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The Effects of the Long-Term Application of Different Nitrogen Fertilizers on Brown Earth Fertility Indices and Fungal Communities

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Abstract: Soil fungi play a crucial role in soil microbes, the composition and variety of whose communities can be altered due to nitrogen constraints, thereby affecting the plant's development. This study aimed to investigate the relationship between the composition of soil fungi communities, fertility index, and the structure of soil fungal communities under varying nitrogen fertilizer conditions, using a long-term positioning test on the brown earth of Northeast China. It examined the impact of 31 years of applying of no fertilizer (CK, 0 kg N hm^{-2} a^{-1}), the single application of inorganic fertilizer (N_2, urea 135 kg N hm^{-2} a^{-1}; N_4, urea 270 kg N hm^{-2}·a^{-1}), the single application of organic fertilizer (M_4, pig housing fertilizer 270 kg N hm^{-2} a^{-1}), and mixed nitrogen fertilizer (M_2N_2, urea 135 N hm^{-2} a^{-1} + pig housing fertilizer 135 kg N hm^{-2} a^{-1}) on the fertility index and fungal community structure of brown earth. The findings indicated the following: Long-term non-fertilization and the single application of chemical nitrogen fertilizer reduced the soil pH value and increased the soil bulk density. The application of organic fertilizer reduced soil bulk density and slowed down the reduction of soil fungal richness caused by nitrogen fertilizer application. The long-term application of different nitrogen fertilizers did not alter the dominant fungal phylum, showing that the dominant phylum in all treatments was Ascomycota. The pH, organic matter, total phosphorus, available phosphorus, total nitrogen, alkaline nitrogen, and available potassium were the main soil factors affecting the structural diversity of soil fungal communities. Total phosphorus explained the greatest differences in soil fungal communities.

Keywords: brown earth; different nitrogen fertilizer; soil fertility; fungal diversity; high-throughput sequencing

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1. Introduction

Nitrogen is a crucial element influencing agricultural yield. Enhancing nitrogen levels can augment the nitrogen required for plant development and boost soil fertility. In China, there is a significant need for nitrogen fertilizer in agriculture, with annual consumption hitting 30 million tons, representing roughly 30% of global nitrogen fertilizer use [1]. Nonetheless, the use of nitrogen fertilizer in China is unjustified, with the issues of high application and low conversion rates being particularly alarming [2]. Resource wastage can result in soil hardening and salinization [1]. Moreover, the overuse of nitrogen fertilizer hinders crop formation and ongoing production [3], pollutes the soil and the environment [4,5], diminishes soil microbial diversity, inhibits resistance to diseases and

insects, and heightens the risk of diseases and pests. Besides, it substantially raises soil H^+ concentration and hastens soil acidification [6,7]. Since chemical nitrogen fertilizer is the sole nitrogen source, it does not supply all essential nutrients for crop growth, leading to early nutrient loss and late-stage crop fertility [8], the diminution of soil compaction, and an increase in soil porosity [9].

Unlike the single application of chemical nitrogen fertilizers, the use of organic fertilizers significantly improves soil nutrients. This leads to a large increase in soil nitrogen, phosphorus, and potassium. Moreover, utilizing organic fertilizers also enhances the fertility, as well as the physical, chemical, and biological characteristics of the soil, consequently boosting crop production [10–15]. Furthermore, within the soil microbes, soil fungi are important decomposers of farmland ecosystems and are widely distributed in soils, maintaining soil fertility and farmland ecosystem stability, and reflecting whether the soil is degraded [16]. Their degradation ability is stronger than that of bacterial [17], and they provide nutrients for plant growth [18]. At the same time, soil fungi are also closely linked to soil-borne diseases, crop growth, and organic matter decomposition, and can be used as a measure of soil quality [19]. Human agricultural activities affect soil fungi, of which fertilization is particularly important. Soil fungal communities are sensitive to nitrogen input [20]. Prolonged nitrogen utilization leads to soil acidification, reduced fungal diversity in the soil, and altered fungal composition [21], elevating pathogenic bacteria levels, and heightening soil disease risks [22]. In addition, different methods of nitrogen application have different effects on soil fungal communities [23], which will lead to differences in soil microbial structure and diversity [24]. For instance, prolonged nitrogen utilization typically results in a uniform soil or root endophytic fungal community in crops [25], shifting the soil from a "bacterial type" to a "fungal type" [26]. Consequently, it is important to conduct long-term experiments on different fertilizer treatments affecting soil properties and the soil fungal community, as they can induce relatively stable differences among different soil fertility conditions affected by the long-term application of different fertilizer treatments.

Based on the above, we explore the effects of different long-term nitrogen fertilizers with different forms of nitrogen—a single chemical nitrogen fertilizer, a single organic fertilizer, a chemical nitrogen fertilizer, and an organic fertilizer—on soil fertility conditions and soil fungal community diversity. A 31-year long-term positioning test station on brown earth in the Shenyang Agricultural University of Northeast China was selected to investigate the following: (1) the difference and relationship between the main fertility properties of brown earth under different treatments; (2) the soil fungi composition and diversity of brown earth after over 30 years of fertilizer application; (3) the relationship between the fertility index and the fungi diversity of brown earth.

2. Materials and Methods

2.1. Study Site

The tested soil samples were collected in the long-term positioning test station (41.82° N, 123.57° E) of brown earth in the Shenyang Agricultural University of Northeast China (Figure 1). This site was flat with an elevation of 75 m and a northern temperate monsoon climate, showing cold and dry winter conditions and concentrated high temperature and rainy summer conditions. The average annual temperature was 6.2–9.7 °C and the annual precipitation was 600–800 mm. The annual frost-free period was 155–180 days. Precipitation was concentrated in summer. The local soil type was classified as brown earth (Hapli-Udic Alfisol according to the U.S. soil taxonomy), which is developed from loess-like parent material, with no hydrochloric acid reaction in the whole profile. The crop was continuous corn (varieties commonly used locally). Sown around 25 April and harvested around 25 September each year. The basic physical and chemical characteristics of the soil in 1987 were as follows: an organic matter content of 15.6 g kg^{-1}, a total nitrogen content of 1.0 g kg^{-1}, a total phosphorus content of 0.5 g kg^{-1}, a total potassium content of 21.56 g kg^{-1}, an alkaline nitrogen content of 67.4 mg kg^{-1}, an available phosphorus content

of 8.4 mg kg^{-1}, an available potassium content of 99.7 mg kg^{-1}, a pH value of 6.39, a bulk density of 1.22 g cm^{-3}. The tested site area is 9.6 m long, 7.2 m wide, and has an area of 69 m^2, with three replications for each site of fertilizer application treatment.

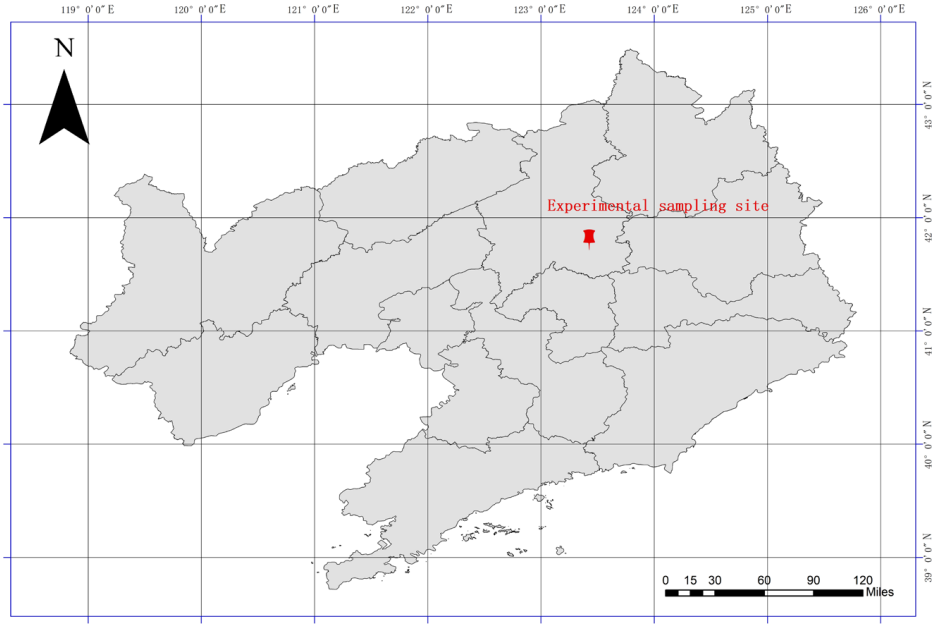

Figure 1. Study site location in the Liaoning Province of China.

2.2. Sample Collection

In this study, the topsoil (0–20 cm) samples were selected on 30 July 2018, from the treatments of a single application of chemical nitrogen fertilizer (urea 135 kg N hm^{-2} a^{-1}, noted as N_2 in this study, and urea 270 kg N hm^{-2} a^{-1}, noted as N_4), chemical nitrogen fertilizer with organic fertilizer (urea 135 kg N hm^{-2} a^{-1} + pig manure 135 kg N hm^{-2} a^{-1}, noted as M_2N_2), a single application of organic fertilizer (270 kg N hm^{-2} a^{-1}, noted as M_4), and a control (no fertilizer, noted as CK). Fertilizers are shown in Table 1. After removing impurities, parts of those samples were air-dried and passed through a 2 mm sieve for the determination of soil properties and other fresh samples were put in a dry-ice box to extract soil DNA and measure soil fungal diversity using high-throughput sequencing technology.

Table 1. Nitrogen application amount.

Treatment	Fertilizer Amount/kg N hm^{-2} a^{-1}
CK	0
N_2	135 (Urea)
N_4	270 (Urea)
M_2N_2	135 (Urea) + 135 (Organic fertilizer)
M_4	270 (Organic fertilizer)

2.3. Test Methods

2.3.1. Determination of Soil Properties

Soil bulk density was determined by the cutting-ring method [27]. Soil pH value was determined by the potentiometer method (soil to water ratio 2.5:1), and Phs-3B type was selected by a pH-meter [28]. Soil total nitrogen content was determined using an elemental analyzer (Vario MACRO cube, Langenselbold, Germany). The Alkaline nitrogen solution determination was performed using the NaOH diffusion plate method [29]. Total phosphorus was determined by the specific method of NaOH molten molybdenum antimony [30]. A wavelength of 700 nm was colorimetric, with contrast absorbance. Available phosphorus was determined by the molybdenum antimony anti-colorimetric method [31].

Total potassium was determined using a NaOH melting flame photometer method [32]. Available potassium was determined using a NH_4OAc flame photometer method [33]. Soil organic matter was determined by soil organic carbon measured by an element analyzer (Elementar Vario EL III, Hanau, Germany) multiplied by the coefficient of 1.724 [29].

2.3.2. Determination of Soil Fungal Diversity

Fungal DNA extraction and ITS high-throughput sequencing: the total DNA of the samples to be tested was extracted according to the instructions of E.Z.N.A.® soil DNA kit (Omega Bio-tek, Norcross, GA, USA), and the extracted DNA was detected using NanoDrop 2000 (Thermo Fisher Scientific NanoDrop2000, Waltham, MA, USA). Random preexperiments on representative samples ensured that the vast majority of samples were able to amplify products at appropriate concentrations [34]. PCR products were amplified and recycled [35], the recovered products were purified, washed down with Tris-HCl, and 2% agarose was detected by electrophoresis. Then, detection quantification was performed. Purified and amplified fragments were constructed in the Library (PE 2 × 300) using standard operating procedures from the llumina MiSeq platform (llumina, San Diego, CA, USA). Sequencing used the llumina platform (all operations were performed by Meji Biomedical Technology Co., Ltd., Shanghai, China). The raw data were unloaded to the NCBI SRA database (BioProject ID: PRJNA1114458).

2.4. Data Processing

Primitive sequences were processed using Trimmomatic and low-quality reads were removed with Readfq, and high-quality reads were sewn up with FLASH (Fast Length Adjustment of Short reads, CCB, USA). Each sequence had a taxonomy with an RDP taxonomic description aligned to the database Unite8.0/ITS_fungi, with the alignment taxonomic confidence threshold set at 70% and community composition calculated for each sample at taxonomic levels. The alpha diversity index was calculated based on OTU analysis using analysis software mothur [36].

Statistical analysis of various soil fertility properties was performed by univariate analysis of SPSS 19 software (SPSS, Chicago, IL, USA). Mean ± standard error and significant difference were obtained for three replicates for each treatment. RDA analysis was performed by Canoco4.5 (Shanghai Cabe Information Technology Co., Ltd., Shanghai, China), and correlation analysis and variance decomposition were performed by R software (R version 4.3.3) [37].

The Chao1 index is calculated as follows:

$$S_{chao1} = S_{sobs} + \frac{n_1(n_1 - 1)}{2(n_2 + 1)}$$

where S_{chao1} = the estimated OTU number; S_{Sobs} = the number of actual observed OTU; n_1 = number of OTUs containing only one sequence (such as "singletons"); n_2 = number of OTU with only two sequences (such as "doubletons").

The Shannon Index is calculated as follows:

$$H_{shannon} = -\sum_{i=1}^{S_{sobs}} \frac{n_i}{N} \ln \frac{n_i}{N}$$

where Sobs = actual number of OTU; n_i = the number of sequences contained in the i-th OTU; N = the number of all of the sequences.

3. Results and Analysis

3.1. Effect of Different Nitrogen Fertilizers on the Main Fertility Properties of Brown Earth

The total potassium content of all treatments decreased after the application of organic fertilizers (Table 2). And the higher the concentration of organic fertilizer, the slower the decline of total potassium content. Moreover, the single application of organic fertilizer for

31 consecutive years increased the total phosphorus, available phosphorus, and available potassium in the soil by 330%, 3850%, and 277%, respectively. The application of chemical nitrogen fertilizer or organic fertilizer significantly increased the content of alkaline nitrogen in the soil, and the application effect of chemical nitrogen fertilizer was stronger than that of organic fertilizer. The application of different nitrogen fertilizers increased the total soil nitrogen content, but the effect was not significant. After 31 years of the application of different nitrogen fertilizers, the pH value of each treatment showed different decreasing trends. Meanwhile, the decrease in soil pH value increased with the increase in the amount of chemical nitrogen fertilizer application. The organic matter content of soil treated with different nitrogen fertilizers increased at different levels. The application of organic fertilizer had a significant improving effect, while treatment without fertilization or with the single application of nitrogen fertilizer did not yield significant changes.

Table 2. Differences in soil chemical indices after different nitrogen fertilizer treatments.

Treatment	SOM g kg^{-1}	pH	BD g cm^{-3}	TN g kg^{-1}	TK g kg^{-1}	TP g kg^{-1}	AN mg kg^{-1}	AK mg kg^{-1}	AP mg kg^{-1}
CK	16.6 ± 0.28 c	5.9 ± 0.66 a	1.2 ± 0.26 a	1.3 ± 0.01 c	14.8 ± 0.10 b	0.5 ± 0.01 c	78.2 ± 4.70 c	34.6 ± 0.58 e	3.4 ± 0.58 e
N_2	16.0 ± 0.17 c	4.6 ± 0.35 b	1.2 ± 0.10 a	1.2 ± 0.09 d	14.7 ± 0.25 b	0.5 ± 0.01 c	167.3 ± 3.11 ab	40.0 ± 0.33 d	40.0 ± 0.58 d
N_4	16.2 ± 0.13 c	4.3 ± 0.26 b	1.2 ± 0.69 a	1.3 ± 0.01 c	16.5 ± 0.75 a	0.5 ± 0.02 c	180.4 ± 7.13 a	54.6 ± 0.58 c	53.3 ± 1.20 c
M_2 N_2	20.0 ± 0.25 b	4.7 ± 0.19 b	1.2 ± 0.12 a	1.6 ± 0.06 b	15.3 ± 0.38 ab	1.2 ± 0.06 b	178.5 ± 4.75 a	76.6 ± 1.15 b	73.0 ± 0.58 b
M_4	28.8 ± 0.40 a	6.3 ± 0.15 a	1.2 ± 0.61 a	2.0 ± 0.09 a	14.8 ± 0.13 b	2.2 ± 0.46 a	160.4 ± 1.61 b	128.3 ± 0.33 a	134.3 ± 1.20 a

Note: SOM, soil organic matter; BD, bulk density; TN, total nitrogen; TK, total potassium; TP, total phosphorus; AN, alkaline nitrogen; AK, available potassium; AP, available phosphorus. Different lower-case letters represent significant differences between nitrogen treatment conditions ($p < 0.05$, $n = 3$).

3.2. Effect of Different Nitrogen Fertilizers on Soil Fungal Community Structure and Diversity

3.2.1. Effect of Different Nitrogen Fertilizers on Diversity in Soil Fungi

After quality control, 728,335 optimized sequences were obtained. OTU clustering of non-repetitive sequences was removed during the clustering process, and 663 OTU representative sequences were obtained through alignment: one domain, four boundaries, 11 phyla, 30 classes, 70 orders, 145 families, 244 genera and 311 species. The Venn diagram reflected the number of species shared and unique species in multiple samples and could also intuitively show the degree of similarity between different samples [38]. As shown in Figure 2, there were eight phyla in different nitrogen fertilizer treatments. The species composition of soil fungi at different phylum levels was similar, with the largest number of soil fungi, 12 species in N_2 treatment and 13 species in N_4 treatment. The N_4 treatment contained one unique phyla, unclassified_k_Chromista. Different nitrogen fertilizer treatments contained 95 common bacterial genera. The CK treatment had 12 unique genera, the N_4 treatment contained 11 unique genera, the M_2N_2 treatment contained 7 unique genera, the N_2 treatment contained 4 unique genera, and the M_4 treatment contained 2 unique genera. Moreover, the number of fungal species with different nitrogen fertilizer treatment levels varied greatly. Compared with the control, the different nitrogen fertilizer species were reduced.

With a coverage index exceeding 99.7% for the tested samples, it is evident that these samples accurately represented the variety of soil fungi. The α diversity indices show that (Table 3), in contrast to the control group, various nitrogen fertilizer treatments markedly decreased the Sobs index, suggesting that the prolonged use of diverse nitrogen fertilizers markedly lessened fungal diversity. In addition, the M_2N_2 treatment had the highest level of fungal richness, indicating that integrating organic and nitrogen fertilizers could mitigate the reduction in soil fungal diversity resulting from nitrogen fertilizer use [39].

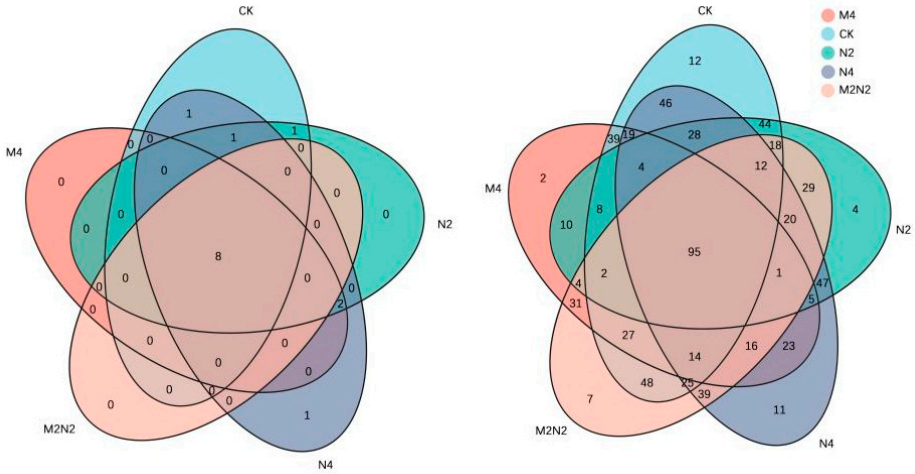

Figure 2. Venn analysis of soil fungi in phylum and genus level with different nitrogen fertilizer application. Note: the left panel shows the phylum level, and the right panel shows the genus level. CK, no fertilizer; N_2, single application of chemical nitrogen fertilizer (urea 135 kg N hm^{-2} a^{-1}); N_4, single application of chemical nitrogen fertilizer (urea 270 kg N hm^{-2} a^{-1}); M_2N_2, chemical nitrogen fertilizer with organic fertilizer (urea 135 kg N hm^{-2} a^{-1} + pig manure 135 kg N hm^{-2} a^{-1}), M_4, single application of organic fertilizer (270 kg N hm^{-2} a^{-1}).

Table 3. α diversity index in different nitrogen fertilizer treatments.

Treatment	Sobs	Shannon	Chao1	Coverage
CK	350 ± 10.48 a	2.91 ± 0.22 ab	436 ± 12.76 a	0.997 ± 0.0001 b
N_2	242 ± 27.51 b	2.83 ± 0.45 b	307 ± 44.69 b	0.998 ± 0.0002 ab
N_4	246 ± 36.45 b	3.26 ± 0.23 a	291 ± 36.71 b	0.998 ± 0.0002 ab
M_2 N_2	259 ± 22.64 b	2.64 ± 0.32 ab	343 ± 42.75 ab	0.998 ± 0.0003 ab
M_4	214 ± 3.53 b	2.09 ± 0.08 b	266 ± 5.43 b	0.998 ± 0.001 a

Note: different lower-case letters represent significant differences between nitrogen treatment conditions ($p < 0.05$, n = 3).

3.2.2. Effect of Different Nitrogen Fertilizer Applications on the Abundance and Composition of Soil Fungal Communities

In this study, 11 phyla were identified, merging with a relative abundance below 0.01% into additional ones, culminating in the discovery of five recognized phyla and two phyla whose taxonomic classifications remain unknown. There were five recognized phyla: *Ascomycota*, *Basidiomycota*, *Chytridiomycota*, *Mortierellomycota*, and *Mucoromycta*. Within this group, *Ascomycota* was predominant, with the prevalence of various nitrogen fertilizers exceeding 50%.

Furthermore, 246 genera were identified, merging with under 0.01% relative abundance into different ones, resulting in 17 recognized genera and 12 genera of indeterminate taxonomic status. A total of 12 genera of indeterminate taxonomic status were merged into the unclassified category to chart the genus level. The illustration (Figure 3) reveals a significant prevalence of genera: *Guehomyces* treated with CK showed a relative abundance of 28.97%, *Pseudogymnoascus* in the treatments of N_2 and N_4 accounted for 33.09% and 17.43%, respectively, and *Pseudaleuria* in the treatments of M_2N_2 and M_4 accounted for 24.92% and 36.67%, respectively. Among the 17 recognized genera, *Talaromyces*, *Chaetomidium* showed an increased trend when nitrogen was applied. In contrast to the control, the *Guehomyces* diminished with the addition of nitrogen fertilizer. With various nitrogen fertilizers at both phylum and genus levels, *Ascomycota* were revealed as the predominant flora in these treatments.

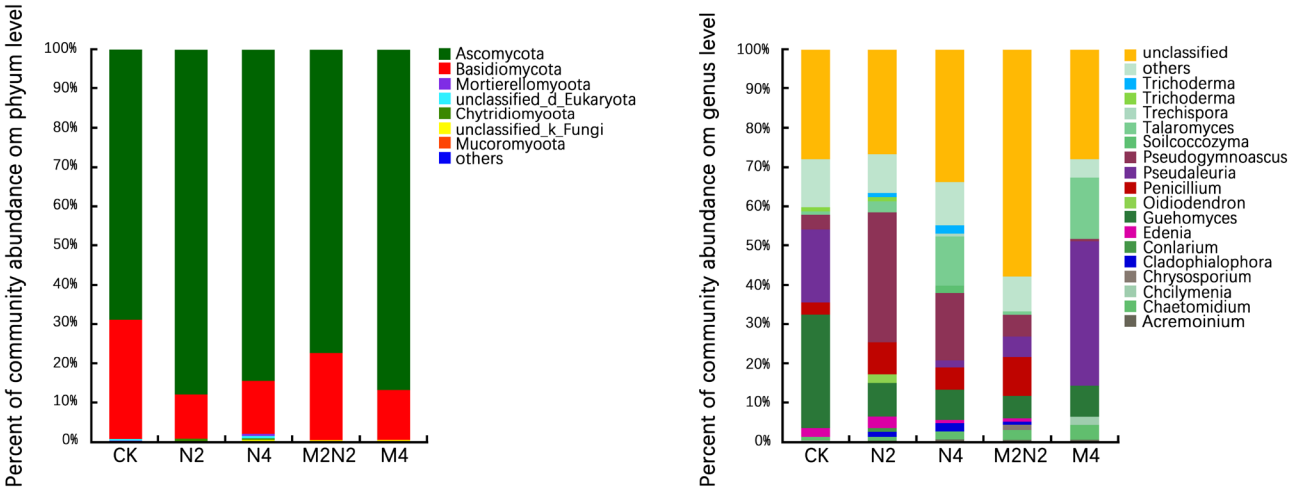

Figure 3. Composition of soil fungi community in different nitrogen fertilizer treatments. Note: CK, no fertilizer; N_2, single application of chemical nitrogen fertilizer (urea 135 kg N hm^{-2} a^{-1}); N_4, single application of chemical nitrogen fertilizer (urea 270 kg N hm^{-2} a^{-1}); M_2N_2, chemical nitrogen fertilizer with organic fertilizer (urea 135 kg N hm^{-2} a^{-1} + pig manure 135 kg N hm^{-2} a^{-1}), M_4, single application of organic fertilizer (270 kg N hm^{-2} a^{-1}).

3.3. Correlation Between the Fertility Properties of Brown Earth and the Fungal Community Structure

3.3.1. Effects of Soil Fertility Properties on Soil Fungal Structure

Table 4 shows the principal component analysis results, indicating a good significance with a p less than 0.01, except for total potassium and bulk density. In addition, the redundant analysis (RDA) of soil fungi and soil fertility properties (Table 5) shows that RDA 1 explained 37.09% of the variation, RDA 2 explained 15.07% of the variation, and the first two components jointly explained 52.16% of the variation. Visualized in Figure 4, the sharp angles between available phosphorus, available potassium, total phosphorus, total nitrogen, and organic matter indicate that they had a positive correlation and a certain synergistic effect. Moreover, pH had a great influence on the soil fungal communities' structural diversity. Total nitrogen, available phosphorus, and alkaline nitrogen had relatively little impact on the structural diversity of soil fungal communities.

Table 4. Principal component analysis of soil properties.

Soil Property	PC1	PC2	r^2	p
AN	0.8462	−0.5329	0.8805	0.001
TN	−0.1672	−0.9859	0.8255	0.001
AP	0.2089	−0.9779	0.6732	0.001
TP	−0.1914	−0.9815	0.6729	0.003
AK	−0.029	−0.9996	0.6598	0.002
SOM	−0.2439	−0.9698	0.6202	0.002
pH	−0.9518	−0.3067	0.6163	0.007
TK	0.5241	−0.8516	0.3669	0.073
BD	0.414	0.9103	0.0321	0.837

Note: The values of PC 1 and PC 2 in the table indicate the correlation between soil properties and sorting axis. The r^2 indicates the correlation coefficient between the environmental factors and the distribution of species. The p is the significance level. Note: SOM, soil organic matter; BD, bulk density; TN, total nitrogen; TK, total potassium; TP, total phosphorus; AN, alkaline nitrogen; AK, available potassium; AP, available phosphorus.

Table 5. Redundant analysis (RDA) of soil properties.

Soil Property	RDA1	RDA2	r^2	*p*
pH	−0.951	0.310	0.812	0.001
SOM	−0.977	0.211	0.596	0.004
TP	−0.999	0.043	0.534	0.005
AK	−0.988	0.154	0.513	0.030
TN	−0.985	0.173	0.406	0.037
AP	−0.994	0.112	0.227	0.233
AN	0.949	−0.316	0.181	0.333

Note: The values of RDA 1 and RDA 2 in the table indicate the correlation between soil properties and sorting axis. The r^2 indicates the correlation coefficient between the soil properties and the distribution of species. The *p* is the significance level. Note: SOM, soil organic matter; BD, bulk density; TN, total nitrogen; TK, total potassium; TP, total phosphorus; AN, alkali nitrogen; AK, available potassium; AP, available phosphorus.

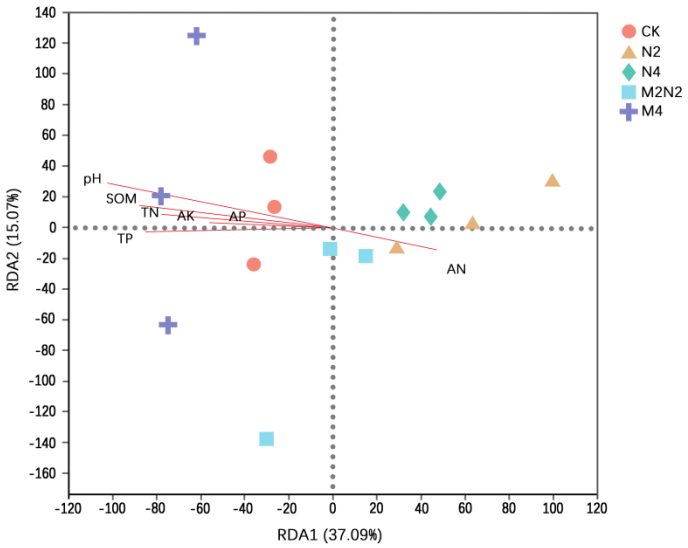

Figure 4. Redundancy analysis between soil fungi at genus level and the physical and chemical properties in different nitrogen fertilizer treatments. Note: SOM, soil organic matter; BD, bulk density; TN, total nitrogen; TK, total potassium; TP, total phosphorus; AN, alkaline nitrogen; AK, available potassium; AP, available phosphorus. CK, no fertilizer; N_2, single application of chemical nitrogen fertilizer (urea 135 kg N hm^{-2} a^{-1}); N_4, single application of chemical nitrogen fertilizer (urea 270 kg N hm^{-2} a^{-1}); M_2N_2, chemical nitrogen fertilizer with organic fertilizer (urea 135 kg N hm^{-2} a^{-1} + pig manure 135 kg N hm^{-2} a^{-1}), M_4, single application of organic fertilizer (270 kg N hm^{-2} a^{-1}).

3.3.2. Network Effects of Main Fertility Properties on Soil Fungi

To investigate the link between species and environmental factors, the network map was created using the highest 30 taxonomic levels (that is, those with a relative abundance exceeding 0.01%) and their respective environmental factors [40]. Calculations of Spearman's rank and correlation coefficients were performed to represent the interrelations among species and between species and their environmental factors. Figure 5 shows the network results between soil properties and genus-level fungi, with red lines indicating a positive correlation and green indicating a negative correlation. The thicker line reflected more closely to the species, and the node with an increased number of lines signified a tighter connection. Furthermore, the pH was linked to 25 different genus nodes, showing a positive association with 6 species at the genus level and 19 species at the same level. The species correlation analysis of fungi indicated a significant positive linkage between pH and Holtermaniella, and a pronounced negative association with Cladophialophora. Organic matter showed the most significant positive association with Holtermaniella and the most pronounced negative association with Penicillium. Total phosphorus exhibited the most significant positive correlation with Holtermanniella and the most pronounced

negative correlation with Trichocladium. Moreover, a great positive association was found between available potassium and g-unclassified-c-Agaricomycetes, and a significant negative association with Trichocladium. Holtermanniella gained prominence due to the positive correlation with fertility properties, whereas Trichocladium showed a significantly negative correlation with soil fertility properties. Specifically, pH, organic matter, phosphorus, and potassium elevated the relative abundance of Holtermanniella, while simultaneously reducing that of Trichocladium.

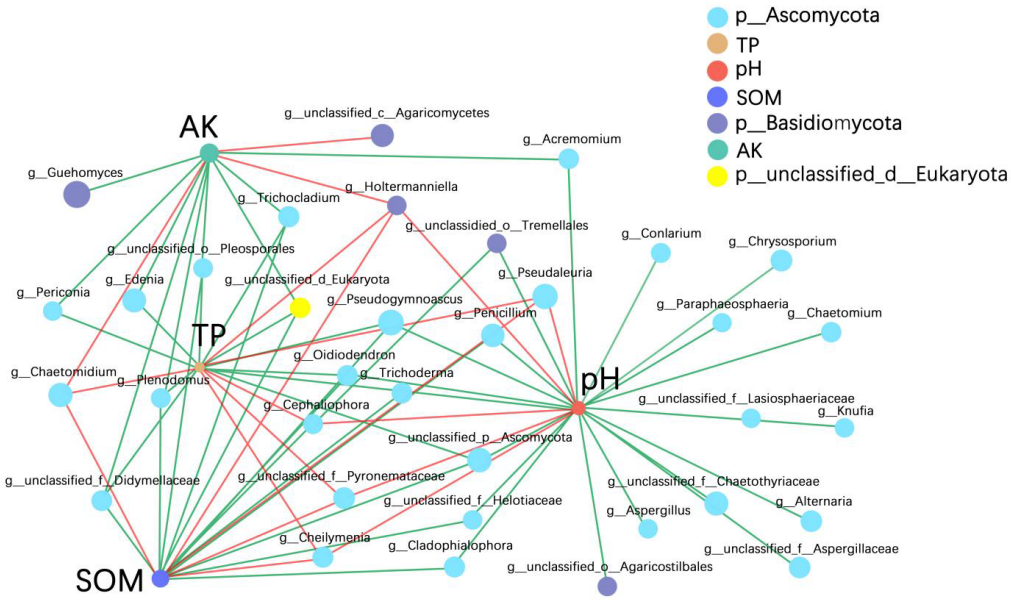

Figure 5. The network analysis of soil physical and chemical properties and genus-level fungi. Note: the species with a *p* less than 0.05 are not visualized in the network. SOM, soil organic matter; BD, bulk density; TN, total nitrogen; TK, total potassium; TP, total phosphorus; AN, alkaline nitrogen; AK, available potassium; AP, available phosphorus. Red lines indicate a positive correlation, and green lines indicate a negative correlation.

SOM links to 19 nodes, showing a positive correlation with 5 species at the genus level and a negative one with 14 species. TP has a connection to 17 nodes, showing a positive correlation with 6 species at the genus level, a negative correlation with 11 species at the same level. AK has a connection with 12 nodes, and a positive correlation with three species and nine species at the genus level.

3.4. FUN Guild Prediction of Fungal Function by the Long-Term Application of Different Nitrogen Fertilizers

FUNGuild (Fungi Functional Guild) is a taxonomic analysis of fungal communities by microecological guild [41], which is a concept in microecology. FUNGuild fungal function prediction is derived from the current published literature or authoritative website data. The fungi are divided into the following three categories: (1) pathological trophic type; (2) symbiotic trophic type; (3) saprophytrophic type. The fungal lifestyles of these three nutrient types are as follows: (1) the destruction of host cells, including the phagocytic fungus phagotrophs; (2) material exchange with the host cells; (3) the degradation of dead host cells to obtain nutrients. Based on these three nutritional types, our results about fungi were further subdivided into 15 guilds, i.e., arbuscular mycorrhizal fungi, rhododendron mycorrhizal fungi, ectomycorrhizal fungi, animal pathogens, plant pathogens, lichen parasitic fungi, lichen symbiotic fungi, leaf endophytes, undefined root endophytes, undefined saprophytic fungi, wood saprophytic fungi, and three types of special fungi of yeast, facultative yeast, and thallus. In the different nitrogen fertilizer treatments of our study, most soil fungi were Animal Pathogen-Soil Saprotroph, Animal Pathogen-Dung-Saprotroph-Endophyte-Epiphyte-Plant, Saprotroph-Wood Saprotroph, and Undefined

Saprotroph. The relative abundances of these saprotrophic fungi were greater than 50% in each treatment (Figure 6).

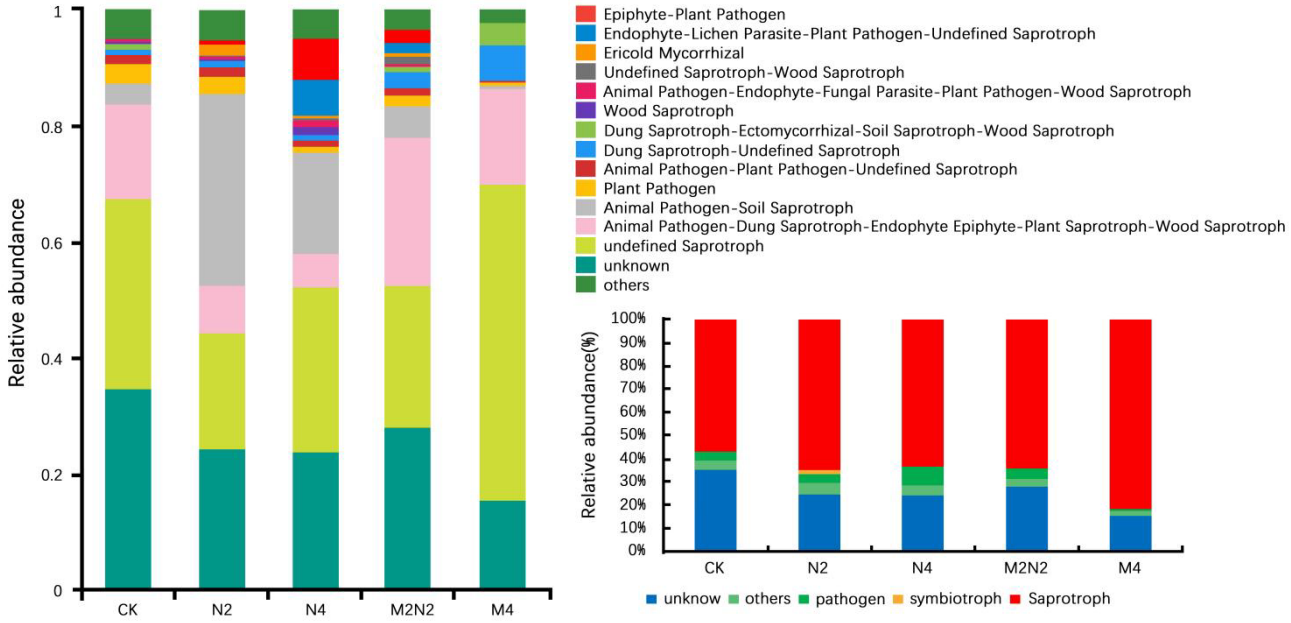

Figure 6. Soil fungi function prediction in different nitrogen fertilizer treatments classified by the FUNGuild system. Note: The abscissa represents the different nitrogen fertilizer treatments, and the ordinate indicates the proportion of microbial community abundance. CK, no fertilizer; N_2, single application of chemical nitrogen fertilizer (urea 135 kg N hm^{-2} a^{-1}); N_4, single application of chemical nitrogen fertilizer (urea 270 kg N hm^{-2} a^{-1}); M_2N_2, chemical nitrogen fertilizer with organic fertilizer (urea 135 kg N hm^{-2} a^{-1} + pig manure 135 kg N hm^{-2} a^{-1}), M_4, single application of organic fertilizer (270 kg N hm^{-2} a^{-1}).

4. Discussion

4.1. Effect of Long-Term Application of Different Nitrogen Fertilizers on Soil Fertility

The single application of chemical nitrogen fertilizer (N_2, N_4) reduced the soil pH, because the hydrolysis and nitrification of urea could produce NO^{3-}, which combined with H^+ in the soil, leading to the decrease of soil pH [42]. After 31 years of these treatments, the soil pH with the treatment of organic fertilizer plus chemical fertilizer could be lower than that of single chemical fertilizer, indicating that organic fertilizer enhanced soil buffering and slowed down the soil acidification process [43]. Moreover, total nitrogen content showed an increasing trend due to the long-term addition of organic fertilizer. This could be due to the fertility release of organic fertilizer, which is relatively slow and could effectively provide a nitrogen supply for the soil for a long time [44]. Compared with CK treatment, the content of total phosphorus in different nitrogen fertilizer treatments except N_2 were increased. Meanwhile, it was highest in the M_4 treatment due to the organic fertilizer containing a large number of nutrients and microorganisms, which increased the content of total phosphorus in the soil [45]. And the content of total potassium in different fertilization treatments decreased, with the lowest and highest in the M_4 treatment. Generally, the total potassium content of soil was mainly related to the parent matter and clay minerals [46]. In this study, there was no potassium fertilizer input, and the parent material of the brown earth was loess-like, in which there should generally have evolved 2:1 clay minerals such as illite, montmorillonite, and vermiculite, and strong potassium supply potential [47]. Thus, the available potassium content was mainly affected by total potassium and the addition of fertilizer. After the application of organic fertilizer, the fixation of clay minerals would be reduced to potassium, and the organic acid produced in the decomposition process would promote the release of mineral potassium, increasing the potassium content of the

soil [48,49]. During the treatments, the content of available potassium with the single application of chemical nitrogen fertilizer decreased mostly, showing that these treatments should lack exogenous potassium to limit plant growth. Moreover, the organic matter content with the single addition of chemical nitrogen fertilizer could be increased due to its promotion of plant production, thereby the root would be returned to form more organic matter [49]. This should be similar with our treatment of N_4, compared to CK treatment. In conclusion, organic fertilizer can compensate for some of the defects of applying nitrogen fertilizer alone.

4.2. Effects of Long-Term Application of Different Nitrogen Fertilizers on the Structure and Function of Soil Fungal Community

In this study, the dominant fungi phyla were all *Ascomycota*, a type of saprophytic fungus, which is known to break down soluble materials in the soil [50]. The rapid growth of *Ascomycota* might be strongly influenced by nitrogen content [51,52]. A greater quantity of *Ascomycota* was observed when treated with a single nitrogen fertilizer compared to organic fertilizer, suggesting that chemical nitrogen fertilizer more effectively stimulated *Ascomycota* [52]. As is known, *Ascomycota* induced rot in roots, stems, ears, and deceased branches [53]. Under the condition of low concentration of nitrogen fertilizer, and treatment with organic fertilizer combined with nitrogen fertilizer, the soil had the lowest relative abundance of *Ascomycota.* And organic fertilizer and nitrogen fertilizer had the lowest probability of soil fungal diseases. Thus, utilizing various nitrogen fertilizers could enhance the organic matter in the soil and boost the proportional presence of *Ascomycota*. The concentration of fungi in the soil varied along with the prevalent species, in which *Chaetomidium*, *Guehomyces*, and *Pseudogymnoascus* were the primary fungi species. *Chaetomidium* and *Guehomyces* showed different sensitivities, indicating that the former proliferated while the latter diminished when nitrogen fertilizer was added [54].

In addition, based on the FUNguild prediction in this study, the majority (exceeding 50% in quantity) of soil fungi nutritional types were Animal Pathogen-Soil-Saprotroph, Animal Pathogen-Dun-Saprotroph-Endophyte-Epiphyte-Plant, Saprotroph-Wood Saprotroph, and Undefined Saprotroph saprophytic trophic fungi. This indicated that soil-stable accessible carbon sources might fall short for soil fungal life needs, although saprophytic fungi had the capacity to decompose organic material in the soil. This would lead to a higher likelihood of engaging in life activities, thereby increasing the saprotrophic fungi amount [55]. Consequently, the number of saprotrophic fungi could be enriched with organic fertilizer, in this case, M_4 treatment in this study was the greatest. Furthermore, the treatment of N_4 resulted in the greatest concentration of pathography, suggesting that an overabundance of nitrogen might contribute to the physiological function of plant roots, resulting in harmful fungal proliferation [56]. So, we should be aware that excessive nitrogen fertilizer will increase the content of soil pathogens and cause crop diseases.

4.3. The Effects of Long-Term Application of Different Nitrogen Fertilizers on Soil Fungal Diversity

Studies have indicated that soil fertility properties have the potential to affect the community composition of soil fungi [57,58]. It had been revealed that soil fungi community composition was primarily influenced by pH and organic substances [59,60]. Meanwhile, pH could affect soil nutrient availability, and subsequently alter soil fungal populations. The composition and spread of soil fungi are governed by the organic matter in the soil as well, to a certain degree [61]. Organic matter, serving as the primary material and energy source for the majority of soil fungi, significantly affects the community composition of soil fungi [61]. Moreover, the use of fertilizers markedly alters the soil fungi, making soil fungi vulnerable to these changes. Utilizing organic fertilizers could diminish the presence of harmful fungi species in the soil [62]. Soil total phosphorus and available phosphorus markedly enhance the abundance of ectomycorrhizal fungi and plant pathogens [63]. Nonetheless, the interpretation of fungal communities is minimally influenced by soil organic matter, due to the abundant organic matter in various nitrogen

fertilizers [64], offering ample carbon sources for fungal life. In contrast to the control treatment, there was a notable reduction in the Sobs index with the addition of nitrogen fertilizers, suggesting a substantial decrease in soil fungal diversity over time [65]. The reason for this should be the extended duration of nitrogen application in this study, offering a more accurate representation of the impact of prolonged nitrogen utilization on soil [25].

Furthermore, despite having the highest Sobs index, this study revealed that using both organic and nitrogen fertilizers could decelerate the reduction in soil fungal diversity resulting from nitrogen fertilizer application. Compared to the control treatment, the Shannon index exhibited the greatest reduction in the treatment of M_4, while in N_4 treatment it rose marginally. This should be consistent with the result that there was no noticeable influence on the black soil after 34–35 years of nitrogen fertilizer application [66]. Nonetheless, nitrogen application might diminish soil fungus types; the soil's nitrogen build-up in this study was within the soil fungi's tolerance spectrum [67], therefore not greatly affecting fungal diversity. Generally, the long-term application of combined nitrogen fertilizers primarily influenced the soil fungi's community composition by altering the soil's physical and chemical characteristics. Soil pH and organic matter should be the key properties impacting the soil fungal population.

5. Conclusions

This study investigated the effects of the long-term application of different nitrogen fertilizers on brown earth fertility properties and fungal communities based on an over 30-year fertilization site of brown earth in Northeast China. It showed that the combination of chemical nitrogen fertilizer and organic fertilizer was the most beneficial treatment for alleviating soil acidification, increasing the content of alkaline nitrogen, and alleviating the decrease of microbial richness caused by nitrogen fertilizer application. After applying combined nitrogen fertilizer, soil fungi were mostly saprophytic fungi. With the increase in the amount of chemical nitrogen fertilizer, the content of soil–plant pathogens should also be increased. Therefore, the high-concentration application of nitrogen fertilizer should be avoided in agricultural production and the combined application of organic fertilizer and chemical nitrogen fertilizer will have a better effect. In the future, it will be necessary to conduct research on the functions and metabolic pathways of the dominant soil fungi so as to develop more professional agricultural fertilization programs.

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