities between each replicate of degraded or degraded + AMF and the corresponded four replicates of unsterilized were averaged as the IRI of composition for each pot. The IRIs of abundance, richness, and composition of all six genes were standardized using Z-score transformation and then averaged to obtain a multirecovery index (MRI) for each pot.

It should be noted that identifying specific and unambiguous indicators of recovery is confounded even for plant communities (Ghazoul & Chazdon, 2016), as an attempt to reveal the recovery of N-cycling microbial communities in the present study, we justified the better recovery condition as higher abundance and richness, and more similar community compositions to their referred unsterilized soil state.

2.7 | Statistical analyses

Mixed model analyses were used to test the overall effects of different soil states, plant diversity and their interactions on the abundance, richness, and IRIs of N-cycling microbes. In the mixed model analyses, soil state, plant diversity and their interactions were set as fixed factors, replicated plots were considered as random factor. The differences of the abundances and richness of six N-cycling genes among degraded, degraded + AMF and unsterilized were calculated using one-way analysis of variance (ANOVA) with least significant difference (LSD) post hoc test. The influence of AMF inoculation on IRIs and MRI were estimated using independent t test. The influence plant diversity levels on the abundances, richness, IRIs and MRI of six N-cycling genes were calculated using one-way analysis of variance (ANOVA) with least significant difference (LSD) post hoc test.

Non-metric multidimensional scaling (NMDS) analysis based on Bray-Curtis distances was performed to determine the community compositions (β -diversities) of the six N-cycling microbial communities based on the genes T-RFLP profiles, and the stress values were all less than 0.2 ($k = 3$). Permutational multivariate analysis of variance (PERMANOVA) was performed to determine the effects of soil states and plant diversity on the community compositions of N-cycling microbial communities. NMDS and PERMANOVA were performed using the vegan package in R software (R Core Team, 2014). The “core” compositions were identified as highly abundant (top 10% most abundant T-RFs in 36 soil samples of each soil state and each plant diversity level) and ubiquitous (found in more than half of the 36 soil samples) (Delgado-Baquerizo et al., 2018), the shared core composition numbers were counted and visualized using the UpSetR package in R software.

We used structural equation model (SEM) analyses to evaluate the direct and indirect influence of AMF inoculation and plant diversity on IRIs and MRI, respectively. Because soil properties and plant biomass can influence or, in reverse, be influenced by soil microbes (Li, Pan, et al., 2019; Li, Tian, et al., 2019; Heijden et al., 2008), therefore, covariance was constructed between IRIs and soil properties and plant biomass. First, a priori model based on the known effects and relationships among variables was established. All data needed to be standardized using the Z-score transformation before modelling. Next, the insignificant ($p > .05$) relationships and covariance were removed from the model. Some parameters were used to estimate the adequate fit of models as follows: Root mean squared error of approximation (RMSEA) is low (≤ 0.08), and model fit chi-square value (χ^2) is low (Schermele-Engel et al., 2003). SEM analyses were performed using the "lavaan" package in R software.

2.8 | Network analyses

We constructed nine networks based on three different plant diversity levels of degraded, degraded + AMF and unsterilized to visualize the interactions among six N-cycling compositions based on their T-RFs profiles. An open network analysis pipeline (Molecular Ecological Network Analyses Pipeline, <http://ieg2.ou.edu/MENA/>) was used to create the networks. In brief, first, the relative abundances of T-RFs components were calculated for each N-cycling gene, and all T-RFs components of the six N-cycling genes were combined together to create the relative abundance matrix. Second, the matrix was submitted to construct the network: only T-RFs present in a minimum of 10 of the total 36 samples were retained ($< 1/4$), missing data was left blank, logarithm was not taken, and Pearson correlation coefficient was chosen. After which, the same threshold value (0.960) was set for all nine networks to make them comparable. Third, network properties were calculated using the "global network properties" and the "individual nodes centrality" commands. The "keystone" species were identified based on the z_i value that higher than 2.5 or p_i values that higher than 0.62, respectively, in the respective network (Guimerà & Nunes Amaral, 2005). The networks were visualized using Gephi version 0.9.2 software (Bastian et al., 2009). More details on the theories, algorithms, pipeline structure and procedures can be found in previous studies (Zhou et al., 2010, 2011). The differences of links, nodes, and "keystone" species' relative abundance among soil states were performed using one-way ANOVA.

3 | RESULTS

3.1 | Abundances of N-cycling microbes

Twenty-two weeks after transplanting, the abundances of *nifH*, AOB-*amoA*, *narG*, *nirK*, and *nosZ* genes in soils of degraded and degraded + AMF reached an order of magnitude comparable to that in soil of unsterilized (Figure 2a). However, the abundances of

AOA-*amoA* gene in soils of degraded and degraded + AMF were significantly lower than unsterilized. In addition, gene copies of *nifH*, AOA-*amoA*, *narG*, *nirK*, and *nosZ* in degraded + AMF were significantly higher than that in degraded (Log_{10} -transformed, LSD post hoc test, $F_{2,321} = 193.8, p < .001$, $F_{2,321} = 19.6, p < .001$, $F_{2,321} = 11.6, p < .001$, $F_{2,321} = 13.8, p = .009$, $F_{2,321} = 11.6, p < .001$, respectively).

As revealed by the mixed model analyses, plant diversity levels only significantly influenced the abundance of AOA (Table S5). One-way ANOVA with LSD post hoc test revealed that higher plant diversity levels had lower abundances of *nifH*, AOA-*amoA*, *narG*, and *nirK* genes in unsterilized soil state (Figure 2a). However, in the degraded soil states (degraded and degraded + AMF), the influence of plant diversity on the abundances of N-cycling microbes was weakened, with plant diversity displaying only significantly influenced the abundance of AOA-*amoA*.

Compared with degraded, degraded + AMF had significantly higher IRIs of *nifH*, AOA-*amoA*, *narG*, *nirK*, and *nosZ* genes abundances (Figure 2b; $t_{214} = 2.9, p = .004$, $t_{214} = 5.2, p < .001$, $t_{214} = 4.0, p < .001$, $t_{214} = 2.2, p = .029$, $t_{214} = 2.8, p = .005$, respectively). In addition, high plant diversity also could increase the IRIs of N-cycling microbial abundances, which could significantly increase the IRIs of *nifH*, *narG*, and *nirK* abundances, but mainly in the degraded + AMF (Figure 2b). In addition, no significant interactive influence was detected between soil state and plant diversity level on the IRIs of N-cycling microbial abundances (Table S5).

3.2 | Richness of N-cycling microbes

The richness of the two *amoA* genes of ammonia-oxidizers was significantly higher in degraded soil than that in degraded + AMF and unsterilized soils (Figure 3a; LSD post hoc test, $F_{2,321} = 139.2, p < .001$ for AOA, $F_{2,321} = 158.3, p < .001$ for AOB, respectively). In contrast, richness of the other four genes in the degraded soil were significantly lower than that of degraded + AMF (LSD post hoc test, $F_{2,321} = 32.6, p < .001$ for *nifH*, $F_{2,321} = 8.7, p = .001$ for *narG*, $F_{2,321} = 23.5, p < .001$ for *nirK*, $F_{2,321} = 37.3, p < .001$ for *nosZ*, respectively). Plant diversity had weak effects on the richness of N-cycling microbes as revealed by mixed model analysis (Table S5). We further separated the T-RFs into "abundant group" and "rare group" (Figure S1), and the results showed that compared with degraded, degraded + AMF had significantly higher richness of the "abundant group" of *narG* gene (LSD post hoc test, $F_{2,321} = 5.3, p = .034$), and the "rare group" for *nifH* ($F_{2,321} = 24.8, p < .001$), *narG* ($F_{2,321} = 15.4, p < .001$), *nirK* ($F_{2,321} = 42.0, p < .001$), and *nosZ* ($F_{2,321} = 33.7, p < .001$) genes, but significantly less richness of "rare group" of AOA ($F_{2,321} = 26.3, p < .001$) and AOB ($F_{2,321} = 162.8, p < .001$).

The IRIs of N-cycling microbial richness were found to be significantly affected by AMF (Table S5). AMF inoculation significantly decreased the IRIs of AOA and AOB richness (Figure 3b; $t_{214} = -9.0, p < .001$, $t_{214} = -12.2, p < .001$, respectively), while it significantly increased the IRIs of *nifH*, *nirK*, and *nosZ* communities richness ($t_{214} = 6.2, p < .001$, $t_{214} = 7.3, p < .001$, and $t_{214} = 5.0, p < .001$,

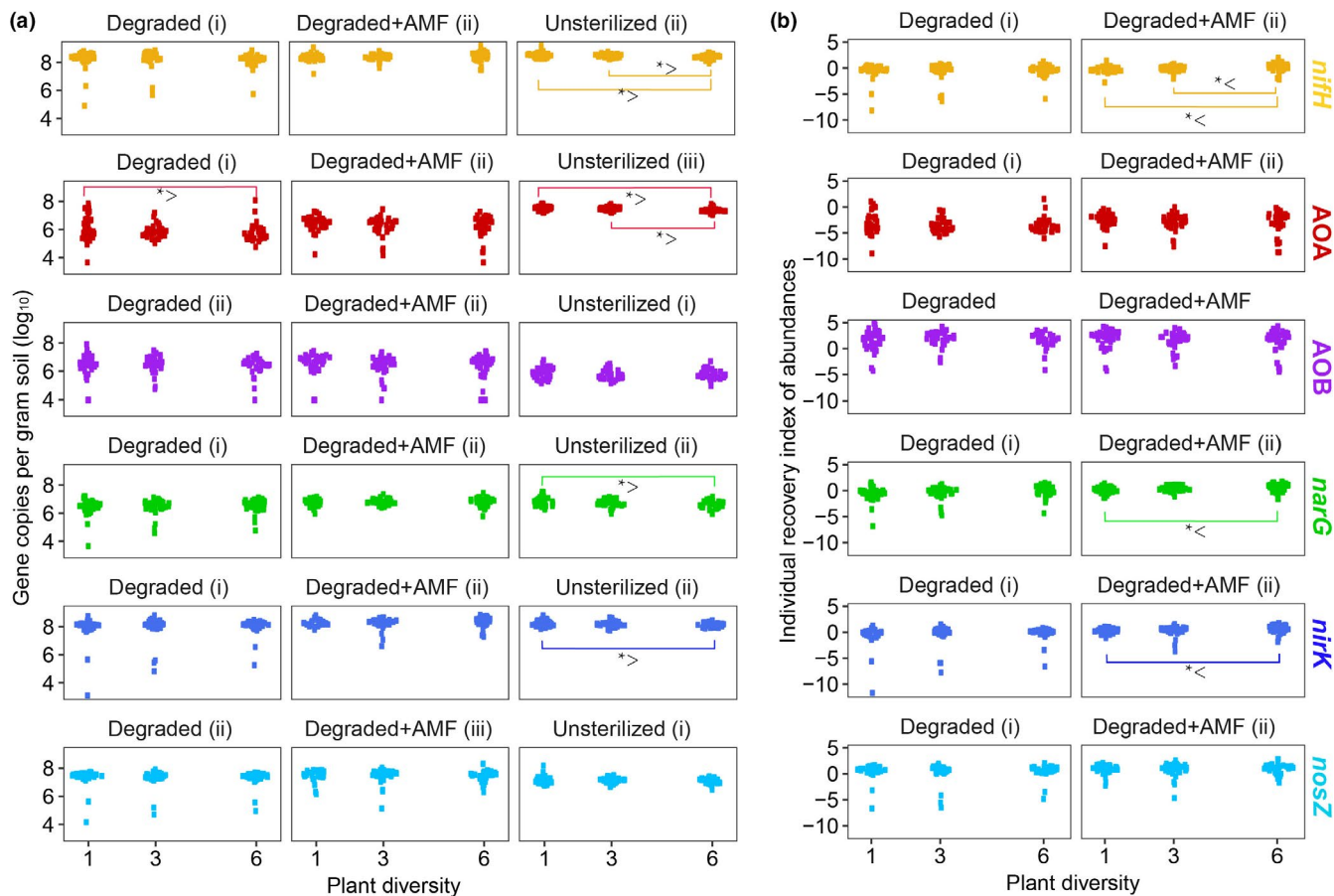


FIGURE 2 (a) Abundances of N-cycling microbial genes; (b) Individual recovery indexes of N-cycling microbial abundance. Different numbers (i, ii, iii) in parentheses indicate significant difference between soil states (LSD post hoc test, $p < .05$). Significant differences between plant diversities are labelled within the figure, “>*” indicates the left significantly higher than the right (LSD post hoc test, $p < .05$), “<*” indicates the left significantly lower than the right (LSD post hoc test, $p < .05$) [Colour figure can be viewed at wileyonlinelibrary.com]

respectively). Additionally, in the degraded + AMF soil state, high plant diversity significantly decreased IRI of AOA richness. In contrast, the IRIs of AOB and *nirK* richness were significantly increased in high plant diversity levels in degraded and degraded +AMF (Figure 3b).

3.3 | Community compositions of N-cycling microbes

The NMDS combined with PERMANOVA analyses showed that the community compositions of N-cycling microbes were significantly different between degraded, degraded + AMF and unsterilized (Figure 4a and Table S6). However, plant diversity had very weak impact on the compositions, no significant difference was detected among plant diversity levels (Figure 4a). In details, the highly abundant and ubiquitous compositions were identified as the “core” compositions, and the results showed that degraded and degraded + AMF had significantly higher core T-RF numbers than unsterilized (Figure S2). Although 15 core T-RFs were shared by different soil states with different plant species (one-way ANOVA, $F_{2,6} = 7.4$, $p = .024$), there still amounts of core T-RFs were only shared within respective soil

state (14 T-RFs for unsterilized, 6 T-RFs for degraded, and 5 T-RFs for degraded + AMF, respectively).

On average, IRIs of composition in degraded + AMF for all six N-cycling microbial communities were higher than that of degraded (Figure 4b). Significantly higher IRIs of composition in degraded + AMF than in degraded were only detected for diazotrophic community (*nifH* gene; $t_{214} = 4.6$, $p < .001$). To be noted, higher IRIs of composition in degraded + AMF were also found for AOB, *narG* and *nosZ* genes, which were marginally not significant ($t_{214} = 1.7$, $p = .097$, $t_{214} = 1.7$, $p = .089$ and $t_{214} = 1.8$, $p = .079$, respectively). Plant diversity could significantly influence the IRIs of composition of *nifH* and *nosZ* genes, higher plant diversity levels had significantly higher IRI of *nifH* composition, but inconsistent results for the *nosZ* composition (Figure 4b; Table S5).

3.4 | Drivers for the recovery of N-cycling microbes

As revealed by SEM based on the IRIs of N-cycling microbial abundances, richness and compositions ($\chi^2 = 188.643$, $p = .054$, $df = 159$, $RMSEA = 0.029$), we found that inoculation of AMF and plant diversity

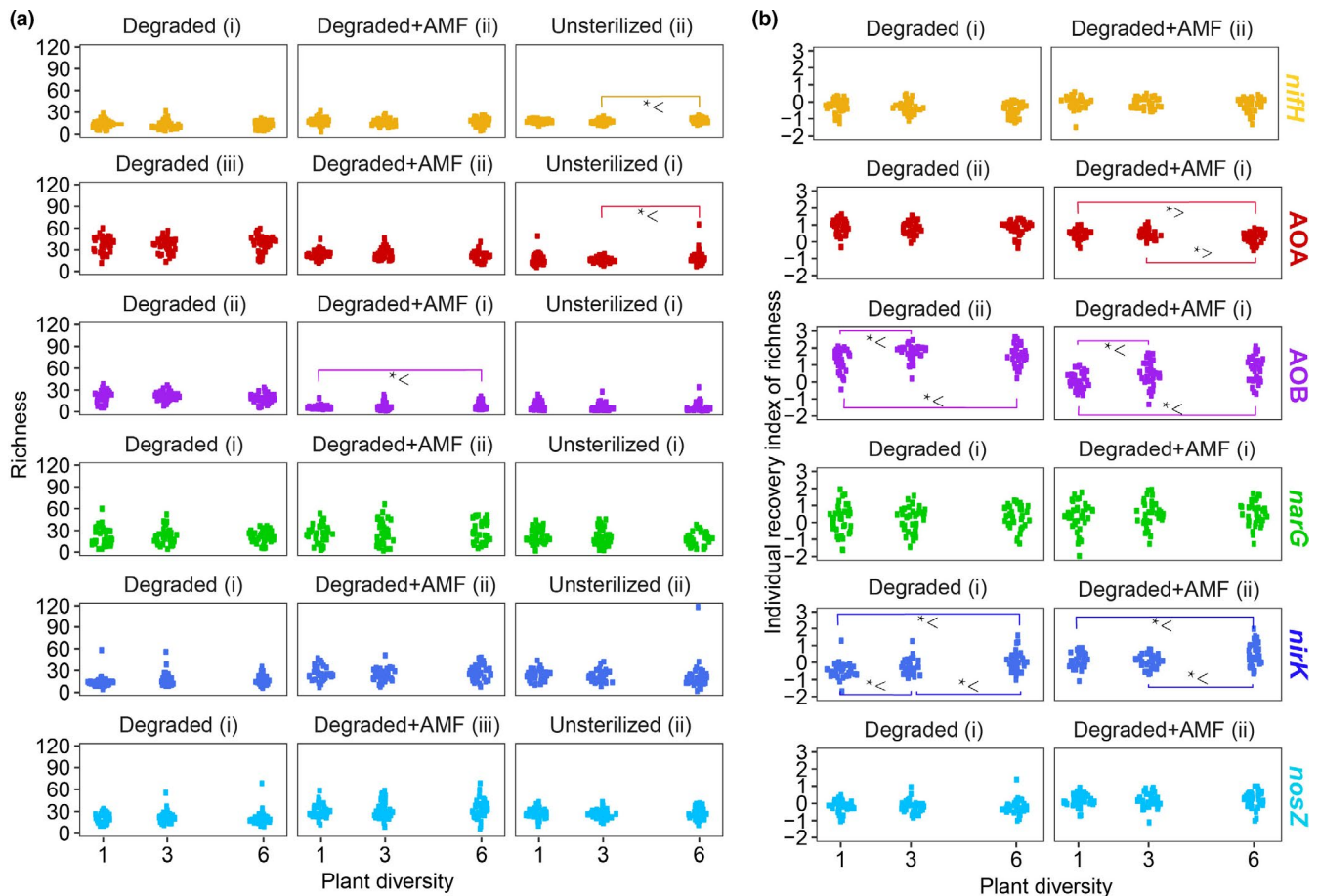


FIGURE 3 (a) Richness of N-cycling microbial communities. (b) Individual recovery indexes of N-cycling microbial richness. Different numbers (i, ii, iii) in the parentheses indicate significant difference between soil states (LSD post hoc test, $p < .05$). Significant differences between plant diversities are labelled within the figure, “>*” indicates the left significantly higher than the right (LSD post hoc test, $p < .05$), “<*” indicates the left significantly lower than the right (LSD post hoc test, $p < .05$) [Colour figure can be viewed at wileyonlinelibrary.com]

drove the restoration of N-cycling microbes (Figure S3 and Table S7). Statistical analyses revealed that AMF inoculation could significantly increase MRI ($t_{214} = 4.1$, $p < .001$), while plant diversity had weak influence on MRI as revealed by one-way ANOVA and mixed model analyses (Figure 5a, Table S5). Nevertheless, MRI was highest in degraded + AMF with six plant species and lowest in degraded with one plant species (LSD post hoc test, $F_{5, 210} = 3.9$, $p < .001$). We further performed a SEM based on MRI data (Figure 5b; model fit $\chi^2 = 22.022$, $p = .231$, $df = 18$, $RMSEA = 0.032$), and results revealed that MRI of N-cycling microbes was significantly and positively driven by both inoculation of AMF and plant diversity, and the impact of AMF inoculation ($p < .001$) was stronger than that of plant diversity ($p = .027$).

Soil properties and plant biomass also play roles in driving the recovery of N-cycling microbes (Figure 5b, Figure S3 and Table S7). SOM contents of unsterilized were significantly lower than that of degraded and degraded + AMF (LSD post hoc test, $F_{2, 321} = 97.55$, $p < .01$, Table S8), and SOM contents were significantly co-varied with MRI and IRIs of *nifH*, AOA, *narG*, *nirK*, *nosZ* abundance, and IRI of *nirK* composition (Figure 5b, Table S7). Soil pH values in degraded + AMF were significantly higher than that in degraded (LSD post hoc test, $F_{2, 321} = 97.55$, $p = .038$) and unsterilized (LSD post hoc

test, $F_{2, 321} = 97.55$, $p = .040$), and soil pH values were significantly covaried with the IRIs of *nosZ* abundance, *nifH* richness and *nosZ* richness. Soil TP contents were significantly co-varied with MRI and IRIs of *nifH* abundance, AOA abundance, *nirK* abundance, *nosZ* abundance, AOB richness and *nosZ* richness, while soil TP contents were not influenced by soil state and plant diversity. Soil TN contents in one plant species pots were significantly higher (LSD post hoc test, $F_{2, 105} = 2.177$, $p = .049$) than that in six plant species pots in degraded soil state, and soil TN contents were significantly co-varied with the IRI of *nirK* richness. Plant biomass in six plant species pots was significantly higher (LSD post hoc test, $F_{2, 105} = 3.425$, $p = .018$) than that in one plant species pots in degraded state, and plant biomass was significantly covaried with the IRI of *nosZ* richness.

3.5 | N-cycling microbial co-occurrence networks

Soils inoculated with AMF had more complex N-cycling co-occurrence networks with significantly more network total links than soils without AMF inoculation (Figure 6; LSD post hoc test, $F_{2, 6} = 22.0$, $p < .05$). In addition, compared with unsterilized, two degraded soil

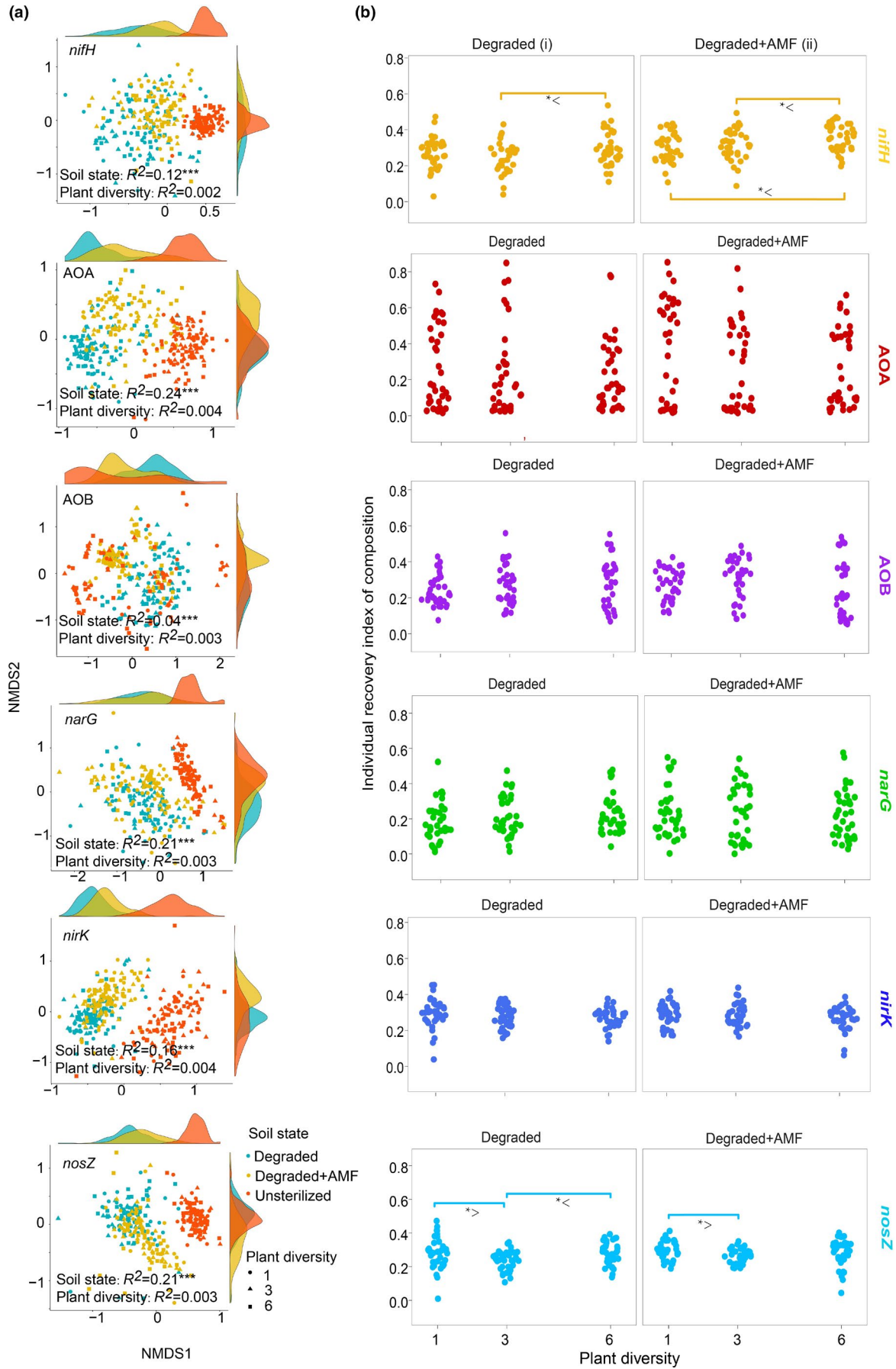


FIGURE 4 (a) N-cycling microbial community compositions. (b) Individual recovery indexes (Bray-Curtis similarities) of N-cycling microbial compositions. Nonmetric multidimensional scaling (NMDS) combined with permutational multivariate analysis of variance (PERMANOVA) were used to determine the influence of the three soil states and plant diversity levels on microbial communities (** $p < .001$). The stress values of all six NMDS analyses were less than 0.2 ($k = 3$). Different numbers (i, ii, iii) in the parentheses indicate significant difference between soil states (LSD post hoc test, $p < .05$). Significant differences between plant diversities are labelled within the figure, ">*" indicates the left significantly higher than the right (LSD post hoc test, $p < .05$), "<*" indicates the left significantly lower than the right (LSD post hoc test, $p < .05$) [Colour figure can be viewed at [wileyonlinelibrary.com](#)]

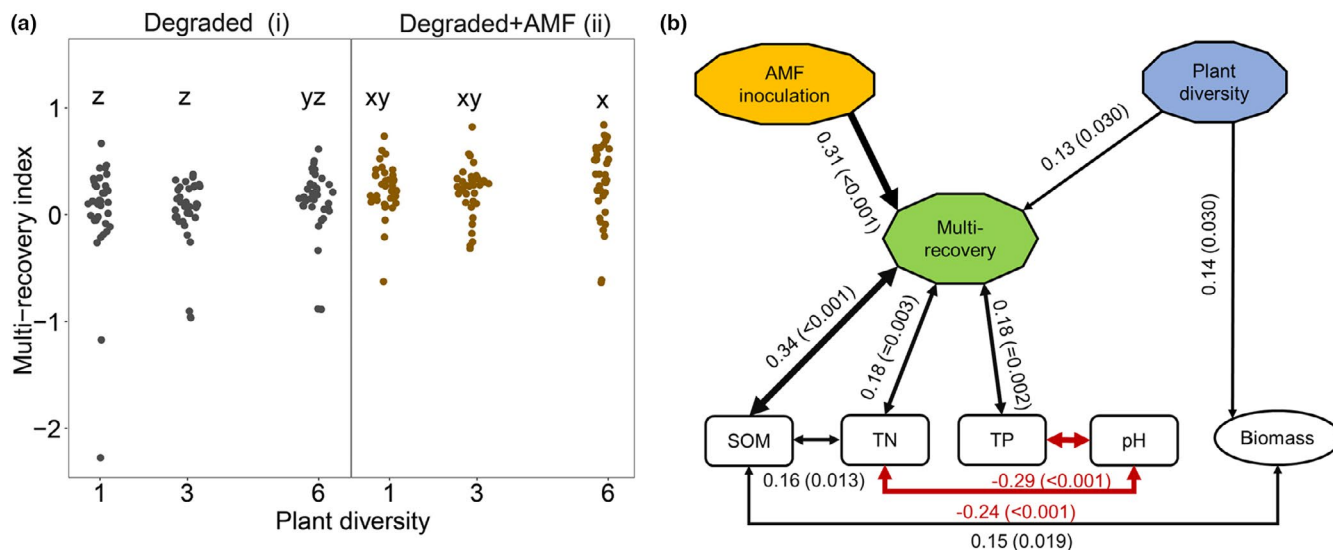


FIGURE 5 (a) Multirecovery index based on the averaged Z-score values of individual recovery indexes of different soil states and different plant diversity levels. Different numbers (i, ii) indicate significant difference ($p < .05$) between soil states using an independent t test. Different letters (z, y, x) indicate significant difference ($p < .05$) of multirecovery index among the six groups using one-way ANOVA with LSD post hoc test. (b) Structural equation model (SEM) shows the direct and indirect effects of AMF inoculation, plant diversity levels, plant biomass, and soil properties for multirecovery index of N-cycling microbes. Numbers adjacent to arrows indicate the effect-size (following p -value) of the relationships. The indicative meanings of arrows in two structural equation models are the same. AMF, Arbuscular mycorrhizal fungi; Biomass, plant biomass; TP, total phosphorus; TN, total nitrogen; SOM, soil organic matter [Colour figure can be viewed at [wileyonlinelibrary.com](#)]

states had significantly more links (LSD post hoc test, $F_{2,6} = 22.0$, $p < .05$) and nodes (LSD post hoc test, $F_{2,6} = 15.8$, $p < .05$) in the N-cycling co-occurrence networks. Plant diversity had a weaker effect on the co-occurrence networks than AMF inoculation, no significant difference of nodes and links was detected among three plant diversity levels (one-way ANOVA, $F_{2,6} = 0.248$, $p = .788$ for total nodes, $F_{2,6} = 0.079$, $p = .925$ for total links). In addition, degraded + AMF had similar co-occurrence networks pattern to unsterilized, as exhibited by lower relative proportions of *amoA* genes of ammonia-oxidizers, but higher relative proportions of *narG*, *nirK*, and *nosZ* genes than that of degraded (Figure 6d). Moreover, a large number of species were not shared for the networks of these three soil states, especially for the "keystone" species in the networks (Figure 6e), and the majority of these "keystone" species were significantly different in relative abundance among three soil states (Figure 6f).

4 | DISCUSSION

Many previous studies have demonstrated that soil microbes (Harris, 2009; Wubs et al., 2016), including AMF (Asmelash et al., 2016;

Renker et al., 2004), could steer the restoration of plant communities after degradation. Here, for the interactive relationships between aboveground ecosystems and soil microbial community, we investigated how AMF and plant communities drove the restoration of soil microbes. Considering the important ecosystem functions of N-cycling processes and their related microbes, and that N-cycling and biodiversity loss have crossed the thresholds of a safe operating space for humanity (Rockström et al., 2009), we therefore focused on the restoration of N-cycling microbial communities. To the best of our knowledge, this is the first experimental study investigating the restoration of soil N-cycling microbial communities under the interactive impacts of AMF and different plant diversity levels.

In the pedosphere, intricate interactions take place between aboveground and belowground organisms, which can be classified as positive, negative, and neutral interactions (Singh et al., 2004). In the present study, higher plant diversity levels could decrease all the six N-cycling microbial abundances in the unsterilized soil state, which indicated that the competitive interactions might be stronger than the mutually beneficial interactions (Heijden et al., 2008). Because of niche complementary effects (De Deyn et al., 2009; Vries & Bardgett, 2012), high plant diversity levels can reduce soil

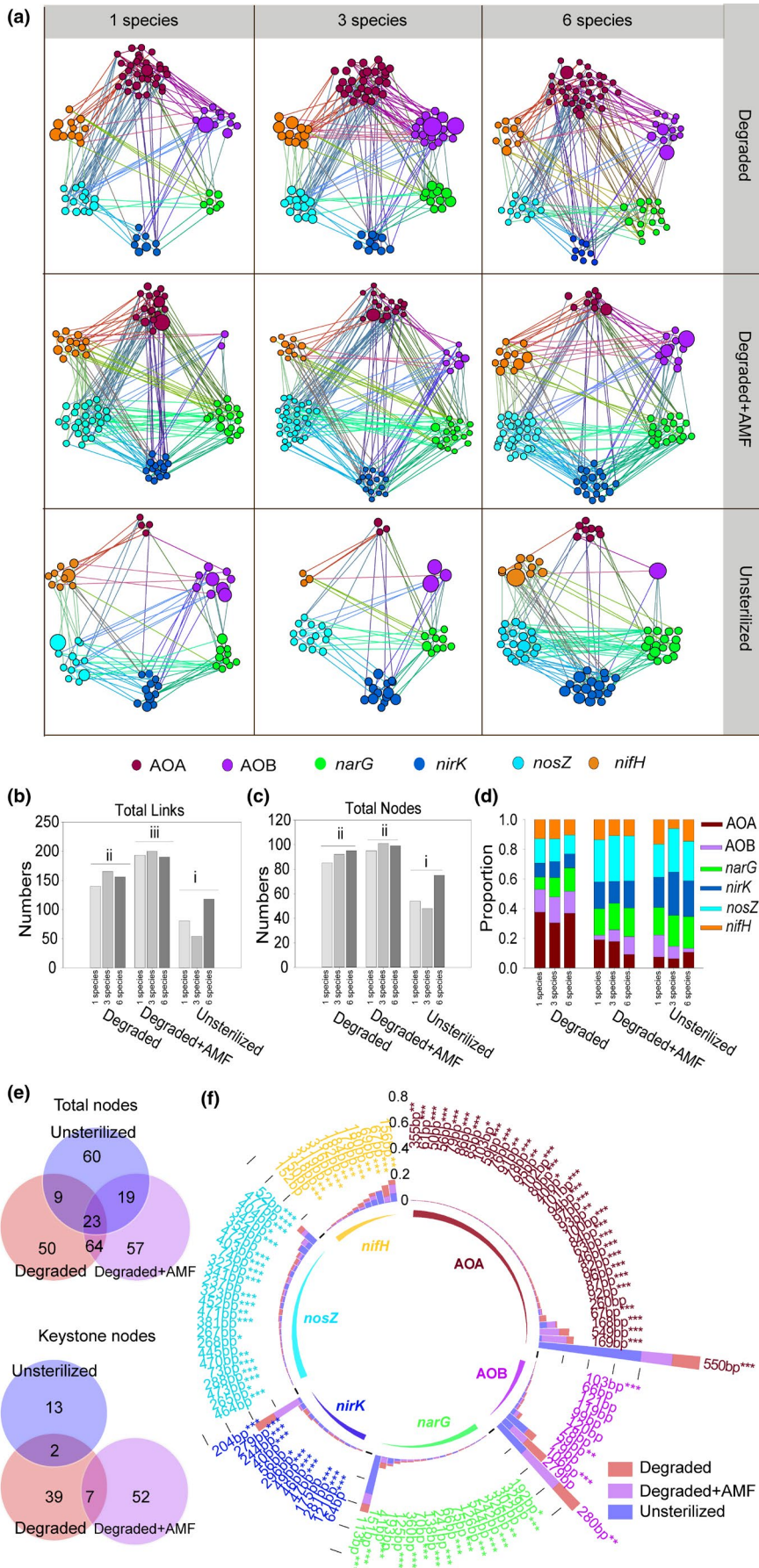


FIGURE 6 (a) Network analyses reveal the interactions within N-cycling microbial compositions of three soil states and different plant diversity levels. (b) Numbers of total links derived from the networks. (c) Numbers of total nodes derived from the networks. (d) Relative proportions of N-cycling microbial genes in the networks. (e) Number of shared and unshared total nodes and keystone nodes in the networks of three soil states. (f) Relative abundance of keystone nodes in the networks of three soil states. Node size in a network indicates the relative abundance of each species. Different numbers (i, ii, iii) of panels (b) and (c) indicate significant difference between the three soil states (LSD post hoc test, $p < .05$). Significant difference of the relative abundance of keystone nodes among three soil states is labelled after name of panel (f), * indicates $p < .05$, ** indicates $p < .01$, *** indicates $p < .001$ [Colour figure can be viewed at wileyonlinelibrary.com]

N availability and indirectly decrease the size of N-cycling microbial populations, such as ammonia-oxidizers and denitrifiers. Rhizobium N-fixers have been reported as beneficial for the diversity and growth of legumes (Li, Pan, et al., 2019; Li, Tian, et al., 2019; Heijden et al., 2008), yet free-living N-fixers were investigated and none of the plants in the present study were legumes, therefore, it was not unreasonable for the contrary result. Also, for the intricate interactions between plants and soil microbial communities, their relationships would vary based on different soil conditions, such as nutrient available (Heijden et al., 2008) and external environmental factors (Nunan et al., 2005).

However, in the degraded soil states, plant diversity had weaker influence on the abundances of N-cycling microbial communities. This result implied that the disturbance events of degradation altered the associations between plants and soil microbes (Hahn & Quideau, 2013), and contributed to a weak relationship or an equilibrium dynamic between competition and facilitation. We speculated that because the N-cycling microbial communities were establishing themselves (Smith & Ogram, 2008) and the plants were growing in the initial stage, their interactions were not completely established, thus weak influence of plant diversity on soil N-cycling microbial communities were detected in the degraded soil states. Nonetheless, little research is available concerning the relationships between plants and microbes in degraded soil ecosystem, therefore more research and direct evidence are needed, which may supply new insights or challenges into the interactions of belowground and aboveground.

The results of qPCR showed that the abundances of AOA in both degraded soil states were far lower than that in the unsterilized, which supported that soil microbial communities were disrupted in the present study. Also, due to the extremely slow growth rates of AOA (Könneke et al., 2014; Tournet et al., 2011), they were not fully re-established within the 22-week scope of this study. While the gene copies of the other five N-cycling microbes in the degraded soil states were similar in order of magnitude to that in the unsterilized soil state, indicating that different N-cycling microbes had different recovery rates. Although the majority of the microbial abundances and their richness were generally restored from degradation, the community compositions of all six N-cycling microbes in both degraded and degraded + AMF differed significantly from unsterilized. A comprehensive meta-analysis reported that soil biological communities have no consistent trend towards recovery even 10 years after disturbance by fires (Pressler et al., 2018). Our findings based on the microorganisms which grow fast and act as pioneers of ecosystems support evidence that ecosystem restoration is a long-term process (Moreno-Mateos et al., 2020), and as such are critically important for environmental protection.

To rescue soil N-cycling microbes from degradation, we investigated the inoculation of AMF spores and introduction of more plant species. The results of SEM analyses proved that both AMF inoculation and plant diversity could significantly drive the recovery of N-cycling microbial communities, positive impacts on MRI indicated the generally stimulative roles that AMF inoculation and plant

diversity played in the restoration of N-cycling microbial communities. The promotion of restoration could be explained by the more complex mycorrhizosphere under AMF inoculation and high plant diversity levels, which improved nutrient cycling and mass exchange and provided more links within soil ecosystem (Barea et al., 2002; Richardson et al., 2009). Because soil N-cycling microbes form an inseparable microbial N loop, the metabolite of the previous step is also the substrate for the next step (Knops et al., 2002; Kuypers et al., 2018), enhance the transport of soil nutrients and the exchange of microbial metabolites by the mycorrhizosphere would improve the restoration of soil N-cycling microbial communities, particularly in the early stage of degradation, during which the microbial N loop is damaged. Additionally, the densities of the root systems and the mycorrhizosphere can be increased by increasing the number of plant species (Kroon et al., 2012) and the growth of mycorrhizal hyphae, with the latter playing a far greater role (Chapman et al., 2006). Therefore, the effects of plant diversity were less than the effects of AMF inoculation, as revealed by SEM analyses. Even so, more plant species can host diverse AMF as the reason that the majority of plants have obligate mycorrhizal associations (Brundrett, 2009; Henning et al., 2018; Wang & Qiu, 2006). Therefore, higher plant diversity also helped rescue soil N-cycling microbes, which contributed to the result that degraded + AMF with six plant species had the highest MRI. These results supported our hypothesis that inoculation of AMF spores could improve the restoration of soil N-cycling microbes, and higher plant diversity reinforced this promotion.

The results of network analyses showed that more complex networks in degraded + AMF than degraded might facilitate the metabolism of microbes and transport of soil N as discussed above, which resulted in a decreased inhibition of own metabolites accumulation and increased activities of individual species (Wolin et al., 1997). Interactions among microbes are crucial for the re-establishment of N-cycling microbes when soil microbial communities had been disrupted. While in the already-established microbial consortia of unsterilized soil, various soil microbes could contribute to nutrient cycling and mass exchange in the social microbial network (Zengler & Zaramela, 2018), which might weaken the interactions among these N-cycling microbes, and cause the looser N-cycling networks observed in unsterilized soil.

Interestingly, although the inoculation of AMF could improve MRI, IRIs, and co-occurrence network complexity of N-cycling microbes, different results were observed in the recovery of ammonia-oxidizers. The IRIs of ammonia-oxidizers abundances were increased by the inoculation of AMF, however, the IRIs of their richness exhibited contrasting results. As reviewed previously, AMF could help plants absorb soil available N, which would directly compete for soil ammonium with ammonia-oxidizers (Tao et al., 2019; Veresoglou et al., 2012). The increased IRIs of the whole population sizes of ammonia-oxidizers indicated that the dominant roles of AMF were promotion, instead of inhibition, of the restoration. Combined with the results of the decreased IRIs of richness of ammonia-oxidizers, AMF might compete with some less competitive members of the ammonia-oxidizers, such as the slow-growing or rare species. This

explanation was partly proved by our results that significantly less richness of "rare group" in degraded + AMF than degraded was only found in AOA and AOB communities (Figure S1b). Moreover, these results indicated that competition also exists in the degraded ecosystem and could strongly steer the restoration of N-cycling microbes.

In conclusion, our finding provided strong evidence that inoculation of AMF could promote the restoration of most N-cycling microbes and enhance interactions within the N-cycling microbial loop. Plant diversity also played a role in driving the restoration of the soil N-cycling microbial communities, although this effect was lower in importance compared with AMF inoculation. By analysing the relationships between belowground and aboveground organisms in degraded soil states, we detected that the negative influence of plant diversity on N-cycling microbes became weaker compared with the unsterilized soil state. These findings extend our knowledge and also pose challenges in exploring the cascading relationships among belowground organisms, aboveground individuals, and the AMF communities of degraded ecosystems, which are becoming increasingly common on our rapidly changing planet.

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AUTHOR CONTRIBUTIONS

J.W., J. W. and Y.G. designed the project; J.W., J.W. and N.-H.Q. performed experiments; J.W., J.-Z.H., Y.-G.Z. and Y.G. wrote the manuscript. All authors commented and approved the content of the manuscript.

DATA AVAILABILITY STATEMENT

The abundance, richness, IRI, MRI and T-RFLP properties of six N-cycling microbial communities and soil properties of 324 samples have been made available on Dryad under <https://doi.org/10.5061/dryad.cjsxksn41>.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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