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Long-term N addition reduced the diversity of arbuscular mycorrhizal fungi and understory herbs of a Korean pine plantation in northern China

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With the development of agriculture and industry, the increase in nitrogen (N) deposition has caused widespread concern among scientists. Although emission reduction policies have slowed N releases in Europe and North America, the threat to biodiversity cannot be ignored. Arbuscular mycorrhizal (AM) fungi play an important role in the establishment and maintenance of plant communities in forest ecosystems, and both their distribution and diversity have vital ecological functions. Therefore, we analyzed the effects of long-term N addition on AM fungi and understory herbaceous plants in a Korean pine plantation in northern China. The soil properties, community structure, and diversity of AM fungi and understory herbaceous plants were detected at different concentrations of NH_4NO_3 (0, 20, 40, 80 kg N ha^{-1} $year^{-1}$) after 7 years. The results showed that long-term N deposition decreased soil pH, increased soil ammonium content, and caused significant fluctuations in P elements. N deposition improved the stability of soil aggregates by increasing the content of glomalin-related soil protein (GRSP) and changed the AM fungal community composition. The *Glomus* genus was more adaptable to the acidic soil treated with the highest N concentration. The species of AM fungi, understory herbaceous plants, and the biomass of fine roots were decreased under long-term N deposition. The fine root biomass was reduced by 78.6% in the highest N concentration treatment. In summary, we concluded that long-term N deposition could alter soil pH, the distribution of N, P elements, and the soil aggregate fractions, and reduce AM fungal and understory herb diversity. The importance of AM fungi in maintaining forest ecosystem diversity was verified under long-term N deposition.

KEYWORDS

nitrogen deposition, AM fungi, herbs, biodiversity, soil aggregates, glomalin-related soil protein

1 Introduction

The dramatic increase in atmospheric emissions of reactive nitrogen (N) from human activities since the Industrial Revolution (industrial and agricultural activities and fossil fuel combustion) has led to a rapid increase in N deposition to terrestrial and aquatic ecosystems (Ackerman et al., 2019). N deposition provides a new source of fertilizer for plants, but excessive N input can also affect biogeochemical cycles and alter ecosystem structure and function (Yu et al., 2019). Forest ecosystem species diversity is an important indicator reflecting the relationship between plants and the environment, determining the structure and ecological functions of forest communities, which are mutually constrained and synergistic with environmental factors. Moderate N input will alleviate forest N limitation to a certain extent and promote vegetation growth. On the other hand, excessive N deposition induces ecological problems such as soil acidification, reduction of biodiversity, and degradation of forest functions (Vuorenmaa et al., 2018; Gao Y. et al., 2019; Hong et al., 2019). The results of a worldwide network of long-term monitoring of N deposition showed that China has faced more severe N deposition than the United States, European countries, and other countries in East Asia since the twentieth century (Zhang et al., 2021). N deposition remains a threat to the biodiversity and stability of forest ecosystems (Weldon et al., 2022).

Herbaceous plants have the highest species diversity in forest ecosystems and contribute significantly to forest ecosystem structure and function, but are more sensitive to atmospheric changes and N deposition (Gilliam, 2007; Thrippleton et al., 2016; McDonnell et al., 2022). It has been demonstrated that excessive N input can have complex effects on herbaceous community structure and plant biological characteristics, such as reducing herbaceous diversity and altering herbaceous root chemical components and biomass (Li et al., 2015). However, most studies have focused on tropical forests and temperate grasslands, and not enough studies have been conducted on herbaceous plants in temperate forests.

The functions of soil microorganisms in forest ecosystems should not be underestimated. Among these, arbuscular mycorrhizal (AM) fungi can establish symbiotic relationships with most herbaceous plants and play an important role in the establishment and maintenance of plant communities (Smith and Read, 2008; Mariotte et al., 2013; Wang et al., 2019). AM fungi can promote plant growth (Hoeksema et al., 2010; Xie et al., 2022) and improve plant resilience (Ruiz-Lozano et al., 2016; Chen et al., 2018). Glomalin-related soil protein (GRSP) in mycelium secretions can also promote soil aggregation (Gao W. et al., 2019) and affect host plants in a range of direct and indirect ways. Most AM fungi depend on the host plant for existence and reproduction, and AM fungi are thought to be susceptible to environmental conditions, such as climate (Compant et al., 2010), plant species (Kivlin et al., 2011), and soil properties. Excessive N input indirectly affects the structure and diversity of AM fungi through changes in soil factors (Ma et al., 2021; Boeraeve et al., 2022) and thus affects the ecological function of AM fungi. The results of the studies are not the same around the world, which is due to the influence of experimental conditions. A global meta-analysis showed that the negative effect of N addition on AM fungi was mainly a reduction in total AM fungal abundance as well as the diversity and structure of AM fungi (Han et al., 2020). However, in a tropical simulated N deposition experiment, it was found that N addition mainly reduced the diversity and abundance of AM fungi (Camenzind et al., 2014). AM fungi have been shown to contribute to the maintenance of forest herbaceous diversity under N application conditions (Smith and Stephan, 2021).

In this experiment, AM fungi, herbaceous plants, and soil properties were investigated in a temperate Korean pine plantation ecosystem in northeastern China that was subjected to a 7-year N addition experiment. The AM fungi and the understory herbaceous plants that coexisted with them were investigated by morphological identification and high-throughput sequencing, respectively. We hypothesized that: (1) long-term N fertilization would change the soil pH value, increase soil GRSP content, and thus improve the stability of soil aggregates; (2) long-term N addition would change the structure and diversity of AM fungal communities, thus affecting the ecological functions of AM fungi; and (3) long-term N addition would change AMF diversity directly by reducing pH and indirectly by changing herbaceous plant composition.

2 Materials and methods

2.1 Study site

The experiment sample site was located in a Korean pine plantation in Liangshui National Nature Reserve, Heilongjiang Province, China (128°53′20″E, 47°10′50″N); natural N deposition is 12.93 kg N ha^{-1} $year^{-1}$. The area has a distinct temperate continental monsoon climate with an average annual temperature of –0.3°C, of which the average annual precipitation of 676 mm, a frost-free period of 100–120 d, and an annual snowpack period of 130–150 d. The Korean pine plantation in this test site was established in 1954 after a clear-cutting of the mixed broadleaved-Korean pine forest. The dominant plant species are Korean pine and undergrowth-associated herbs (*Oxalis corniculate* and *Mitella nuda*).

2.2 Experimental design

The simulated N deposition experiment commenced in 2014, and the sample plots were treated with N application by understory manual spraying from June to September (four times per year). Each sample plot was 5 m × 30 m, bounded by a 10 m wide buffer strip. The required NH_4NO_3 was dissolved in 20 L water when it was needed. There were four treatments as follows: CK (0 kg N ha^{-1} $year^{-1}$), LN (20 kg N ha^{-1} $year^{-1}$), MN (40 kg N ha^{-1} $year^{-1}$), and HN (80 kg N ha^{-1} $year^{-1}$), which were approximately twice, four times, and eight times the level of bulk N deposition in the region, respectively (Song et al., 2019). There were three replicates in each treatment with a completely randomized design.

2.3 Soil sample collection and pre-treatment

Soil samples were collected in July 2020. After further removal of the top layer of plastic material, three small random sample squares (1 m×1 m) were set in each sample plot, and five random samples were collected from each plot and mixed into a composite sample (15 cm^3, a total of 12 composite samples). Soil samples were sieved and divided into three parts: one part was stored at −80°C for soil microbial DNA extraction, another part was air-dried and used for soil chemical property determination, and the remaining part was used to determine the composition of soil aggregates and measures the content of GRSP.

2.4 Soil physicochemical properties

The air-dried soil samples were sieved through soil sieves with apertures of 2 mm, 1 mm, and 0.25 mm to obtain four groups of soil samples (>2 mm, 1–2 mm, 0.25–1 mm, <0.25 mm) (Muruganandam et al., 2009). Each group of soil samples was weighed. Soil structural stability was evaluated by the mean weight diameter (MWD) of water-stable aggregates (Zhao et al., 2017). The contents of easily extractable glomalin-related soil protein (EE-GRSP) and total glomalin-related soil protein (T-GRSP) were determined (Janos et al., 2008). EE-GRSP was extracted from 0.5 g of soil with 4 ml of 20 mmol L^{-1} sodium citrate (pH=7.0) and autoclaved at 121°C for 30 min. T-GRSP was extracted from 0.5 g of soil with 4 ml of 50 mmol L^{-1} sodium citrate (pH=8.0) and autoclaved at 121°C for 60 min. The supernatant was collected at 4°C, 10,000 rpm for 10 min. T-GRSP extraction was performed six times until the solution turned straw-colored (Gao et al., 2017). The GRSP content was determined using a spectrophotometer (595 nm) with bovine serum albumin as the standard.

The soil pH of the air-dried soil samples was determined using a pH meter (FiveEasy FE20) on a mixture with a water-to-soil ratio of 2.5:1. TC (total carbon), TOC (total organic carbon), and total N (TN) were determined by an analyzer (multi N/C 2100 TOC/TN Analyze, Analytik Jena, Germany). Soil ammonium N (NH_4^+-N) and nitrate N (NO_3^-N) were measured using a continuous flow analyzer (BRAN+LUEBBEAA3, Germany). Soil total phosphorus (TP) and available phosphorus (AP) were determined using the molybdenum antimony colorimetric method determined by the molybdenum antimony anti-colorimetric method.

2.5 Herbaceous plant diversity, root biomass, and AM fungal colonization rate

We explored herbaceous plant diversity in July 2020. Three small random sample squares (1 m×1 m) were set in each sample plot, and the species of herbaceous plants, the number of individuals of the species (counted as a single plant in the above-ground part), height, and cover were investigated. The fine roots (<2 mm in diameter) of herbaceous plants were selected, and the root biomass was measured (Li et al., 2021). We determined the AM fungal colonization rate of roots from herbs by a modified aniline blue staining method (Zubek et al., 2011). In addition, we randomly selected 50 mixed herbaceous roots from each sample site for the infestation rate experiment.

2.6 AM fungal community structure and diversity

2.6.1 Isolation and identification of AM fungal spores

Spores were obtained by wet sieving (three-layer soil sifters are 100, 200, and 400 mesh) method from the soil (10 g) around the roots of herbaceous plants. The spores were placed under a stereomicroscope for count, observation, and preliminary classification. The spores were counted and transferred to polyvinyl alcohol-lactic acid-glycerol (PVLG) to observe the morphological characteristics, including spore color, shape, diameter size, sporiferous saccule, spore wall thickness, spore wall type, and subtending hyphae width and shape. The spores were identified according to the photos and feature descriptions from the VA mycorrhizal fungi identification manual (Schenck and Perez, 1990), INVAM (https://invam.ku.edu/), and international AM fungal classification system (http://www.amf-phylogeny.com/).

AM fungal spore density, species richness, relative abundance (RA), frequency, importance value (I), and species alpha diversity index were calculated per 10 g of air-dried soil sample. The degree of AM fungal dominance was assigned into four categories according to their important values (I): I > 50% means dominant species, 30% < I ≤ 50% means subdominant species, 10% < I ≤ 30% means accompanying species, and I ≤ 10% means rare species.

2.6.2 High throughput analysis of AM fungi

Total DNA was extracted from mixed soil (0.5 g) using Fast DNA® Spin Kit for Soil. Then AM fungus-specific primers AML1 and AML2 were selected as the first round of primers (Lee et al., 2008), and AMV4.5NF (AAGCTCGTAGTTGAATTTCG) and AMDGR (CCCAACTATCCCTAT-TAATCAT) were selected as the second round of primers for amplification with the nested PCR amplification method (Sato et al., 2005). PCR was performed using TransGen AP221-02: TransStart Fastpfu DNA Polymerase, 20 μl reaction system. The PCR amplification products were detected and recovered by 2% agarose gel electrophoresis, and Illumina's MiseqPE300 platform was used for sequencing (commissioned by Shanghai Meiji Bio-Pharmaceutical Technology Co). Raw sequences were uploaded to the NCBI Sequence Read Archive (SRP426700).

2.7 Statistical analysis

One-way ANOVA and Duncan's multiple tests ($P < 0.05$) were performed using IBM SPSS Statistics 26 (IBM Corporation, New

York) software to compare the means of plant, AM fungi, and soil physicochemical property data. The correlation between soil physicochemical properties and herbaceous plant diversity was calculated by Pearson's test (two-tailed) at two significant levels, $P < 0.05$ and $P < 0.01$. RDA analysis was used to reveal the effect of soil properties on the community composition of AM fungi. Structural equation modeling (SEM) was used to predict causal pathways of N addition, key soil physicochemical indicators, and AM fungi on herbaceous plant diversity. The fit of models that had been previously built was evaluated using chi-squared test, root mean square error of approximation (RMSEA), and goodness-of-fit index (GFI).

3 Results

3.1 Soil physical and chemical properties

3.1.1 Soil chemical properties

The soil pH gradually decreased with the increase of N addition concentration, and the pH value in HN treatment significantly decreased to 5.24 ($P < 0.05$) (Table 1). TP content was significantly increased by 16.7% in the MN treatment. The AP content was significantly reduced after N addition. There were significant changes in NH_4^+-N and NO_3^-N content. The NH_4^+-N content increased with the concentration of N addition, and there was a significant increase in MN and HN treatments. The NO_3^-N content was significantly increased in the LN treatment and significantly decreased in the HN treatment.

3.1.2 Soil aggregate and glomalin-related soil protein

The weight percentage of soil aggregate varied with its size fractions (Figure 1). In all N addition treatments, MWD values ranged from 1.48–1.74 mm. The content of large aggregates (2–5 mm) and MWD value were significantly higher in the HN treatment than in the CK treatment.

N addition exerted a significant effect on the EE-GRSP and T-GRSP content of soil aggregate. The contents of EE-GRSP ranged from 1.36 to 4.29, and T-GRSP ranged from 3.73 to 13.50 $mg{\cdot}g^{-1}$ (Figure 2). GRSP content varied according to the amount of N addition. N addition increased the content of EE-GRSP and T-GRSP in soil aggregates and reached the maximum in the MN treatment. N addition increased the EE-GRSP content of soil aggregates by 60.6% to 118.7%, 12.0% to 77.2%, 5.0% to 60.5%, and 16.3% to 56.2% for each particle size (<0.25 mm, 0.25–1 mm, 1–2 mm, and 2–5 mm), respectively. N addition increased the T-GRSP content of soil aggregates by 45.5% to 87.6%, 25.5% to 88.4%, 44.5% to 105.2%, and 51.1% to 86.4% for each particle size, respectively.

3.1.3 Relationship between the stability of soil aggregates and the content of GRSP content under N addition conditions

In the present study, Korean pine plantation soils were dominated by medium aggregates (1–2 mm), with MWD values ranging from 1.48 to 1.74 mm, which has been identified as an indicator of soil stability, and long-term (7 years) HN treatment (80 kg N ha^{-1} $year^{-1}$) increased the soil macro-aggregates content and thus improved soil stability. Pearson correlation analysis of undisturbed soil GRSP content and MWD showed that both EE-GRSP and T-GRSP were significantly positively correlated with MWD values ($P < 0.01$) (Figure 3), where R^2 is the coefficient of determination.

3.2 Understory herbs

3.2.1 Herb plants communities

There were 33 species of herb plants were investigated in this study (Table S1). N addition significantly altered the dominance of five plants in Table S1 ($P < 0.05$): *Chrysosplenium sinicum*,

TABLE 1 Effects of N addition on the soil properties of a Korean pine plantation.

N addition	CK	LN	MN	HN
pH	5.73 ± 0.13 a	5.55 ± 0.09 ab	5.47 ± 0.10 ab	5.24 ± 0.19 b
TP (g kg^{-1})	0.54 ± 0.03 b	0.55 ± 0.05 b	0.63 ± 0.01 a	0.55 ± 0.04 b
AP (mg kg^{-1})	1.18 ± 0.07 a	0.76 ± 0.10 c	0.84 ± 0.02 bc	0.91 ± 0.03 b
TN (g kg^{-1})	6.67 ± 0.09 ab	6.54 ± 0.05 b	6.78 ± 0.13 a	6.79 ± 0.06 a
NH_4^+-N (mg kg^{-1})	5.28 ± 0.57 b	5.49 ± 0.58 b	8.40 ± 0.51 a	9.24 ± 0.64 a
NO_3^-N (mg kg^{-1})	3.79 ± 0.15 b	4.04 ± 0.08 a	3.98 ± 0.07 ab	3.40 ± 0.17 c
TC (g kg^{-1})	72.1 ± 8.94 a	66.87 ± 6.60 a	73.27 ± 2.18 a	71.47 ± 11.5 a
SOC (g kg^{-1})	62.73 ± 7.92 a	60.33 ± 7.65 a	64.23 ± 3.46 a	61.8 ± 5.90 a
C/N ratio	10.81 ± 1.28 a	10.22 ± 1.05 a	10.81 ± 0.52 a	10.52 ± 1.63 a

The value represents the mean ± SD (n = 3). Different letters in the same row of data in the table indicate significant differences among different N addition treatments (a,b,c) at $P < 0.05$. TP represents soil total phosphorus, AP represents soil effective phosphorus, TN represents soil total nitrogen, TC represents soil carbon, SOC represents soil organic carbon, CK represents the control, LN represents N addition with 20 kg N ha^{-1} $year^{-1}$, MN represents N addition with 40 kg N ha^{-1} $year^{-1}$, HN represents N addition with 80 kg N ha^{-1} $year^{-1}$.

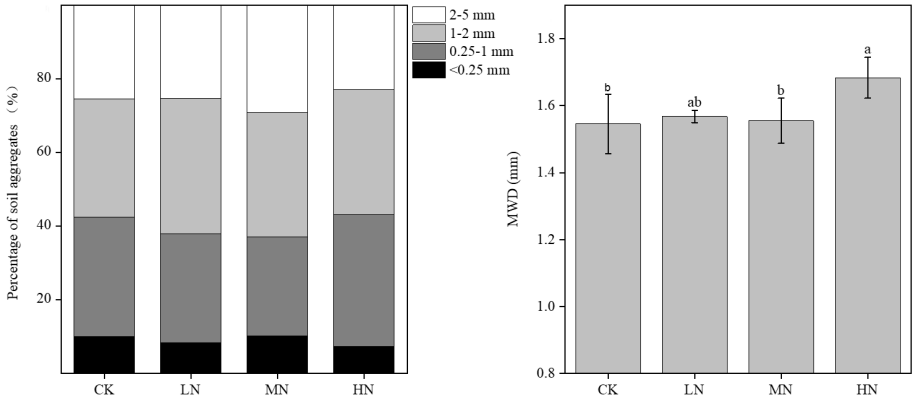

FIGURE 1
Soil aggregate size fractions and MWD value under different N addition treatments. Percentage of soil aggregate size fractions and MWD (mean weight diameter) value. Mean (n=3) of each parameter followed by a different letter is significantly different between four treatments (a, b) at $P < 0.05$.

Aegopodium alpestre, *Dryopteris crassirhizoma*, *Eranthis stellate*, and *Phryma leptostachya*. The species of herb plants were not detected in the LN treatment (9.1%), MN treatment (27.3%), and HN treatment (33.3%) compared with the CK treatment.

3.2.2 Herb diversity, fine root biomass, and AM fungal colonization rate

The Shannon index and Simpson index of herb plants decreased gradually with increasing N addition concentration, and the MN and HN treatments were significantly lower than CK (Table 2). The root biomass and AM fungal colonization rate decreased significantly with the increase of N addition concentration. The biomass decreased by 34.43 g/m^2 in the HN treatment. The AM fungal colonization rate decreased significantly by 21.95% in HN treatment. This indicated that herb diversity and AM fungal colonization rate were significantly reduced when the applied N concentration exceeded 40 kg N ha^{-1} year^{-1} in this experiment. The fine root biomass of herb plants was also significantly reduced when the applied N concentration exceeded 20 kg N ha^{-1} year^{-1}.

3.3 AM fungal community structure and diversity

3.3.1 Morphological identification of AM fungi

There were 4,461 AM fungal spores isolated from all soil samples, and nine genera and 20 species were identified morphologically. There were seven species belonging to *Acaulospora*, accounting for 35% of the total number of species, followed by four species of *Glomus* genus, accounting for 20% of the total number of species.

The relative abundance (RA) and importance values (I) of AM fungal spores were analyzed under the N application treatment (Table 3). The soil samples had the most abundant spore species in the CK treatment, with 17 AM fungal species. Moreover, *Acaulospora spinosa* and *Claroideoglomus lamellosum* species were only found in the CK treatment. The spore species in soil samples were gradually reduced with increasing N addition concentration. There were 14 AM fungal species in the LN treatment and MN treatment. *Sclerocystis sinuosa* was only found in the MN treatment. However, there were only 10 AM fungal species in the HN treatment.

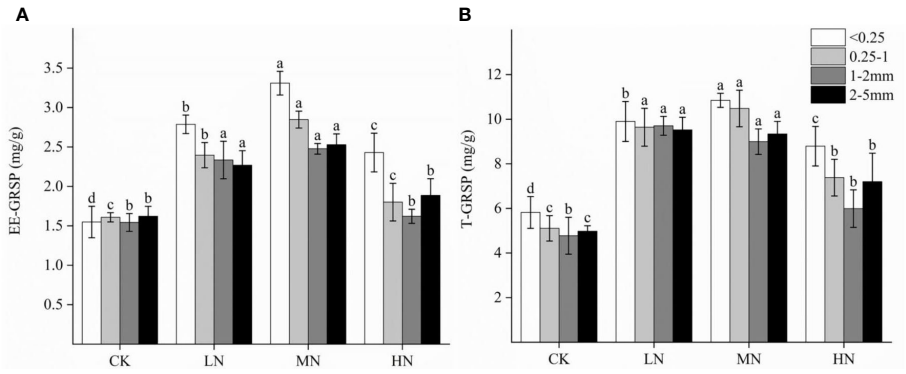

FIGURE 2
Effects of different N application rates on EE-GRSP **(A)** and T-GRSP **(B)** content in soil aggregates of each particle size. EE-GRSP represents easily extractable glomalin-related soil protein, and T-GRSP represents total glomalin-related soil protein. Mean (n=3) of each parameter followed by a different letter is significantly different between four treatments (a, b, c, d) at $P < 0.05$.

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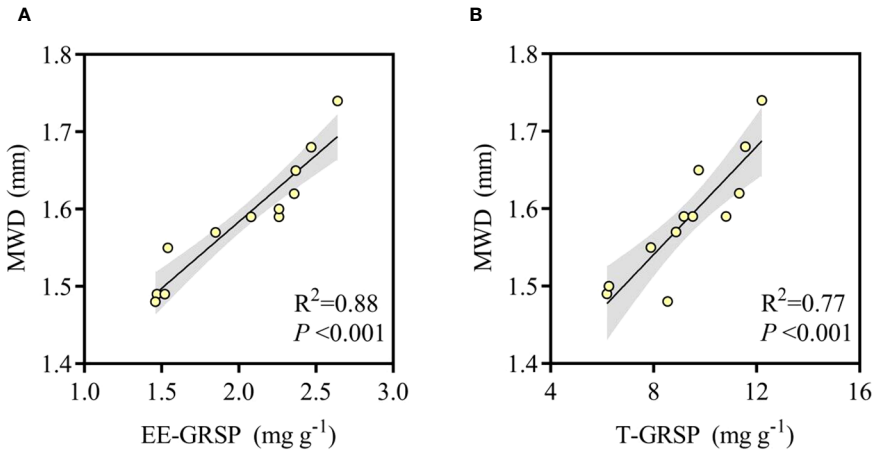

FIGURE 3
Relationships between the MWD and GRSP under different treatments. MWD represents mean weight diameter, EE-GRSP represents easily extractable glomalin-related soil protein **(A)**. T-GRSP represents total glomalin-related soil protein **(B)**.

3.3.2 Spore density, species abundance, and diversity index of AM fungi

The highest AM fungal spore density was found in the MN treatment, with 40.13 spores g^{-1} (Table 4), followed by the HN treatment, LN treatment, and CK treatment with 36.9, 36.43, and 35.27 spores g^{-1}, respectively. With increasing N addition concentration, the species abundance of AM fungi decreased gradually. There were only 8 species g^{-1} in the HN treatment. The diversity of AM fungal species was significantly different between the N addition treatments and the CK treatment, which had the highest Simpson's index of 0.76. The LN treatment had a significantly lower diversity index than the CK and MN treatments, while the HN treatment had the lowest Simpson's index of 0.49. Similarly, the CK treatment had the highest Shannon index of 1.85, whereas the HN treatment had the lowest Shannon index of 1.02.

3.3.3 Bioinformatical analyses

In the present study, we identified 398,299 sequences from the total dataset, which optimized to 391,960 sequences. The coverage of operational taxonomic units (OTUs) in all soil types was more than 99%. The largest OTU richness of AM fungi was in the CK treatment (Table 5). The highest diversity of AM fungi was found in the CK treatment indicated by Shannon and Simpson's indices, which were 2.27 and 0.59, respectively. The lowest diversity of AM fungi was found in the HN treatments, which were 0.84 and 0.16, respectively.

The results of the community structural composition showed that the majority of OTU sequences belonged to *Glomus* (Figure 4). The relative abundance of *Glomus* increased with N addition and was significantly higher in the HN treatment than in the CK treatment. In contrast, the relative abundance of *Paraglomus* was reduced by 96.71% in the HN treatment compared to the CK treatment.

The AM fungal community composition and relative abundance at the species level are shown in Figure 4. The dominant species was *Glomus*-Glo3-VTX00074 in the CK treatment, *Acaulospora*-VTX00272 in the LN treatment, *Diversispora*-unclassified in the MN treatment, and *Glomus*-MO-G5-VTX00219 in the HN treatment. N addition had a significant effect on AM fungal community composition in the Korean pine plantation. *Glomus*-MO-G18-VTX00064 was only found in the CK treatment. *Glomus*-group-B-*Glomus*-acnaGlo7-VTX00057 was only found in the LN treatment. *Glomus*-MO-G22-VTX00125 and *Glomus*-Wirsel-OTU13-VTX00140 were unique species in the MN treatment.

TABLE 2 Effects of N addition on the diversity of undergrowth herb plants, root biomass, and infection rate of AM fungi.

N addition	CK	LN	MN	HN
Shannon index	2.49 ± 0.23 a	2.28 ± 0.13 a	1.97 ± 0.07 b	1.87 ± 0.05 b
Simpson index	0.9 ± 0.03 a	0.87 ± 0.02 a	0.83 ± 0.01 b	0.82 ± 0.02 b
Pielou index	0.52 ± 0.04 a	0.49 ± 0.03 a	0.5 ± 0.01 a	0.49 ± 0.03 a
Root biomass (g/m^2)	43.8 ± 3.36 a	22.77 ± 3.63 b	18.33 ± 5.33 b	9.37 ± 1.96 c
Colonization (%)	91.11 ± 5.09 a	83.33 ± 3.33 ab	76.67 ± 3.33 b	71.11 ± 10.72 b

The value represents the mean ± SD (n = 3). Different letters in the same row of data in the table indicate significant differences among different N addition treatments (a,b,c), at $P < 0.05$.

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TABLE 3 Relative abundance (RA) and importance value (I) of Mycorrhizal fungal spores under four N addition treatments.

	CK		LN		MN		HN	
	RA(%)	I(%)	RA(%)	I(%)	RA(%)	I(%)	RA(%)	I(%)
Acaulospora mellea	1.98	50.99	0.64	16.99	1.58	50.79	1.45	34.06
Acaulospora spinosa	0.28	16.81	0.00	0.00	0.00	0.00	0.00	0.00
Acaulospora tuberculata	0.00	50.00	0.73	33.70	1.33	34.00	0.00	0.00
Acaulospora bireticulata	2.65	34.66	0.00	0.00	0.00	0.00	0.54	16.94
Acaulospora gedanensis	1.70	34.19	0.00	0.00	1.83	50.91	1.36	34.01
Acaulospora rehmii	3.30	34.99	0.82	33.75	0.00	0.00	0.00	0.00
Acaulospora rugosa	1.98	50.99	2.38	51.19	2.16	51.08	0.81	33.74
Claroideoglomus claroideum	17.48	58.74	11.71	55.86	18.87	59.43	26.56	63.28
Claroideoglomus lamellosum	4.06	52.03	0.00	0.00	0.00	0.00	0.00	0.00
Glomus glomerulatum	1.13	33.90	0.64	33.66	1.16	33.92	0.54	33.61
Glomus versiforme	0.28	16.81	0.00	0.00	0.00	0.00	0.18	16.76
Glomus ambisporum	4.54	52.27	1.01	33.84	1.08	17.21	0.00	0.00
Claroideoglomus candidum	45.27	72.64	70.63	85.32	61.10	80.55	63.23	81.62
Sclerocystis rubiformis	0.00	0.00	0.37	16.85	0.08	16.71	0.00	0.00
Sclerocystis sinuosa	0.00	0.00	0.00	0.00	0.25	16.79	0.00	0.00
Paraglomus brasilianum	3.78	51.89	0.73	33.70	1.33	34.00	1.08	33.88
Racocetra fulgida	0.00	0.00	1.65	34.16	1.16	33.92	0.00	0.00
Rhizophagus intraradices	1.13	17.23	1.37	34.02	0.00	0.00	0.00	0.00
Dominikia aurea	4.54	35.61	2.84	51.42	3.82	51.91	2.26	34.46
Funneliformis mosseae	2.65	34.66	2.01	34.34	2.08	34.37	0.00	0.00
Unidentified	3.21	51.61	2.47	34.57	2.16	34.42	1.99	34.33

TABLE 4 Spore densities, species richness, Shannon index, and Simpson index of AM fungi in four N addition treatments.

N addition	CK	LN	MN	HN
Spore densities g^{-1}	35.27 ± 2.97 a	36.43 ± 1.53 ab	40.13 ± 1.02 b	36.9 ± 2.93 ab
Species richness	15.67 ± 0.58 a	12.00 ± 1.00 b	11.67 ± 1.52 b	8.00 ± 1.00 c
Shannon index	1.85 ± 0.05 a	1.15 ± 0.03 c	1.34 ± 0.06 b	1.02 ± 0.05 d
Simpson index	0.76 ± 0.05 a	0.51 ± 0.03 c	0.59 ± 0.03 b	0.49 ± 0.04 d

The value represents the mean ± SD (n = 3). Different letters in the same row of data in the table indicate significant differences among different N addition treatments (a,b,c,d) at $P < 0.05$.

TABLE 5 Diversity index of AM fungal community in four N addition treatments.

N addition	CK	`LN	MN	HN
Shannon index	2.27 ± 0.37 a	1.54 ± 0.22 b	1.12 ± 0.05 bc	0.84 ± 0.15 c
Sob index	30.67 ± 11.68 a	26.33 ± 4.93 ab	12.67 ± 1.53 b	21.33 ± 7.02 ab
Simpson index	0.59 ± 0.04 a	0.36 ± 0.02 b	0.31 ± 0.08 b	0.16 ± 0.04 c
ACE index	31.92 ± 12.17 a	29.82 ± 7.84 a	15.16 ± 4.02 a	24.86 ± 9.66 a

The value represents the mean ± SD (n = 3). Different letters in the same row of data in the table indicate significant differences among different N addition treatments (a,b,c), at $P < 0.05$.

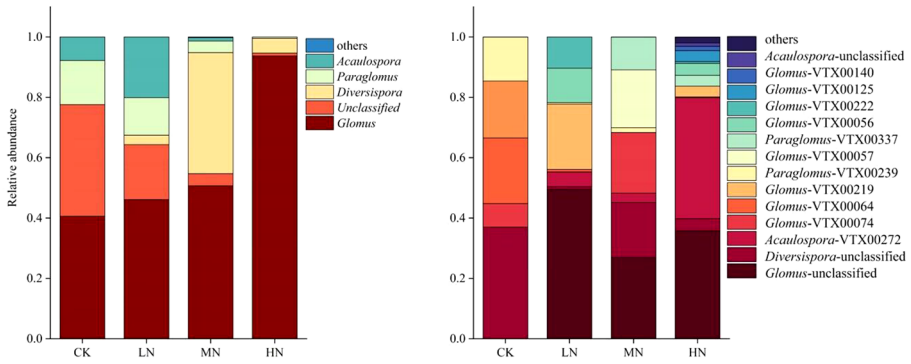

FIGURE 4
Relative abundances of AM fungal communities in Korean pine plantations with different N addition at genus and species level.

3.3.4 The morphological and molecular identification of AM fungi

The result of morphological identification showed that there were nine genera and 20 species of AM fungi after wet sieving, while high-throughput sequencing amplified and sequenced the DNA extracted from the mixed soil from each small sample, and four genera and 11 virtual species were identified. The genus *Diversispora* was detected only by high-throughput sequencing in contrast to the morphological identification, while morphological identification revealed six genera that were not identified by high-throughput sequencing. However, the AM fungal diversity index had a similar change trend by high-throughput sequencing and morphological identification under different treatments.

3.4 Relationship between soil physicochemical properties and AM fungal community composition under N addition conditions

RDA analysis showed that six soil physicochemical factors explained 91.12% of the variation in the AM fungal community, as shown in Figure 5. Soil pH and NO_3^-N, AP, NH_4^+-N, and TN content significantly influenced the distribution of AM fungal species. In addition, *Glomus* was negatively correlated with pH and positively correlated with NH_4^+-N and NO_3^-N content. *Paraglomus* was positively correlated with AP content and pH value.

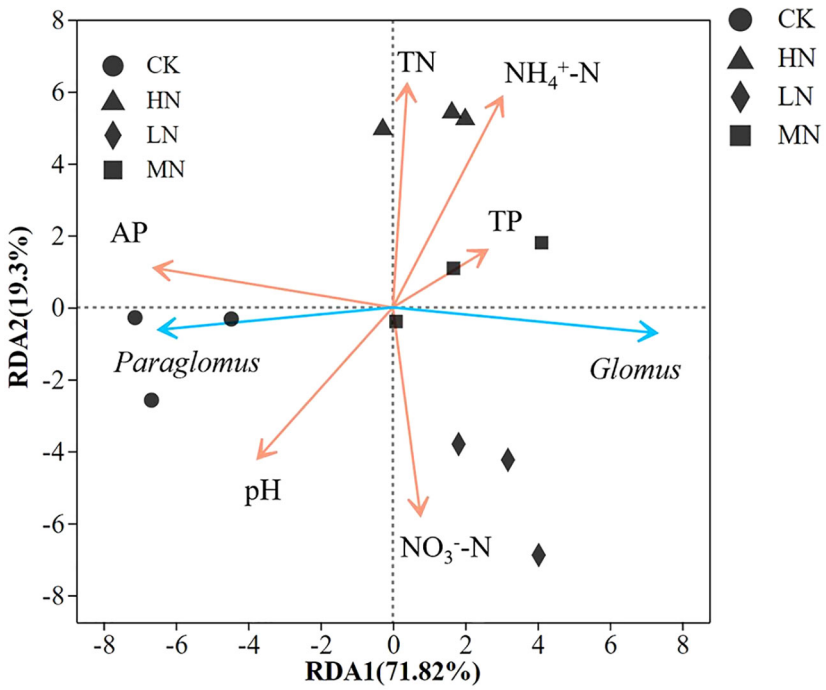

FIGURE 5
Redundancy analysis (RDA) of AM fungi species, genus distribution, and environmental characteristics. TP represents soil total phosphorus, AP represents soil effective phosphorus, TN represents soil total nitrogen.

3.5 Relationship between environmental factors and herb diversity under N addition conditions

Long-term N addition reduced the diversity of understory herbaceous plants. The correlation was analyzed between α diversity index of herbs and biochemical factors by Pearson correlation. The diversity of understory herbaceous plants was significantly and positively correlated with the diversity of AM fungi ($P < 0.05$, Figure 6) and the colonization rate of AM fungi ($R^2 = 0.66$). The diversity of herbs was significantly and positively correlated with total N content ($P < 0.05$), significantly and negatively correlated with NH_4^+-N content ($P < 0.01$), and significantly and positively correlated with pH ($R^2 = 0.67$). Additionally, herbaceous plant fine root biomass was significantly and positively correlated with their diversity ($P < 0.01$).

After correlation analysis of soil physical and chemical indicators, herbaceous plants, and AM fungi, we built SEM models for important indicators and obtained the following models after multiple fitting (Figure 7). The P-value of χ^2 was higher than 0.05, indicating that the model structure was reasonable. The SEM throughput analysis largely explained the effects of biotic as well as abiotic factors on herb diversity ($R^2 > 90\%$). N addition indirectly affected AM fungal diversity and plant diversity through soil pH value, ammonium N, and AP content. Among these, AM fungal diversity was the strongest pathway affecting plant diversity.

4 Discussion

4.1 Effect of N addition on soil properties

Soil acidification is caused by N deposition, and soil pH decreases linearly with increasing N application (Tian and Niu, 2015). In this study, long-term HN treatment (80 kg N ha^{-1} $year^{-1}$) reduced soil pH, while LN and MN treatments had no significant effect on soil pH. The reason may be that soils in terrestrial ecosystems are more sensitive to N inputs, while forest ecosystems are more stable than other types of ecosystems (Roth et al., 2021).

MN treatment had a significant effect on soil TP content, while AP content decreased to varying degrees with N addition. This may be because soil acidification activates aluminum ions and iron ions in the soil, causing more phosphorus to be absorbed by the soil and resulting in a decrease in AP content. When plants and microorganisms absorb and utilize AP, they get a corresponding supplement from the phosphorus element of other components, which leads to the reduction of AP content.

Soil TN content was stable, while ammonium content increased linearly with N application. The results of Qin et al. (2022) showed that long-term N application can change the soil ammonium and nitrate content. In addition, total N increased significantly with the increase of N addition. The stability of the whole N content in this experiment may be because the forest ecosystem is more stable and can resist a certain concentration of N addition.

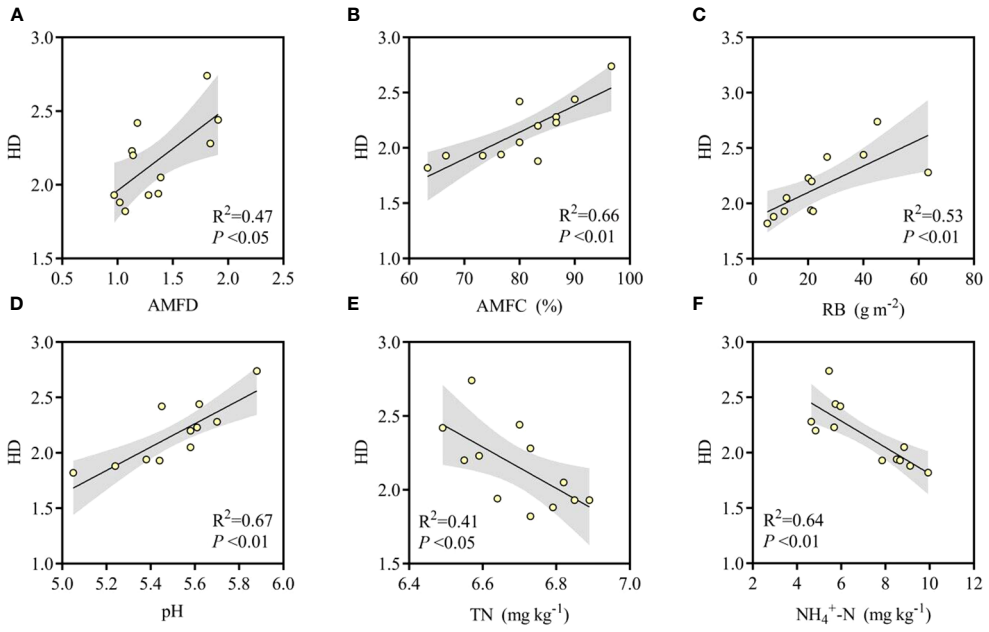

FIGURE 6
Linear correlation between herb Shannon diversity (HD) and **(A)** AM fungi Shannon diversity (AMFD), **(B)** AM fungi colonization (AMFC), **(C)** root biomass (RB), **(D)** soil pH, **(E)** soil total N, **(F)** soil NH_4^+-N.

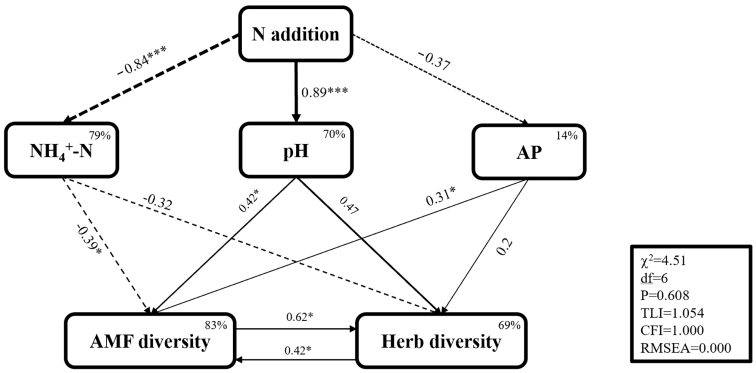

FIGURE 7
Structural equation model simulates the effects of various response variables on plant diversity of Korean pine plantation. Valid paths are indicated by arrows, and continuous and dashed arrows indicate positive and negative relationships, respectively. The numbers next to the paths indicate the normalized path coefficients, positive path coefficients indicate positive relationships, and negative path coefficients indicate negative relationships. The higher the absolute value of the path coefficient, the more important the path is and the thicker the line. * indicates the significance level of each fitting factor: *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. The number in the upper-right corner of the box is R^2 and shows the percentage of explained variance for each endogenous variable. The fit of the model was determined using chi-squared test (c^2), degrees of freedom (df), probability level (P), comparative fit index (CFI), Ticker-Lewis value (TLI), and root mean square error of approximation (RMSEA) expressed. Among them, high P values for c^2 ($P > 0.05$); CFI, TLI > 0.90; RMSEA < 0.05 indicate a well-fitting model.

There was an increased tendency for EE-GRSP content as the N addition concentration was increased, and the T-GRSP content was significantly higher in the HN treatment than in the CK treatment, which was probably due to the significant acidification of the soil (Agnihotri et al., 2022). On the contrary, short-term N addition reduced the GRSP content in the soil (Jia et al., 2021; Huang et al., 2022). The reason might be that short-term N additions could not significantly change soil acidity and alkalinity, but lead to a decrease in GRSP by changing soil dissolved organic carbon and N/P ratio. The proportion of large soil aggregates and GRSP content were significantly elevated in the HN treatment. Pearson correlation analysis of *in-situ* soil GRSP content and MWD showed that both EE-GRSP and T-GRSP were significantly positively correlated with MWD values (Figure 3).

4.2 Effect of N addition on the structure and diversity of AM fungi community

N addition and local soil conditions affect plants and soil microorganisms (Cao et al., 2020; Zeng et al., 2021). In this study, both high-throughput sequencing and traditional spore morphological identification were used to investigate the community structure and diversity of woodland AM fungi. Morphological identification is inconsistent, limited, and subjective, but can be improved by means such as increasing the sample size. High-throughput sequencing technology has been widely used by researchers but still has some limitations. Due to the variety of microorganisms, the current NCBI database cannot cover all the microorganisms, or lacks accurate species information, resulting in the omission of some low-abundance species. Thus, we combined the two methods to accurately reflect the diversity and structural characteristics of AM fungi (Chaudhary et al., 2020). Both high-throughput sequencing and morphological identification results showed that N addition altered AM fungi community composition and reduced AM fungal alpha diversity.

RDA analysis showed that the community composition of AM fungi could be significantly affected by pH in the Korean pine plantation (Figure 5). Studies have reported that soil pH is one of the most important abiotic factors affecting the ecological distribution of AM fungi (Davison et al., 2021). As the N concentration increased, the abundance of *Glomus* gradually increased and was significantly higher in the HN treatment (Figure 4). The main reason for this phenomenon might be that *Glomus* showed better ecological advantages in acidic soils. Studies have shown that AM fungi alleviate the negative effect of nitrogen deposition on ecosystem functions (Kang et al., 2020). In the future, attention should be paid to the ecological functions of *Glomus* under the background of nitrogen deposition. Short-term HN treatment (80 kg N ha^{-1} $year^{-1}$) also reduced fungal diversity and changed community composition (Cao et al., 2020). This phenomenon is mainly due to the changes in phosphorus elements in the soil caused by N addition, causing plants to select AM fungi with high phosphorus uptake ability.

4.3 Effect of N addition on understory herb plants

Cases of reduced plant diversity caused by soil acidification induced by N deposition have been reported (Kimmel et al., 2020). In this study, we found that the diversity of understory herbaceous plants in forests also decreased with lower soil pH. Additionally, excess N can also create nutrient imbalances. Large inputs of exogenous N will break the original stable stoichiometry, causing nutrient imbalances in soil and plants, and leading to the loss of certain plant species. N deposition indirectly affects plant species diversity by changing the composition of soil microbial communities (Bobbink et al., 2010). This study also found that changes in understory plant diversity were linearly positively correlated with AM fungal diversity and AM fungal colonization rate (Figure 6),

which also verified the role of AM fungi in maintaining the stability of understory plants. In addition, long-term N application will significantly reduce plant fine root biomass, while a small amount of N application will increase plant root biomass (Frew, 2022). SEM clearly showed that AM fungal diversity was the strongest influencing pathway affecting herb diversity under N addition conditions (Figure 7), which further proved the ability of AM fungi to maintain understory herb diversity.

5 Conclusion

N addition in temperate Korean pine plantation ecosystems altered the AM fungal community structure, reduced the diversity of AM fungi and herbaceous plants, decreased soil pH, increased inorganic N concentration, and reduced soil available phosphorus content. We showed that N addition could change soil properties, the composition and diversity of soil AM fungi, and the AM fungal communities, which ultimately affected the distribution of soil aggregates. The above biotic and abiotic factors in the underground part affected plant communities and diversity. This paper showed the correlation between the changes of AM fungi and herbaceous plants under the background of N deposition and used SEM to prove the correlation. Future research should address issues such as soil acidification, biodiversity loss, and changes in ecosystem stability due to increased nitrogen deposition. Given the importance of AM fungi in ecosystems, future research should explore the pathways by which AM fungi regulate ecological functions under N addition in order to improve our understanding of the ecological functions of AM communities under conditions of global environmental change.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/, SRP426700.

Author contributions

All authors made significant contributions to this study. FS and GJ provided the conceptualization. FS and WW framed the methodology. WW and YF wrote and prepared the original draft. XW, RW, and XH conducted the review and editing. MZ and KL oversaw the project administration. FS was responsible for funding acquisition. 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.

The reviewer FF declared a shared affiliation with the author GJ at the time of review.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2023.1192267/full#supplementary-material

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