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Far-reaching effects on soil properties and underground microbial ecosystem after the introduction of black locusts in forest

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Black locust (*Robinia pseudoacacia* L.), a common woody afforestation species, may threaten native biodiversity and ecosystems. Its invasive nature has not been examined in Asia, and links between aboveground and underground ecosystems have been inadequately established. Here, to figure out its effect on native ecosystems along the afforestation chronosequence, we selected three age class mixed forests of *Robinia pseudoacacia*/*Platycladus orientalis* stands (M7, M13, and M23) and a pure native forest of *Platycladus orientalis* stands (P0) as the control group on the Loess Plateau in China. The results exhibited that Black locust significantly increased the shrub diversity by 23.24%, herb richness by 10.79%, total carbon by 6.14%, organic carbon by 16.05%, and total nitrogen by 36.24% in 13 years of introduction. While the decrease in the diversity and richness of shrubs and herbs, soil pH, and total phosphorus emerged after the introduction of 13 years. Despite the increasing richness and diversity of soil bacteria, total phosphorus was inferred to be a limiting factor for microbes after 23 years. The partial least squares path models showed strong correlations and remarkable aboveground–underground associations. Moreover, vegetation, the main driving force, had the most decisive influence on soil properties and microbial communities. Taken together, these results will contribute to assessments and management of black locust plantations and provide insights into the biological invasion and aboveground–underground theories.

KEYWORDS

Robinia pseudoacacia (black locust), alien species, native ecosystems, soil bacteria and fungi, chronosequence, aboveground–underground

1. Introduction

Black locust originated in the Appalachian and Ozark regions of North America (Kleinbauer et al., 2010). Due to its light-demanding, vegetative reproduction through root and stem sprouts, wide ecological ranges, and fast growth, this species has always been planted widely in the world, especially for ecological erosion control, vegetation restoration, and reconstruction in fragile ecological zones (Laiolo et al., 2003), which has led to “black locust mania” since the 18th century (Vítková et al., 2016).

In the 1950s, as a major woody afforestation species, black locust was also introduced into the Loess Plateau, China, and now its distribution area is more than 70,000 ha (Liang et al., 2018). Recently, it has been proven to have obvious negative impacts on native vegetation communities, animal communities, and ecosystems (Essl et al., 2011; Vilà et al., 2011; Vítková et al., 2016, 2017; Lazzaro et al., 2017) and is considered as an invasive species in several European countries, such as Norway, the Czech Republic, Germany, and Italy (Vítková et al., 2017). However, there are fewer reports of the ecological effects of the introduction of the alien black locust on the native ecosystems in Asia (Chang-Seok et al., 2004), and its invasive nature has not been finalized. Therefore, a compelling and scientific evaluation of the effects of this species on native ecosystems is necessary for local forest management.

Black locusts greatly enhance nitrogen contents in soil (Macedo et al., 2008), improve forest stand conditions, and alter the biodiversity of vegetation communities (Vítková et al., 2017) and microbial communities in native ecosystems (Kleinbauer et al., 2010). For soil properties, an increasing number of studies provide evidence that black locust plantations markedly raised organic carbon and nitrogen content on degraded agricultural lands (Zeng et al., 2015; Papaioannou et al., 2016; Vítková et al., 2017; Liu et al., 2018b; Zhang et al., 2019a,b), but many other studies find no any increase in nitrogen stocks in the mineral soil (Marco et al., 2013) or even the species loss in nutrient-poor stands (Rice and Federici, 2004). Moreover, the proliferation of this particular species in the central regions of Italy has resulted in a gradual decline in the richness and diversity of native species (Benesperi et al., 2012). However, Motta et al. (2009) assumed that native plant species are likely to outcompete black locusts and dominate in the absence of disturbance stand over several decades. The understory flora caused by black locust invasion were different from those dominated by native trees (Vítková et al., 2017), and their effects on biodiversity are still equivocal. In short, there is no consensus on whether the introduction of black locusts will bring about drastic positive or negative ecological effects on the native vegetation communities, soil properties, and soil microbial communities.

Recently, a number of studies have focused on the aboveground–underground associations, especially the drivers of the relative abundance and diversity of the soil microbial community (Bardgett and Wardle, 2013). Many studies considered that plant and soil properties co-shape the richness of soil microbial communities (Zhang C. et al., 2016; Yang et al., 2017b, 2019a; Xu et al., 2020). Some studies indicated that plant richness and coverage were predicted to play key roles in the abundance of soil microbial communities (Goodness et al., 2016; Jiang et al., 2017), while other studies showed that soil properties such as soil nutrients and humidity made more contributions to the composition and diversity of microorganisms (Reinhart et al., 2016). Yet it remains uncertain whether there are still any aboveground–underground links or associations among vegetation, soil properties, and soil microbial communities in the new ecosystem after the introduction of the alien black locust, and if so, how they interact with

each other. Which one has the biggest contribution to the ecosystem component?

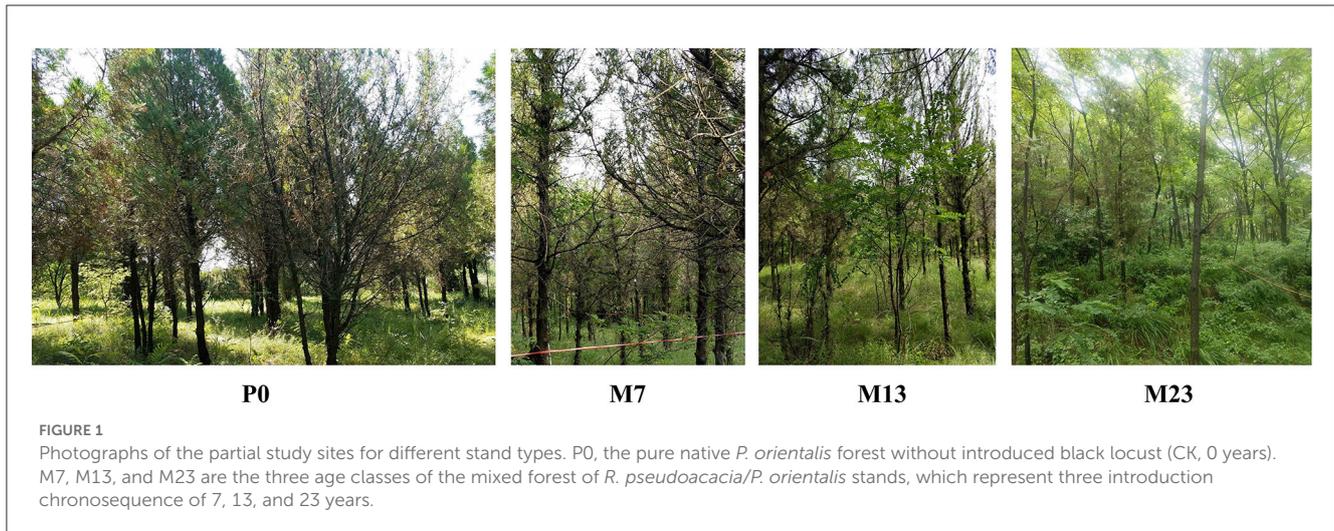
Given that the black locust has been introduced around the world for long history and could have produced far-reaching ecological effects on the local ecosystem through a long succession process, most studies chose only a certain age class of black locust stands to compare with native stands whether in Europe or Asia and did not study the effect of introduction time (Vítková et al., 2017; Lazzaro et al., 2018). Compared with the un-introduced area, plant communities, soil properties, and microbial communities under pure black locust forest showed significant changes during the afforestation years (Liu et al., 2018a; Xu et al., 2020). Thus, we hypothesized that the introduction of alien black locust had effects on native ecosystems along the introduction chronosequence. To verify this hypothesis, we adopted the method of replacing the introduction chronosequence with space and selected three age-class artificial mixed forests of *R. pseudoacacia*/*P. orientalis* stands (7a, 13a, and 23a) and pure native forests of *P. orientalis* stands (CK, 23a) with approximately similar site conditions. The aims of this study were to (1) evaluate qualitatively and quantitatively the effects on vegetation, soil properties, and soil fungal and bacterial communities caused by the introduction of alien black locust following afforestation; (2) explore the aboveground and belowground associations among vegetation, soil properties, and soil microbial communities; and (3) if the relationships do exist, clarify how these ecosystem components affect each other.

2. Materials and methods

2.1. Experimental area

Yongshou County, Shaanxi province, is the ideal area for studying the introduction of black locust as its an area with more than 70 years of afforestation history and is the largest area of black locust forest in China. It belongs to the southern Loess Plateau gully region (34°47′–34°51′ N, 108°05′–108°10′ E). The annual mean temperature is 10.8°C, and the annual mean precipitation is 605 mm, mainly concentrated in August and September every year (Liu et al., 2018a). There is an annual solar radiation hour of 2166.2 h and a frost-free period of 210 days per year. The dominant soil type is loessial (Liu et al., 2018b).

In this region, the original vegetation had been destroyed completely by the 1950s, and only a small area of natural secondary broad-leaved forests such as *Quercus wutaishanica* Mayr and *Populus davidiana* Dode remain (Ma, 1991; Yu et al., 2014). In the 1970s to 1980s, large-scale vegetation afforestation projects were carried out, and the main tree species was exotic black locust, as well as native trees, such as *Pinus tabulaeformis* Carr. and *Platycladus orientalis* (L.) Franco (Yu et al., 2014). However, there was the only mixed forest of *Robinia pseudoacacia*/*Platycladus orientalis* stands with native tree species, a large studied area and a different age class. Moreover, to exclude the influence of stand density on the experiment, we chose the mixed forest of *R. pseudoacacia*/*P. orientalis* stands with a mixed ratio of 6:4 as the research object.



2.2. Experiment design

In July 2021, adopting the method of substituting space for introduction chronosequence, we selected three age classes (7, 13, and 23 years) of the artificial mixed forest of *R. pseudoacacia/P. orientalis* stands and pure native forest of *P. orientalis* stands (23a) with approximately similar elevation, slope aspect, soil type, etc. (Supplementary Table S1 and Figure 1). Among them, the pure native *P. orientalis* forest was 23 years age class, which represented the stage of native stands and no introduced black locust, so it was marked as P0. In the three age classes of mixed forest, *P. orientalis* was the same age class of 23 years, but *R. pseudoacacia* were three age classes of 7, 13, and 23 years. They represented three introduction times of 7, 13, and 23 years and were recorded as M7, M13, and M23, respectively.

Three 20 m × 20 m representative sample plots were established for each stand type (Supplementary Table S1). The distance between replications plots of each age class stand was >1 km, and the distance between plots of different age class stands was more than 2 km. In total, 12 plots were surveyed. In each plot, three 2 m × 2 m subplots and five 1 m × 1 m subplots were arranged along the diagonal of the plot for investigating shrubs and herbaceous composition. For mixed forest stands, the mixed proportion of *R. pseudoacacia/P. orientalis* was 6:4. Numerous ecosystem component indices were chosen (Table 1).

2.3. Plant investigation, soil sampling, and analysis

In each plot, we measured the diameter at breast height (DBH), height, crown diameter, and canopy density (CD) of trees. We also surveyed each species with the name, the number of stems, coverage, frequency, and height using a similar sampling approach that Liu et al. (2018b) described and separated into native or non-native species to calculate the diversity and richness index of shrub, herb, and understory vegetation. The ecotype of

TABLE 1 The different indices of ecosystem components.

Ecosystem component	Indices	Symbol
Vegetation	Canopy density	CD
	Litter biomass	LB
	Species richness of understory vegetation	S_{plant}
	Species diversity of understory vegetation	H_{plant}
	Shrub richness	S_{shrub}
	Shrub diversity	H_{shrub}
	Herb richness	S_{herb}
	Herb diversity	H_{herb}
Soil	Soil pH	pH
	Bulk density	BD
	Soil moisture	SM
	Total phosphorus	TP
	Total nitrogen	TN
	Nitrate-nitrogen	$NO_3^- - N$
	Ammonium-nitrogen	$NH_4^+ - N$
	Total carbon	TC
	Total carbon to total nitrogen ratio	C/N
	Soil organic carbon	SOC
	Microbial biomass carbon	MBC
Bacterial community	Bacterial diversity	$H_{bacteria}$
	Bacterial richness	$S_{bacteria}$
Fungal community	Fungal diversity	H_{fungi}
	Fungal richness	S_{fungi}

vegetation is the most direct and sensitive indicator to reflect the change in the ecological environment. To indicate the changing understory microenvironment, we classified the ecological types

of plants by referring to Flora of Loess Plateau (Chen et al., 2007; Li, 2009) and Flora of China (<http://www.iplant.cn/frps>). According to the needs of water, vegetation ecotypes were divided into xerophytes, mesophytes, and xero-mesophyte. Xerophytes were adapted to dry habitats, while mesophytes were adapted to an average supply of water and temperature environment (Li, 2009). Detailed information about shrubs and herbs is listed in Supplementary Tables S2, S3.

The litter was collected from the soil surface using five 1 m × 1 m quadrats randomly arranged in each plot for calculating litter biomass (LB). We also collected the top 0–20 cm soil at 10 points in each 20 m × 20 m plot according to an S-shaped pattern and mixed them evenly into one sample. The visible plant roots, stones, and litter were removed, and then, soil samples were sifted through the 2-mm sieve, divided into two parts, and transported to the laboratory immediately. Two parts of soil samples were stored in a –80°C refrigerator and at room temperature, respectively, for DNA analysis and nutrient analysis. The analytical methodology of soil physicochemical properties was determined as previously reported (Bao, 2000), including pH, SM, BD, SOC, TC, TP, TN, NO₃⁻-N, NH₄⁺-N, and MBC (Xu et al., 2020).

2.4. Fungal and bacterial community analysis

2.4.1. DNA extraction, PCR, and high-throughput sequencing

Using the MP FastDNA spin kit for soil (MP Biomedicals, Solon, OH, USA), we extracted fungal and bacterial DNA from 0.5 g of soil samples. The V4 region of the bacterial 16S rRNA gene and the ITS1 region of the fungal rRNA gene were amplified using the primers 515F (GTGCCAGCMGCCGCGGTAA) and 806R (GGACTACHVGGGTWTCTAAT) for 16S rRNA and the primers ITS5-1737F (GGAAGTAAAAGTCGTAACAAGG) and ITS2-2043R (GCTGCGTTCTTCATCGATGC) for ITS (Bokulich and Mills, 2013; Mori et al., 2014). PCR products were detected using electrophoresis with 2% agarose gel. Then, a TruSeq[®] DNA PCR-free Sample Preparation Kit was used for library construction, and the target bands were recovered. Finally, qualified sequencing was performed on an Illumina NovaSeq6000 platform (Majorbio Bio-Pharm Technology Co., Ltd. Shanghai, China).

2.4.2. Sequence data processing

First, the raw tags were demultiplexed and quality filtered to obtain effective tags. Then, Uparse v7.0.1001 (<http://www.drive5.com/uparse/>) was operated for effective tags clustering. By default, sequence clustering became OTUs with 97% identity. After clustering, species annotation analysis and taxonomic information at each classification level were performed using the Mothur method and the SSU rRNA database of SILVA138 (<http://www.arb-silva.de/>) (Wang et al., 2007; Edgar, 2013).

2.5. Statistical analyses

We used a one-way analysis of variance (one-way ANOVA) and a least significant difference (LSD) to estimate the significant differences in soil properties, vegetation properties, and microbial communities among different stands using SPSS 20.0 (Chicago, USA). $H_{bacteria}$, $S_{bacteria}$, H_{fungi} , and S_{fungi} indices were calculated as the diversity and abundance of the microbial community at the OTU level using QIIME software (Version 1.9.1). Figures displaying microbial community assemblages were produced using OriginPro2018 software (OriginLab, USA). Non-metrical multidimensional scaling (NMDS) was used for reflecting the inter-group and intra-group differences of samples at different stands. To know the relationship between ecological indices and introduction chronosequence, principal component analysis (PCA) was used first to select the most significant influential indices from all ecological indices and then further regression analysis was performed using the “basicTrendline” package in R-4.0.5 (R Core Team, 2021). The data were normalized using log transformation. Pearson’s correlation analysis was used to understand the relationships among soil properties, vegetation properties, and microbial communities. Furthermore, we used the partial least squares path models (PLS-PM) with the “plsmp” package in R to assess the interaction of vegetation, soil, and microorganisms.

3. Results

3.1. The characteristics of understory flora along the introduction chronosequence

The canopy density (CD), litter biomass (LB), and richness of understory vegetation (S_{plant}) were significantly affected by the introduction chronosequence, except for the diversity of understory vegetation (H_{plant}) (Figure 2). Compared with P0, CD, LB, and S_{plant} significantly increased by 16.67–45.83%, 1.44–7.27%, and 27.43–47.79%, respectively after the introduction of black locust ($p < 0.05$). The richness and diversity of shrubs and herbs (S_{shrub} , H_{shrub} , H_{herb} , and S_{herb}), also changed after the introduction of the black locust. H_{shrub} and S_{herb} increased by 23.24% and 10.79% first and then decreased by 29.46% and 25.52%, with the highest values in M13, but H_{herb} varied later than H_{shrub} with decreasing trends from M13 to M23. In addition, from P0 to M23, the dominant species of shrub changed gradually from *Lespedeza bicolor* to *Rubus parvifolius*, and the dominant species of herb changed from *Tripogon chinensis* and *Carex lanceolata* to *Aster tataricus* and *Patrinia scabiosifolia*, as well as the ecotypes transformed xerophyte into mesophyte (Supplementary Tables S2, S3).

3.2. Soil properties along the introduction chronosequence

Statistically significant differences were found for almost all soil properties except for BD and SM after the introduction of the black locust (Table 2). Carbon-related variables, such as SOC and MBC, increased quickly by more than 79.32% over the introduction

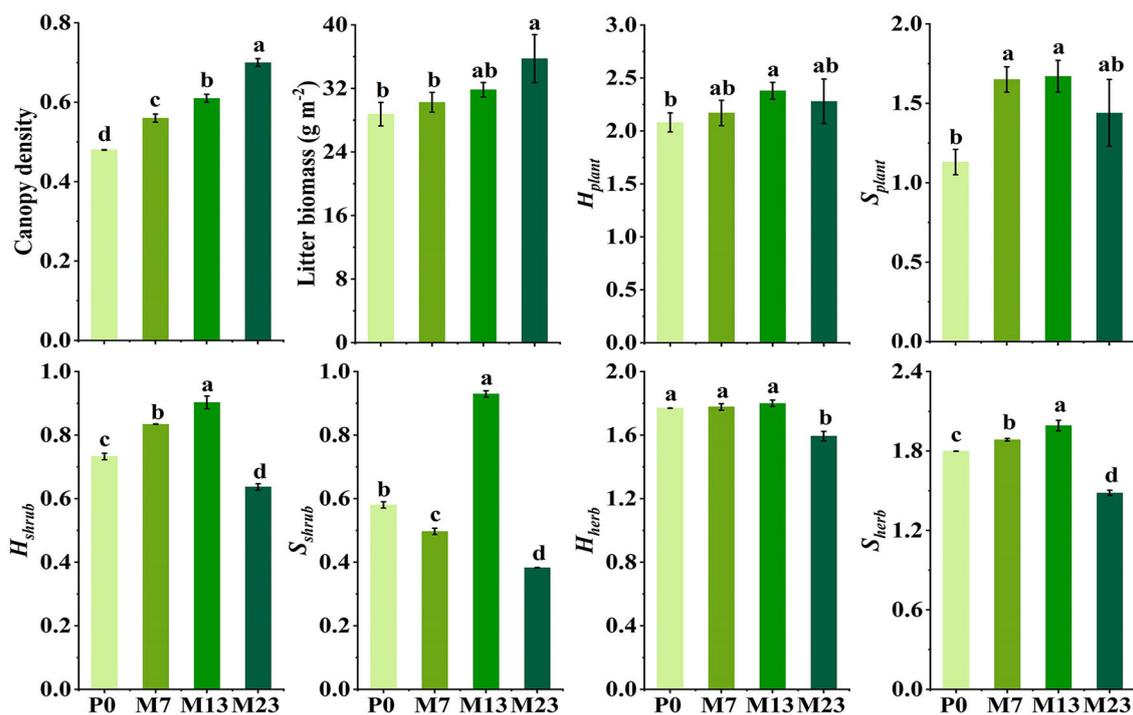


FIGURE 2

The mean (\pm standard error) of characteristics of understory vegetation with introduction chronosequence ($n = 3$). ^{a-d}Different letters indicate that variables were significantly different at $p < 0.05$ in each stand type. P0 is the pure native *P. orientalis* forest without the introduced black locust (CK, 0 years). M7, M13, and M23 are the three age classes of the mixed forest of *R. pseudoacacia*/*P. orientalis* stands, which represent three introduction chronosequences of 7, 13, and 23 years.

chronosequence. The content of TC changed fast, with the decreasing trends from P0 to M7, and increasing trends after the introduction of 7 years. Nitrogen-related variables and pH changed more slowly, with TN, NH_4^+ -N, and pH not showing significant differences relative to pure native *P. orientalis* forest stands until 23 years of the black locust introduction. NO_3^- -N showed differences after 13 years of black locust introduction. TP changed with the increasing trends initially in the early introduction years relative to pure native stands, then decreasing along the introduction chronosequence. On the whole, the introduction of black locust significantly increased the content of NO_3^- -N, NH_4^+ -N, TN, TC, SOC, and MBC by 9.76, 24.94%, 69.05%, 15.56%, 79.32%, and 1.27, respectively, while decreased pH, TP, and C/N by 2.65%, 8.93%, and 32.56%.

3.3. Soil microbial community along the introduction chronosequence

In total, 647,370 bacterial sequences and 766,194 fungi sequences were used for cluster analysis at a 97% similarity level. Based on the results of our analyses, soil fungal and bacterial communities were affected by black locust introduction (Figure 3).

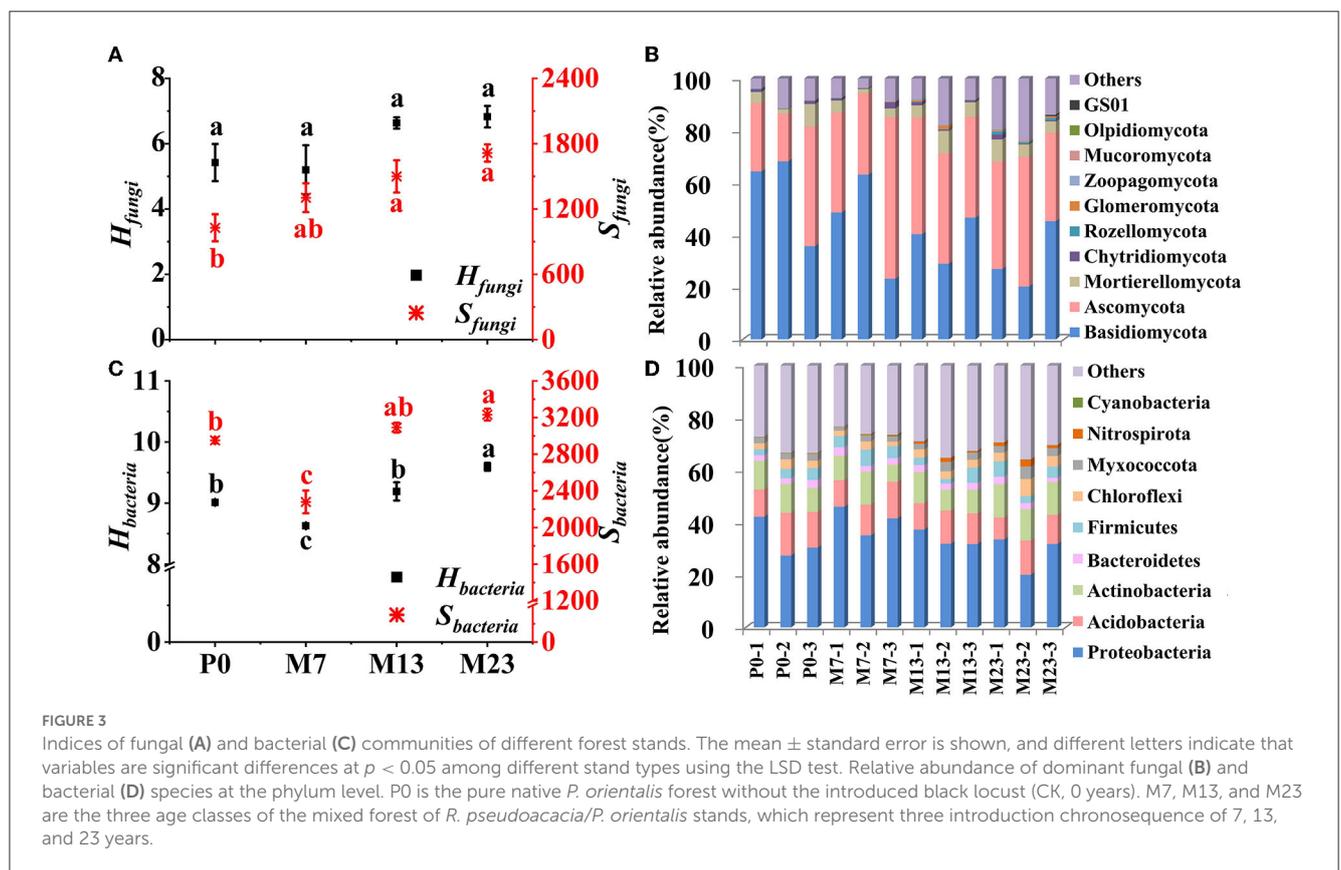
For the fungal community, S_{fungi} increased along the introduction chronosequence, whereas H_{fungi} did not vary significantly after the introduction of the black locust (Figure 3A). For the relative abundance of soil microorganisms in each

stand, fungal communities were dominated by Basidiomycota (42.67%), Ascomycota (39.37%), and Mortierellomycota (5.04%) (relative abundance $>1\%$) (Figure 3B). Along the introduction chronosequence, the relative abundance of Ascomycota increased, but Basidiomycota revealed a reverse trend (Supplementary Table S4). Additionally, the dominant genera were *Hygrocybe* and *Lysurus* at all forest stands, and the abundance of *Hygrocybe* decreased from P0 to M23. *Fusarium* was the dominant genus in M13 and M23 (Supplementary Figure S1a).

$S_{bacteria}$ and $H_{bacteria}$ of soil bacterial community were affected significantly by the introduction of black locust, with the trends decreasing initially in the early introduction years then increasing following introduction chronosequence with the lowest values in M7 (Figure 3C). The dominant phylums of bacterial community were Proteobacteria, Acidobacteria, Actinobacteria, Firmicutes, Chloroflexi, Myxococcota, and Bacteroidetes, with average proportions of 34.14%, 11.97%, 10.42%, 4.03%, 3.24%, 2.66%, and 2.52%, respectively (Figure 3D). The dominant phyla were affected by the introduction of black locust, except for Myxococcota and Bacteroidetes (Supplementary Table S4). Along the introduction chronosequence, the relative abundance of Proteobacteria, Actinobacteria, and Chloroflexi had increasing trends from M7 to M23. Conversely, the abundance of Acidobacteria decreased with the introduction of chronosequence. *Pseudomonas*, *Lysobacter*, *Ramlibacter*, *MND1*, *Sphingomonas*, *Dongia*, *RB41*, and *Bacillus* were the dominant genera in all forest stands (Supplementary Figure S1b).

TABLE 2 Soil properties with introduction chronosequence. The values were mean ± standard error (n = 3). Different letters indicated that variables significantly differed from stand types at p < 0.05.

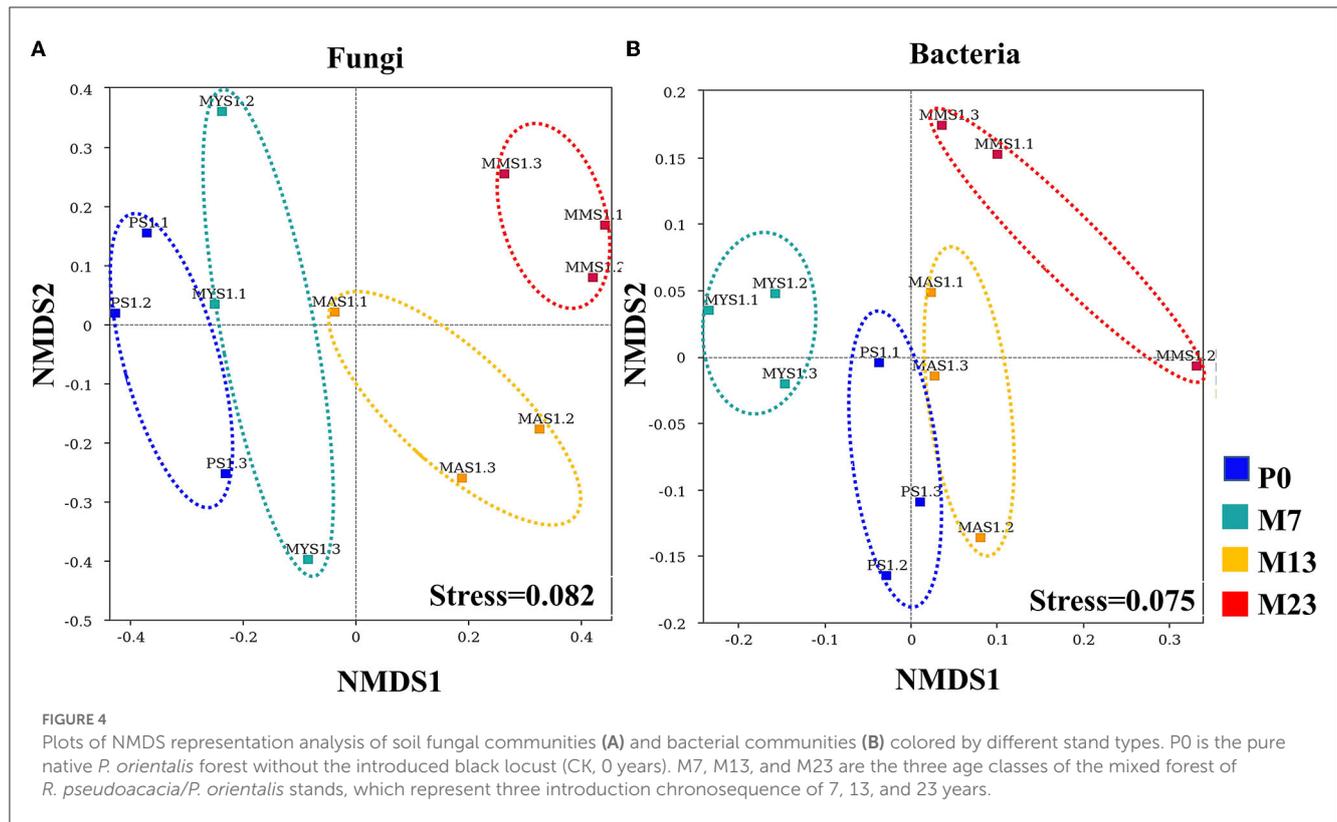
Soil properties	Stand types			
	P0	M7	M13	M23
pH	8.30 ± 0.01 ^a	8.34 ± 0.03 ^a	8.23 ± 0.07 ^a	8.08 ± 0.04 ^b
BD (g cm ⁻³)	1.53 ± 0.05 ^a	1.47 ± 0.01 ^a	1.44 ± 0.06 ^a	1.40 ± 0.03 ^a
SM (%)	21.67 ± 1.01 ^a	22.33 ± 0.99 ^a	22.67 ± 1.33 ^a	21.33 ± 0.87 ^a
TP (g kg ⁻¹)	0.56 ± 0.01 ^b	0.60 ± 0.02 ^a	0.55 ± 0.00 ^b	0.51 ± 0.00 ^c
NO ₃ ⁻ -N (mg kg ⁻¹)	1.83 ± 0.04 ^b	4.88 ± 0.15 ^b	19.33 ± 0.39 ^a	19.69 ± 0.45 ^a
NH ₄ ⁺ -N (mg kg ⁻¹)	3.93 ± 0.03 ^b	3.95 ± 0.10 ^b	4.07 ± 0.03 ^b	4.91 ± 0.07 ^a
TN (g kg ⁻¹)	1.26 ± 0.13 ^b	1.69 ± 0.08 ^b	1.85 ± 0.11 ^b	2.13 ± 0.09 ^a
TC (g kg ⁻¹)	32.96 ± 0.61 ^c	31.38 ± 0.48 ^c	33.14 ± 0.28 ^b	38.09 ± 0.33 ^a
C/N	26.66 ± 2.36 ^a	18.68 ± 1.13 ^b	18.03 ± 1.03 ^b	17.98 ± 1.20 ^b
SOC (g kg ⁻¹)	5.61 ± 0.04 ^c	6.51 ± 0.45 ^b	6.67 ± 0.19 ^b	10.06 ± 0.12 ^a
MBC (mg kg ⁻¹)	102.74 ± 13.25 ^c	122.26 ± 12.18 ^{bc}	136.06 ± 4.34 ^b	233.27 ± 4.97 ^a



NMDS analysis showed that the soil fungal and bacterial communities composition of P0 were distinct from those of M7, M13, and M23 (Figure 4). Furthermore, the soil fungal community of P0 much differed from those of M7, M13, and M23 than the bacterial community.

3.4. Ecological indices response to introduction chronosequence

Through PCA, all ecological indices were divided into four dimensions (Supplementary Table S5). The first axis explained 56%



of the total proportion variance, with several variables having loadings of more than 0.8 (MBC, SOC, NH_4^+ -N, CD, NO_3^- -N, TC, $H_{bacteria}$, C/N, and TP). In addition to these variables, TN, S_{fungi} , H_{fungi} , and pH were also loaded on the first axis. The second axis, third axis, and fourth axis accounted for 15%, 8%, and 6% of the total proportion variance, respectively. After further regression analysis, the results showed that CD, NO_3^- -N, SOC, MBC, TC, and TN were significantly affected by introduction years, with regression coefficients $R^2 \geq 0.80$ (Figure 5). Meanwhile, SOC, MBC, NO_3^- -N, TC, TN, TP, S_{fungi} , pH, $H_{bacteria}$, S_{plant} , and NH_4^+ -N varied quadratically non-linearly along the introduction chronosequence, but CD, C/N, and H_{fungi} varied one-dimensional linearly (Supplementary Table S6). Variables such as TP, pH, and S_{plant} increased initially and then decreased along the introduction chronosequence, while TC and $H_{bacteria}$ revealed reverse trends. CD and H_{fungi} increased with the introduction years, but C/N decreased (Figure 6).

3.5. The relationship among understory vegetation, soil, and microbial community

The network graph was built to explore the correlation of different variables of ecosystem components (Figure 7A). Vegetation is closely related to soil properties, such as the significant positive correlation between LB and NO_3^- -N, TP, NH_4^+ -N, and SOC. Similarly, vegetation characteristics appeared to

be markedly correlated to microbial communities, with H_{plant} being positively correlated with the H_{fungi} , as well as LB being positively correlated with H_{fungi} , S_{fungi} , and $H_{bacteria}$. In addition, NO_3^- -N and SOC were markedly positively correlated with S_{fungi} , but pH and TP were significantly positively correlated with the $H_{bacteria}$, H_{fungi} , and S_{fungi} , which revealed that vegetation had important effects on soil properties and microbial communities.

The path analysis was used to further analyze the relationships among the ecosystem components, such as vegetation, soil chemistry, and microbial phyla, and showed positive response relationships except for the negative responses of soil properties on fungi and bacteria communities (Figure 7B). It also revealed that vegetation had the strongest direct influence on soil chemical properties, as well as a slightly stronger direct and indirect influence on the fungal community than on the bacterial community (Figure 7C).

4. Discussion

Our results showed black locust had effects on native ecosystems along the introduction chronosequence, as well as the strong associations between aboveground and underground on the Loess Plateau. It suggested native ecosystems were affected by afforestation efforts in China, at least from a flora, soil, and microbial perspective.

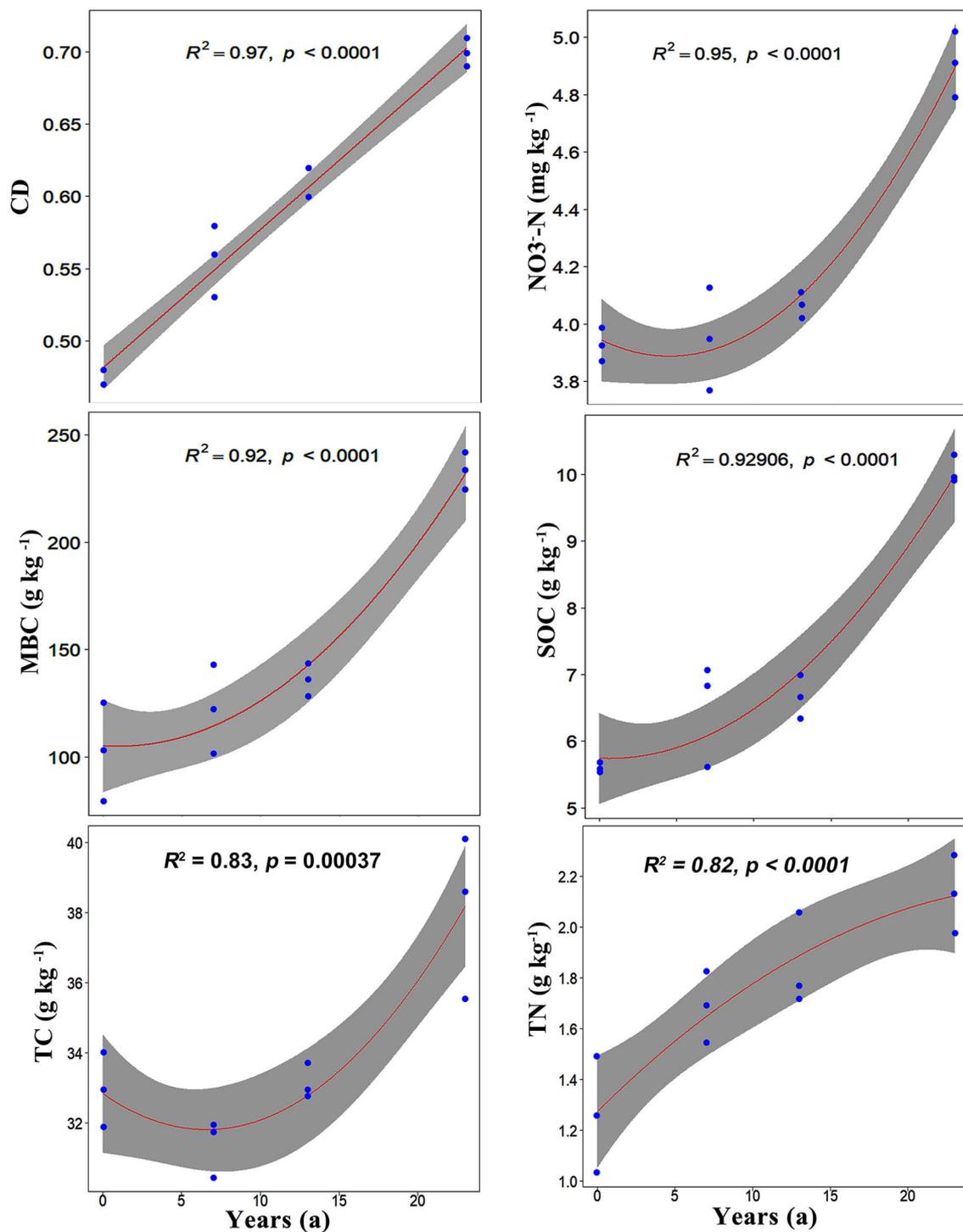


FIGURE 5
 The relationships between ecological indices and introduction chronosequence ($R^2 \geq 0.8$). For all plots, the x-axis is the introduction chronosequence from 0 years to 23 years. The line in each plot represents regression model fit, and the shaded area represents its 95% confidence limits.

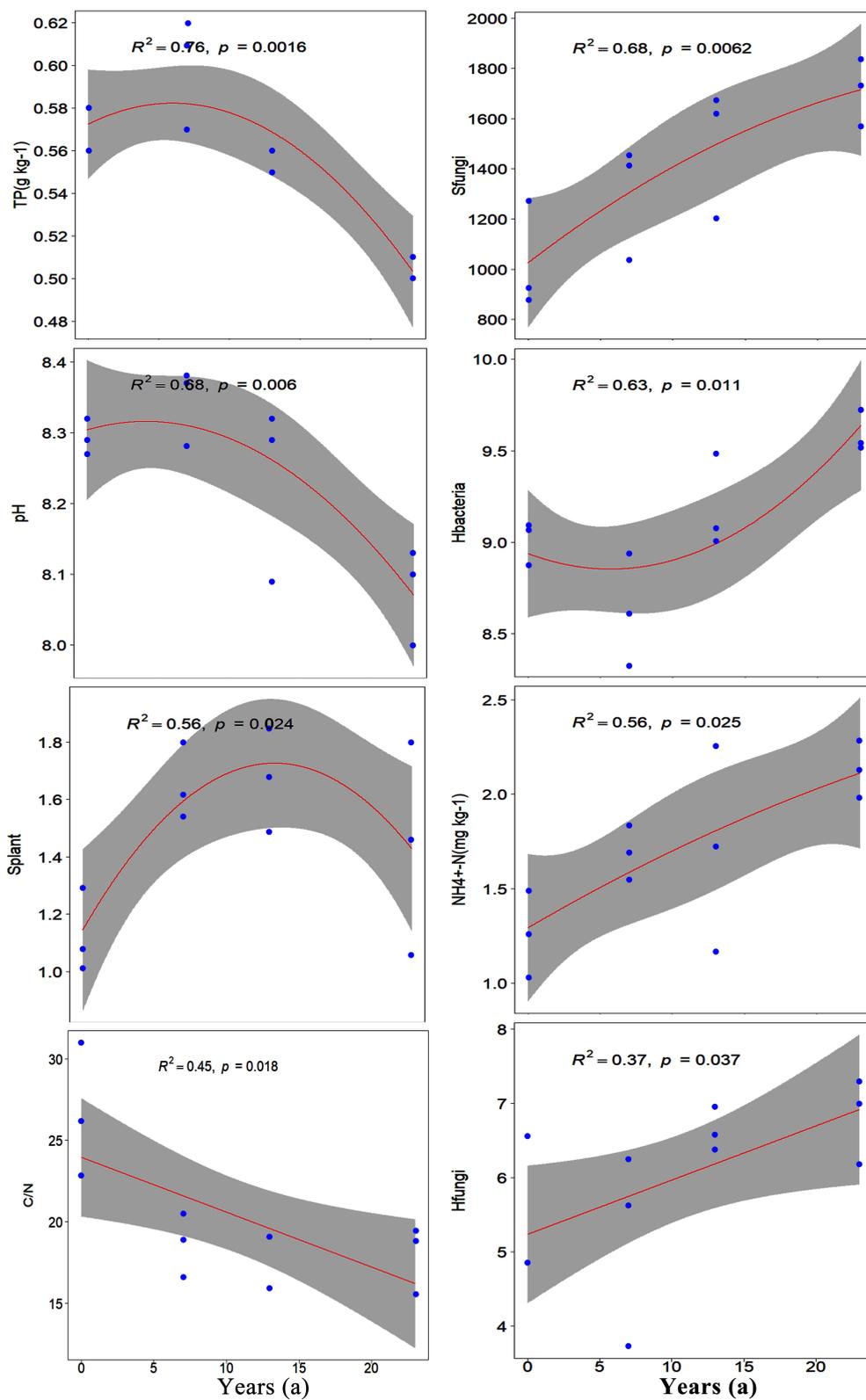


FIGURE 6
The relationships between ecological indices and introduction chronosequence ($R^2 < 0.8$). For all plots, the x-axis is the introduction chronosequence from 0 years to 23 years. The line in each plot represents regression model fit, and the shaded area represents its 95% confidence limits.

4.1. Effects on understory flora after the introduction of alien black locust

The introduction of black locust significantly increased canopy density, litter biomass, and the richness but not the diversity of the understory in our study area (Figure 2). Shrub diversity and herb richness increased to the highest values in M13, then decreased along the introduction chronosequence. However, the diversity of herbs took longer than shrubs to reach the levels where it significantly differed from pure stands with lower values in M23. Other studies also found that black locust formed markedly specific understory vegetation communities, which differ from the native dominant species (Vítková and Kolbek, 2010; Sitzia et al., 2012). The shifts in understory species composition may be from changes in moisture, temperature, and light conditions by the growth of black locust (Liu et al., 2018b; Dyderski and Jagodziński, 2019b). In the early stages of introduction, superior living conditions, such as adequate light conditions and less competition, led to the better growth of vegetation, which caused a higher richness of herbs and higher diversity of shrubs (Xu et al., 2020). Subsequently, the highest H_{shrub} , S_{shrub} , and S_{herb} values were found in M13, which is due to the moderate light intensity and the best forest microhabitat created by the vertical laminated structure of mixed forest stands. However, the growth of black locust and canopy density gradually increased over time, causing lower light conditions and intense interspecific competition over growing space for herbaceous and shrub layers. As a result, in the later stages of introduction, some sun-loving and drought-tolerant undergrowth (such as *Agropyron cristatum* and *Lespedeza cuneata*) were replaced gradually by shade-tolerant, moist-loving species (such as *Aster tataricus*, *Fragaria vesca*, and *Rubus inominatus*), leading to the decrease in H_{shrub} , S_{shrub} , H_{herb} , and S_{herb} (Wei et al., 2013; Kou and Jiao, 2016). The dominant herbaceous species changed gradually from *Tripogon chinensis* and *Carex lanceolata* to *Aster tataricus* and *Patrinia scabiosifolia*, and shrubs species from *Lespedeza cuneata* to *Rubus parvifolius*. Additionally, the ecotypes transformed xerophytes into mesophytes from P0 to M23 (Kirkham, 2005; Li, 2009), which also indicated that the microclimate was changed with the introduction time. In addition, Dyderski and Jagodziński (2019a) showed that black locust forests favored the occurrence of alien species in the undergrowth, but we did not find non-native species of understory that invaded this area, which may be due to the differences in forest age class, afforestation history, natural environment, and so on.

4.2. Effects on soil properties after the introduction of alien black locust

Soil properties were affected in our study area after the introduction of alien black locust except for BD and SM (Table 2). Changes in soil properties associated with carbon appeared significantly higher in mixed stands forests compared with pure stands after 7 years of introduction. Nitrogen-related soil properties and pH might take longer to change, with levels not showing significant differences from pure stands until the introduction year of 13 (NO_3^- -N) or 23 (TN, NH_4^+ -N, pH). As the forest age class increased, the content of soil carbon and nitrogen increased after

the introduction of black locust, but pH decreased (Figures 5, 6), which is in line with the findings by Khan et al. (2010), who found that the leaves of black locust improved the content of soil carbon and nitrogen and decreased soil pH. The higher total nitrogen content under black locust forest was related to litter input and nitrogen-fixing bacteria (Medina-Villar et al., 2016; Anna et al., 2023). Moreover, rhizobia secrete some organic nitrogen into the soil during their lifetime, and the root nodules fall off, thus greatly improving the soil fertility (Babu et al., 2023). It is generally accepted that a nitrogen-fixing species has immediate effects on the N pool in the soils. However, this effect seems to have been delayed for a long time based on our research. This may be because the black locust could not only maintain its needs by nitrogen fixation but also retain most of the soil nitrogen for its root and plant growth of the associated tree species *P. orientalis* (Zhai et al., 1991a; Zhang and Chang, 2006). In the initial introduction stage, black locust was younger with lower nitrogen fixation capacity, and the fixed nitrogen may be used for the growth of *R. pseudoacacia* and *P. orientalis*. With the increase in the introduction chronosequence, the nitrogen fixation capacity of *R. pseudoacacia* increased gradually, as well as the amount of nitrogen (Jiang et al., 2015). In the later stage of the black locust introduction, there was a surplus of soil nitrogen after being utilized by the growth of associated tree species *P. orientalis*, so the soil nitrogen content increased significantly. In addition, the significantly higher NO_3^- -N than NH_4^+ -N may be because of the high rate of nitrification and soil organisms ability to easily absorb NH_4^+ -N (Hou et al., 2015). Leaves of black locust contain more phosphorus than that of *P. orientalis* (Zhai et al., 1991b), and higher phosphorus leaf litter decomposition resulted in a higher TP content of M7 than that of P0. However, the growing root system with more nitrogen-fixing rhizobia of *R. pseudoacacia* and the survival and reproduction of plentiful microbes consumed more phosphorus (Carl et al., 2018), leading to the decreasing trends of TP after 13 years of black locust introduction. Liu et al. (2018b) found the decreasing trends of soil available phosphorus was related to $H_{bacteria}$ in the pure over-mature forest of black locust and inferred phosphorus may be the limit for the diversity of soil bacterial groups in mixed over-mature black locust forest.

4.3. Effects on soil microbial community after the introduction of alien black locust

Similarly, the introduction of black locust affected the properties of the soil microbial community in our study, such as S_{fungi} , $S_{bacteria}$, and $H_{bacteria}$ except for H_{fungi} (Figures 3A, C). The increasing trends of S_{fungi} and $H_{bacteria}$ indices after 7 years of introduction were correlated to litter biomass (Figure 7A). The undergrowth of vegetation and accumulation of litter biomass always lead to more inputs of nutrients to accelerate microbial reproduction (Wang et al., 2010; Vilà et al., 2011). The lowest $H_{bacteria}$ and $S_{bacteria}$ in M7 suggested that in the early stages of introduction, some bacteria species fail to adapt to the new changing environment (Bani et al., 2018). Different patterns of species diversity and richness between fungal and bacterial communities changed along the introduction

chronosequence suggesting an incongruous process between fungi and bacteria succession.

The dominant fungal and bacterial groups were affected significantly after the introduction of black locust, especially Basidiomycota, Ascomycota, Proteobacteria, and Acidobacteria (Supplementary Table S4). This result was consistent with previous studies in pure black locust forests during the afforestation time (Liu et al., 2018b; Yang et al., 2019b). Compared with the native forest of *P. orientalis* stands, Ascomycota were higher in mixed forest stands, but Basidiomycota showed a reverse trend (Supplementary Table S4). The transition of dominant phyla from oligotrophic Basidiomycota-dominant to copiotrophic Ascomycota-dominant community means that soil nutrients and resource availability have been greatly improved after the introduction of alien black locust (Yang et al., 2019b). Proteobacteria are commonly considered copiotrophic bacteria, and their abundance increase in nutrient-rich conditions, while Acidobacteria belong to an oligotrophic group, and their abundance decrease in nutrient-rich conditions (Jangid et al., 2008; Zhang C. et al., 2016). In addition, the relative abundance of Proteobacteria has increased gradually as introduction time increased, whereas that of Acidobacteria showed the opposite trend (Supplementary Table S4). These results further indicated that the introduction of black locust increased the soil nutrient conditions. Moreover, light could affect the metabolism of Acidobacteria all the time (Xu et al., 2020), so increased understory growth may negatively affect them. This may be the reason why Acidobacteria reduced after the introduction of black locust. Due to the abundance of Actinobacteria being positively related to soil carbon and nitrogen contents, they were considered copiotrophic bacteria in some forest soils (Eilers et al., 2010; Nemergut et al., 2011; Ramirez et al., 2012). We also found an increased abundance of Actinobacteria after the introduction of black locust (Supplementary Table S4), which was paralleled by the content of soil carbon and nitrogen. Actinobacteria may have a potential function in carbon and nitrogen cycling in forest soils (Navarrete et al., 2015). Some Chloroflexi have been found to participate in the process of cellulose degradation (Cole et al., 2013; Podosokorskaya et al., 2013) and in the oxidation of NO_2^- (Sorokin et al., 2012, 2014), so the increasing abundance of Chloroflexi may be related to litter decomposition and nitrification in our study area (Figure 3D). In addition, *Lysobacter*, *Ramlibacter*, and *Bacillus* were found in the black locust forest of the Yellow River Delta; *Sphingomonas* was related to nitrogen fixation; *Pseudomonas* and *Bacillus* were related to phosphate-solubilizing microorganisms (Ding et al., 2017).

4.4. Aboveground–underground associations

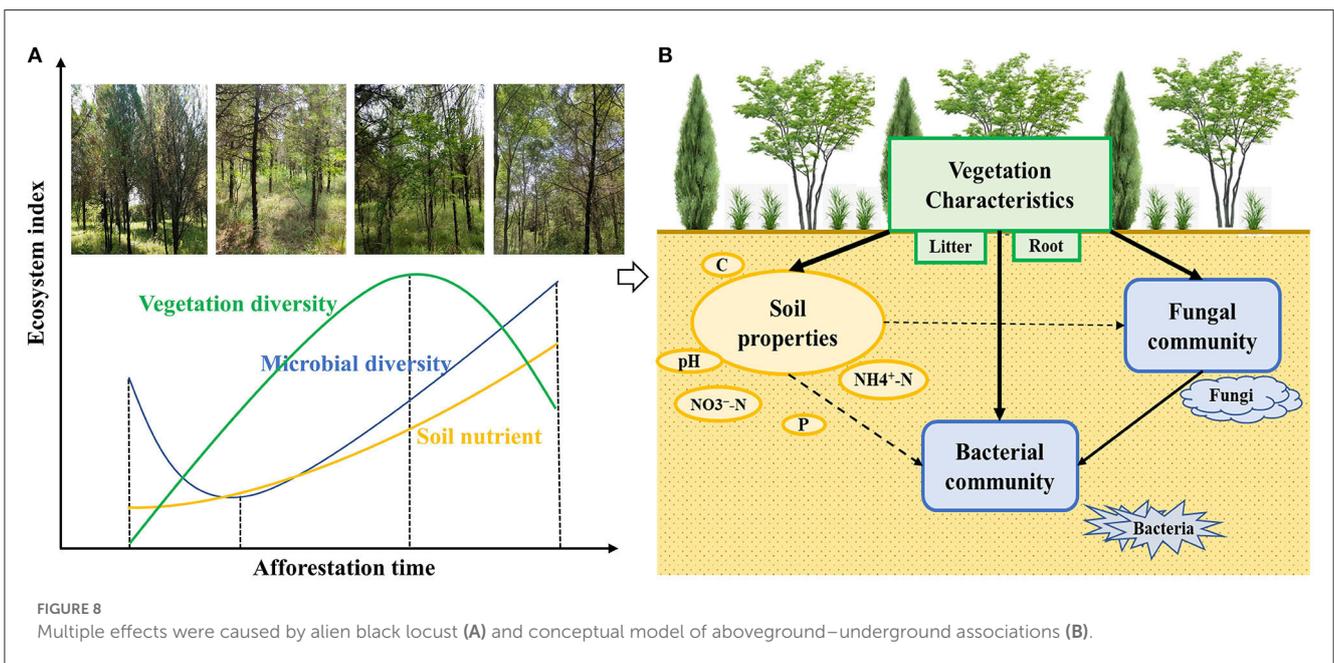
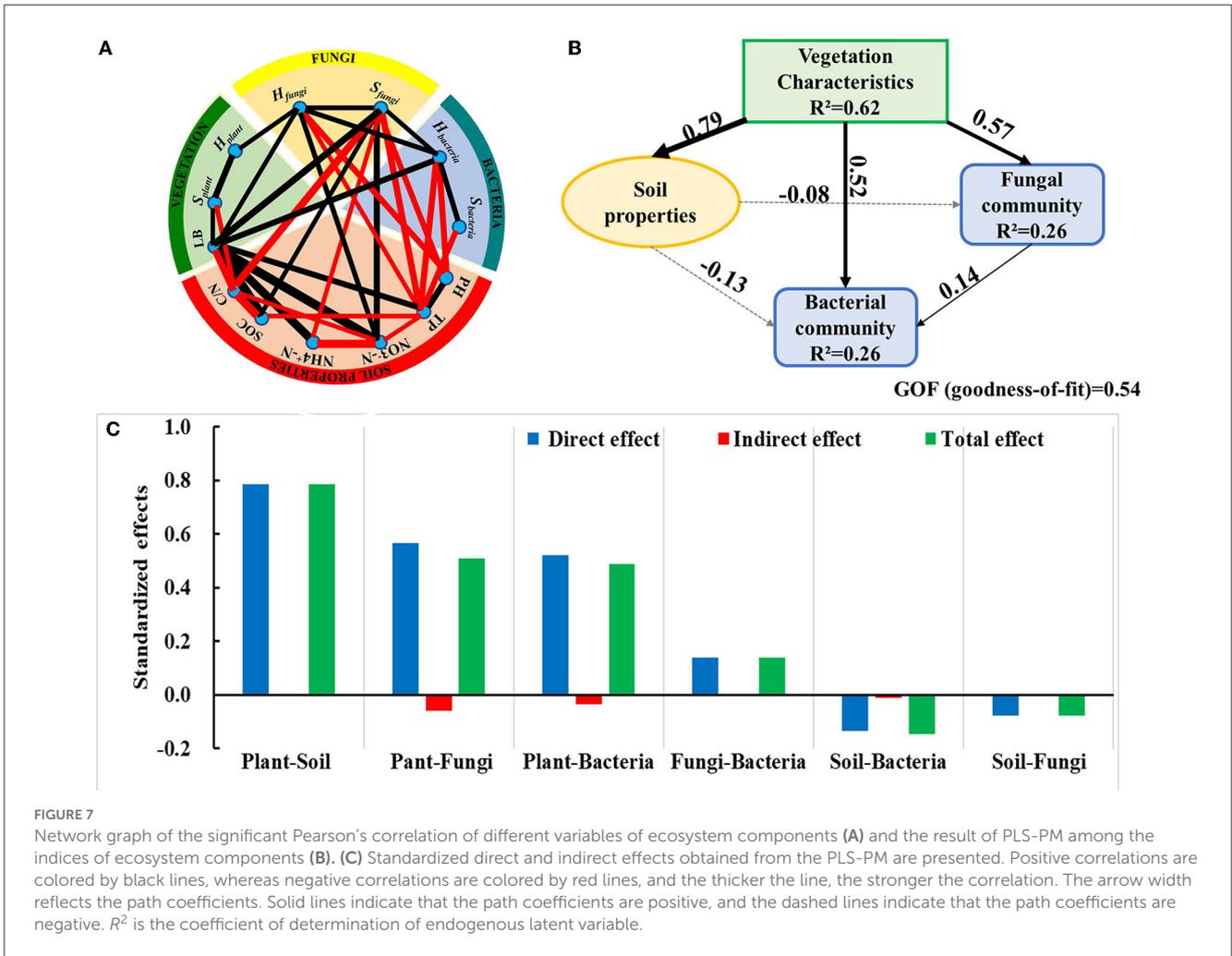
Ecosystem structure and function strongly relied on key associations between aboveground and underground ecological indices (Kardol and Wardle, 2010). Our results showed significant correlations among the ecosystem indices of vegetation, soil, fungi, and bacteria communities in our study area (Figure 8).

Vegetation appeared to have the strongest influence on soil properties after the introduction of black locust and the significant

correlations between litter biomass and soil carbon and nitrogen content in this study (Figure 7B). It may be the introduction of black locust that led to the changes of the microenvironment and understory composition initially, and later had strong far-reaching effects on soil properties through the multiple litters and plant roots (Santos et al., 2016; Reynolds et al., 2017). Black locust, as a leguminous species, has N_2 -fixing capacity and could considerably improve the nitrogen content in soil through the action of root and rhizobia. Additionally, the increase in vegetation communities would affect the microorganisms by accelerating litter decomposition and root biomass accumulation and providing enough energy and nutrients for microbial growth (Zhang Y. et al., 2016).

The vegetation community had stronger effects on the soil microbial community (Figure 7B), which suggested the close associations between aboveground and underground ecosystems (Xu et al., 2020). Vegetation diversity was correlated to fungi diversity rather than bacterial diversity (Figure 7A), suggesting that the diversity of vegetation may have more effects on the diversity of fungi than bacteria, which was consistent with the result of Liu et al. (2018b) and Xu et al. (2020). The reason may be that fungal communities comprise various groups such as pathogens and reciprocal symbiosis fungi (such as ectomycorrhizal and endophytic mycorrhizal fungi) and interact more with the host plant species than bacterial communities under the same environmental factors (Yang et al., 2017a). Moreover, soil fungal pathogens could recruit dominant species and adjust their survival rate to maintain the diversity of the vegetation communities, while fungal reciprocal symbiosis could offer nutrients and protection to benefit the host plants (Lei et al., 2020). Significant correlation between LB and H_{fungi} , S_{fungi} , and H_{bacteria} further indicated that litter is one of the nutrient resources of microbial communities and has significant effects on the richness and diversity of fungi and bacteria communities (Yang et al., 2019b; Zeng et al., 2019).

Soil properties had effects on the richness and diversity of soil fungi and bacterial communities, such as significant positive relationships between SOC and S_{fungi} , NO_3^- -N, and S_{fungi} and H_{fungi} (Figure 7A). The reason is that SOC and NO_3^- -N provided more available nutrition resources for fungal communities during the litter decomposition (Zhang Y. et al., 2016). Labile carbon is an energy source for soil microfauna and a key factor in maintaining soil microbial biodiversity (Babu et al., 2020; Singh et al., 2020). Merrick et al. (1995) and Marzluf (1997) reported soil nitrate N was the preferable N resource for microbes and influenced microbial community richness. The availability of N forms and more nutrition resources activate the fungi community, leading to the increasing diversity of fungi. Furthermore, pH was significantly negatively correlated with bacterial diversity, fungal diversity, and fungal abundance (Figure 7A), suggesting that the environment with the decrease of pH over time of black locust introduction appeared to benefit microbial communities, so there were increasing trends of bacterial diversity, fungal diversity, and richness in mixed forest stands. Xu et al. (2020) thought black locust would provide a more optimal living environment for copiotrophs. Phosphorus is a key chemical element and is related to rRNA synthesis with the growth of soil microbes. Liu et al. (2018b) found available phosphorus had more potent effects on the composition of bacterial phyla, especially for α -proteobacteria.



However, TP was found to be significantly negatively correlated with bacterial diversity, fungal diversity, and fungal abundance in this study (Figure 7A). An explanation for this result is that the increasing microbial communities demanded lots of soil available phosphorus for their growth and development, as well as their metabolisms for producing phosphatases as litter decomposition progresses (Manzoni et al., 2012; Hacker and Oelmann, 2015; Fatemi et al., 2016; Luo et al., 2017). Moreover, the lower abundance of phosphorus-solubilizing microorganisms, such as *Pseudomonas*, *Bacillus*, and *Fusarium*, indicated there was lower available phosphorus in M23 (Supplementary Figure S1). Consequently, we inferred that after the introduction of black locust for many years, the progressively decreasing phosphorus content may restrict the growth and development of soil microorganisms.

In short, the introduction of the black locust after 13 years had negative effects on shrub diversity, herb diversity, and herb richness on the Loess Plateau (Figure 8). Even though there were positive effects on soil nutrients associated with carbon and nitrogen except for phosphorus, as well as microbial diversity and richness, it has been proved that in pure black locust forest with age class from mature to over-mature, soil nutrients and microbial diversity declined (Liu et al., 2018b). The inconsistent results were probably due to the different age classes of black locust forests. Therefore, whether the effects of the introduction of black locust on soil nutrients and microorganisms are positive or negative remains to be further studied in the future.

Additionally, the stronger correlations and influences among vegetation, soil properties, and microbial communities indicated stronger aboveground and underground associations in this study area. Vegetation, the main driving force, affects the structure and composition of soil microbial communities by changing species composition, litter biomass, soil properties, root-soil system components, and other factors (Hacker and Oelmann, 2015; Zhang C. et al., 2016). Soil microorganisms are the link between aboveground and underground components of the ecosystem and play an important role in forest succession and nutrient cycle (Li et al., 2013; Kou and Jiao, 2016). This kind of association among vegetation–soil–microbes can be used for other ecosystems (Reynolds et al., 2017), and the root is also a key and non-negligible component for studying aboveground and underground associations in the forest ecosystem.

5. Conclusion

These results clarified that the introduction of black locust had significant effects on plant communities, soil properties, and soil microbial communities in native forest stands, such as the non-linear trends of the richness and diversity indices of understory vegetation and soil microbial communities along the afforestation chronosequence. Furthermore, the strong correlations among vegetation, soil properties, and soil microbiome suggested that there were remarkable aboveground–underground associations and that vegetation, as the main driving force, had far-reaching

effects on soil properties and soil microbial communities. In short, these results will contribute to the assessments and management of black locust plantations around the world and provide insights into biological invasion and its conservation theories.

Data availability statement

The datasets presented in this study can be found in online repositories. The name of the repository and accession number can be found below: NCBI; PRJNA979340.

Author contributions

YK and LZ conceived this research and designed all experiments. LZ, CW, HL, GL, and HZ performed the experiments. KK, ML, XZ, and GM helped with the investigation work and collection of samples. LZ analyzed experimental data and drafted the manuscript. YK gave comments and suggestions for this article. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fevo.2023.1210498/full#supplementary-material>

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