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Influence of inorganic fertilizer and organic manure application on fungal communities in a long-term field experiment of Chinese Mollisols

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ABSTRACT

The effects of 35 years of manure amendment on fungal communities were evaluated in Mollisols of northeast China. Soil samples from different fertilization regimes were collected, and quantitative PCR analysis of fungal community size and Illumina platform based analysis of the ITS gene were performed to characterize soil fungal abundance and compare community structure and diversity. The treatments were no fertilizer (CK); manure (M); nitrogen, phosphorus and potassium inorganic fertilizer (NPK); and inorganic fertilizer plus manure (MNPK) regimes. Soil fungal diversity was decreased by inorganic fertilizer. Inorganic fertilizer plus manure induced a weak increase in fungal diversity and a slight decrease in size. The predominant phyla were Ascomycota (63.77–78.70%), Zygomycota (8.33–14.80%) and Basidiomycota (4.03–13.47%). At each taxonomic level, the percentages dramatically differed, especially between MNPK and NPK. For example, the numbers of *Fusarium* and *Gibberella* with potential pathogenicity were all higher in NPK than in MNPK; the beneficial genus *Podospora* was the highest in MNPK and the least in NPK. Principal coordinates analysis showed that CK and M were clustered together; the incorporation of NPK with manure improved the fungal structure near to that of CK and separate from that of NPK. Redundancy analysis indicated that fungal community structure was most affected by soil available phosphorus (AP) and organic matter contents, followed by soil pH. Simpson and Shannon indices were closely correlated with soil pH, AP, total phosphorus and total nitrogen. The results indicated that manure application altered soil properties and soil fungal community structure, and manure with inorganic fertilizer counteracted some of the adverse effects of the inorganic fertilizer.

1. Introduction

One gram of soil can contain up to 200 million fungal hyphae as well as up to one billion bacterial cells (Zhang et al., 2014). Microbial parameters are potentially valuable indicators of soil quality. Microorganisms can rapidly respond to changing environmental conditions by modifying microbial biomass and community composition (Krashevska et al., 2015). Recent studies using a variety of molecular approaches have explored how soil microbial community distributional patterns were determined by environmental factors (Thiele-Bruhn et al., 2012; Yu et al., 2015). Soil microbial communities are greatly affected by anthropogenic activities such as agricultural intensification and long-term fertilization. Fertilization is an indispensable agricultural practice for enhancing plant nutrition and achieving high yield, and this also changes the soil environment. Inappropriate fertilization can cause a series of problems, for example, inorganic nitrogen (N) fertilizer decreases microbial respiration rates, lowers extracellular enzyme activity, depresses soil microbial activity (Ramirez et al., 2012) and reduces bacterial diversity (Zhou et al., 2015). Organic amendments, from a variety of sources including agriculture and forestry and urban areas, combined with inorganic fertilizer is one effective way to solve problems caused by inorganic fertilizer, and it is commonly stated that organic amendments from different sources can improve soil biochemical and biological properties (Intanon et al., 2015; Jorge-Mardomingo et al., 2013). Inorganic

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fertilizer with organic amendment affects the structure of farmland bacterial communities (Bonilla et al., 2012; Abubaker et al., 2013; Zhu and Zhu, 2015). In short-term fertilizer experiments, no obvious effect on microbial community occurred (Marschner et al., 2001); however, in long-term fertilizer experiments, the community structure, population and function of soil microorganisms were affected (Cinnadurai et al., 2013). Fungi strongly influence ecosystem structure and function, and are vital components of soil microbial communities with a series of important roles, such as parasitism, decomposition, pathogenesis and symbiosis (Altieri, 1999; Neher, 1999; Van der Putten et al., 2007; Aguilar-Trigueros et al., 2014). Previous reports have shown N fertilization can decrease fungal biomass (Wallenstein et al., 2006) and diversity (Edwards et al., 2011), and alter fungal communities (Paungfoo-Lonhienne et al., 2015). Compared with bacteria, fungi have a stronger ability for N and phosphorus (P) acquisition (Wu, 2011). Zhou et al. (2016) found N fertilizer and N plus P fertilizers could increase the size of fungi, reduce fungal biodiversity and change community composition. Mineral fertilization decreased the development of external mycelium of arbuscular mycorrhizal fungi, while organic fertilization increased it (Gryndler et al., 2006). Although soil chemical and physical parameters show few differences, soils treated with bioorganic fertilizer exhibit greater biological activity and more mycorrhizae than soils treated exclusively with mineral fertilizer (Mäder et al., 2002). The application of organic fertilizer not only changed the composition and abundance of fungal communities, but also significantly reduced the *V. dahliae* population and *Verticillium* wilt disease symptomsin arhizosphere soil (Lang et al., 2011). Although effects of long-term fertilization on abundance and diversity of mycorrhizal fungi have been described in detail (Bradley et al., 2006; Lin et al., 2012), there are few reports concerning inorganic amendment effects on fungal communities in bulk Black soils of China. Investigation on the impact of long-term fertilization on the structure of soil fungal communities, especially the combined application of organic and inorganic fertilizers, will help our understanding of the changes in Black soil quality.

Black soils are a vital soil resource for grain production in China, and are referred to as Mollisols (Xing et al., 2005). In the next 10–15 years, the production of the northeast grain area could increase by 20 million tonnes, representing 50% of the country's future increase (Song et al., 2014). Thus, it is important for China's food security. The long-term use of inorganic fertilizer caused a series of environmental problems in Black soil, for example, high inputs of chemical fertilizers result in serious degradation of soil quality (Yin et al., 2015), and over-use of N fertilizers leads to soil acidification and decreased soil bacterial diversity (Zhou et al., 2015). Soil organic amendments can improve soil biological properties and enzymatic activities (Scotti et al., 2015), and growth and diversity of soil microbial communities (Bulluck et al., 2002). Determining the changes in soil fungal activity, abundance and communities caused by long-term use of inorganic fertilizer and organic amendment would be conductive to maintaining and improving quality of Black soils. The results will provide a theoretical basis for replacement using organic fertilizer and reducing N fertilizer use. We used four different fertilizer treatments to explore their effects on soil properties and fungal community structure and diversity in soils.

2. Materials and methods

2.1. Site description and sample collection

The long-term (35 years) field experiment was located in Harbin, Heilongjiang Province, China (45° 40′N, 126° 35′E). This region has a typical monsoon climate, with a mean annual temperature of 3.5 °C, annual evaporation of 1315 mm and annual precipitation of 575 mm. This site had been in wheat–maize–soybean crop rotation since 1980, with soybean planted in 2014. This site has 96 plots (32 treatments and three replicates per treatment), with each plot of 9 m × 4 m (length × width). In the present study, four fertilization regimes were selected: no fertilization (CK); single manure (M); N, P and potassium (K) inorganic fertilizer (NPK); and N, P and K plus M (MNPK). Dosages of inorganic fertilizers were (all in kg ha^{-1}) 150 N, 75 P_2O_5 and 75 K_2O for wheat and maize plots; and 75 N, 150 P_2O_5 and 75 K_2O for soybean plots. N fertilizer was applied as urea, K fertilizer as potassium sulfate, and P fertilizer as calcium super phosphate and ammonium hydrogen phosphate. Horse manure was the organic amendment applied at approximately 18,600 kg ha^{-1} for all crops in M and MNPK treatments. The soybean growing period started in late April and ended in late September. In late September 2014 (at soybean harvesting stage), we selected three plots of each treatment (a total of 12 plots) for soil sampling.

Soil samples were collected from 10 points at a depth of 5–25 cm (plow layer), and pooled together in a sterile plastic bag for each plot. Soil samples were placed on ice and transported back to the laboratory. Each soil sample was mixed for homogeneity by passing through a 2-mm sieve, impurities were removed and then divided into two parts: one stored at −80 °C prior to biological analysis and the other naturally dried for physical and chemical analysis.

2.2. Analysis of soil properties

Soil pH was determined with glass electrode in a 1:1 (soil: water) suspension (Harter, 1983). Soil nitrate nitrogen (NN) and ammonium nitrogen (AN) were extracted by 2 M KCl solution and subjected to flow injection analysis (Raigón et al., 1992). Followed soil nutritional indices were measured using the methods described by Bao (2011). $NaHCO_3$ solution extraction and analysis by Mo-Sb colorimetric method was used to determine soil available phosphorus (AP). Soil available potassium (AK) was determined using neutral ammonium acetate extraction and analysis by flame photometry. Soil organic matter (OM) was measured using the $K_2Cr_2O_7$-capacitance method. Treating soil with sodium hydroxide solution, total phosphorus (TP) was measured by Mo-Sb colorimetric method and total potassium (TK) by atomic absorption spectrometer. Soil total nitrogen (TN) was determined by the micro-Kjeldahl method.

2.3. DNA extraction and quantitative PCR (qPCR) analysis

A Power Soil DNA isolation kit (MOBIO Laboratories Inc., Carlsbad, CA, USA) was used to extract soil total community DNA (TC DNA), according to the manufacturer's instructions, and increased an incubation step at 65 °C for 10 min (Fierer et al., 2012). To obtain sufficient DNA (whole community DNA via random sequencing, not just sequencing of specific, targeted genes) for the next analyses, we conducted six replicate extractions for each soil sample, and pooled these replicates. The TC DNA was verified by 1.0% agarose gel and using a Nano Drop ND-1000 UV–vis Spectrophotometer (Thermo Scientific, Rockwood, TN, USA) to evaluate DNA quality and concentration (A_{260}/A_{280}). All extracted soil DNA was stored at −80 °C until use.

The recovery of fungi was determined using a qPCR assay specific for the fungal ITS region of the ribosomal RNA gene with primers ITS4/ITS5 (ITS4: 5′-TCCTCCGCTTATTGATATGC-3′, ITS5: 5′-GGAAGTAAAAGTCGTAACAAGG-3′) (Schoch et al., 2012; Zhou et al., 2016). Quantification of the fungal ITS gene was performed by the ABI 7500 Real-Time PCR detection system (Applied Biosystems, Waltham, MA, USA) and the reaction mixture (20 μL) contained

2 × FastFire qPCR PreMix (FastFire qPCR PreMix, Tiangen Biotech, China), 1 × ROX Reference Dye, 1 μL of 1/10 diluted DNA and 10 nM of each primer. Optimized conditions for amplification were as follows: a preliminary denaturation at 95 °C for 1 min, 40 cycles at 95 °C for 5 s and 60 °C for 32 s, followed by melting curve analysis. The standard curve was generated using 10-fold serial dilutions of a plasmid containing the ITS gene insert. All qPCRs were performed in triplicate.

2.4. Illumina Miseq platform sequencing and processing the sequence data

The set of primers ITS1F/ITS2 (Buee et al., 2009; Smith and Peay, 2014) (ITS1F: 5′-CTTGGTCATTTAGAGGAAGTAA-3′, ITS2: 5′-GCTGCGTTCTTCATCGATGC-3′) was used to amplify the fungal ITS1 region. The two primers were tagged with adapter, pad and linker sequences. We added a typical barcode sequence (12 mer) to every sample reverse primers, so that one run of Illumina Miseq sequencing allowed for pooling of multiple samples. PCR amplification conditions were 2 min at 94 °C, followed by 30 cycles at 94 °C for 30 s, 55 °C for 30 s and 72 °C for 30 s, and final elongation for 5 min at 72 °C. PCR products were purified and combined in equimolar ratios with the quantitative DNA binding method in order to create a DNA pool that was further used for sequencing. Following the manufacturer's instructions, the DNA library was sequenced using Illumina MiSeq platformat Personalbio (Shanghai, China).

Using the Quantitative Insights into Microbial Ecology (QIIME) toolkit (Caporaso et al., 2010) (version 1.7.0, http://qiime.org/) to process raw sequence reads. Barcodes were removed from the sequences, and any sequences < 150 bp or with ambiguous bases were removed. After removal of chimeras, operational taxonomic units (OTUs) were generated. OTUs were defined by clustering at 97% similarity. Final OTUs were taxonomically classified using blast against the UNITE database (Release 5.0, http://unite.ut.ee/index.php) with a minimal 80% confidence estimate (Bokulich and Mills, 2013; Liu et al., 2015b). All sequences were deposited in the National Center for Biotechnology Information (NCBI) Short Reads Archive database (SRP062512).

Fungal α-diversity was evaluated using Mothur (Schloss et al., 2009) (version 1.31.2, http://www.mothur.org/), following four metrics: diversity indices (Shannon and Simpson) and richness indices [Chao1 and the abundance-based coverage estimator (ACE)]. Patterns of sample dissimilarity were shown by principal coordinate analysis (PCoA) (Marsh et al., 2013). Based on the Weighted Fast UniFrac distances between the samples, coordinates were used to draw graphical outputs of PCoA.

2.5. Statistical analysis

Statistical effects were calculated using analysis of variance, and means segregated by Tukey's multiple comparison test at $P < 0.05$ (Vargas-Gastélum et al., 2015). The relationships between soil properties with fungal relative abundance and diversity indices were tested by Pearson's correlation coefficient analyses (Ramirez et al., 2012). The above two analyses were performed with SPSS for Windows (SPSS, v.19, Chicago, IL, USA). The correlation of multiple variations between environmental factors and community composition was shown by a redundancy analysis (RDA) using CANOCO 5.0 (Zhou et al., 2015). The manual forward-selection procedure was used in the RDA to determine significance of environmental variables ($P < 0.05$) using a Monte Carlo test with 499 permutations.

3. Results

3.1. Soil properties and soybean yield

Physicochemical properties were determined for the soil samples collected from different fertilization regimes (i.e. CK, M, MNPK and NPK). Soil pH (soil: water = 1:1) and soil nutrient concentrations are shown in Table 1. Fertilizer application increased soil nutrient concentrations including AP, AK, NN, AN, OM, TP and TN. The AP, AK, NN, OM and TP increased in the MNPK compared with NPK treatment; for example, the AP contents were 103.1 and 94.59 mg kg^{-1}, respectively. The concentrations of inorganic nutrients (AP, AK, NN, OM and TP) were greater following fertilizer treatments compared with CK, and were further enhanced by use of organic amendment with the inorganic fertilizers (Karami et al., 2012; He et al., 2015).

The soil pH of NPK (pH 5.53) and MNPK (pH 5.88), indicated that manure in the MNPK increased soil pH. Addition of inorganic fertilizer NPK resulted in a lower pH (pH 5.53) than for CK (pH 6.48). This beneficial action of manure on raising pH may have been due to carbonates and bicarbonates in the manure, and also because organic acids with carboxyl and phenolic hydroxyl groups can buffer soil acidity and increase pH of acid soils (Garcıa-Gil et al., 2004). In addition, the soybean yield for the M (2553.80 kg ha^{-1}) treatment was the highest, and that for MNPK (2421.73 kg ha^{-1}) was greater than for NPK (2283.67 kg ha^{-1}) (Table 1). Inorganic N fertilization can diminish soybean biological N fixation (Gelfand and Robertson, 2015); thus, compared with NPK, the soybean yield for M treatment was highest, and that for MNPK was higher than NPK. However, inorganic fertilizer is necessary for this wheat–maize–soybean rotation cropping system. For example, maize yield is closely related to N fertilization (Quemada et al., 2014), so it

Table 1
General physicochemical properties and soybean yield (mean ± standard deviation*) of soil for each treatment.

Variable	CK	M	MNPK	NPK
AP (mg kg^{-1})	2.89 ± 0.90^{a}	13.86 ± 0.98^{a}	103.1 ± 20.48^{b}	94.59 ± 7.01^{b}
AK (mg kg^{-1})	157.17 ± 29.27^{a}	190.2 ± 1.48^{a}	171.78 ± 9.30^{a}	166.88 ± 15.02^{a}
NN (mg kg^{-1})	2.36 ± 1.02^{a}	4.44 ± 0.62^{b}	6.84 ± 0.63^{c}	4.72 ± 1.12^{bc}
AN (mg kg^{-1})	34.85 ± 0.57^{a}	37.47 ± 6.41^{a}	41.27 ± 4.82^{a}	35.80 ± 7.46^{a}
OM (g kg^{-1})	24.46 ± 0.25^{a}	27.67 ± 0.12^{c}	25.65 ± 0.31^{b}	24.92 ± 0.32^{a}
TK (g kg^{-1})	31.54 ± 0.74^{a}	31.98 ± 0.37^{a}	30.62 ± 1.29^{a}	30.42 ± 1.09^{a}
TP (g kg^{-1})	0.44 ± 0.02^{a}	0.49 ± 0.03^{ab}	0.77 ± 0.17^{c}	0.70 ± 0.03^{bc}
TN (g kg^{-1})	1.18 ± 0.02^{a}	1.23 ± 0.06^{ab}	1.36 ± 0.11^{ab}	1.42 ± 0.08^{b}
pH (1:1H_2O)	6.48 ± 0.06^{c}	6.59 ± 0.05^{c}	5.88 ± 0.16^{b}	5.53 ± 0.03^{a}
Soybean yield (kg ha^{-1})	1811.63 ± 45.10^{a}	2553.80 ± 51.57^{c}	2421.73 ± 43.56^{bc}	2283.67 ± 135.95^{b}

Note:*Values followed by different letters are significantly different ($P < 0.05$) according to Tukey's multiple comparison test.
Abbreviations: AP, Available phosphorus; AK, Available potassium; NN, Nitrate nitrogen; AN, Ammonium nitrogen; OM, organic matter; TK, Total potassium; TP, Total phosphorus; TN, Total nitrogen.CK, without fertilizer; M, single manure; NPK, inorganic fertilizer; MNPK, inorganic fertilizer with manure.

is necessary to apply inorganic fertilizer to increase crop yield (Zhou et al., 2015).

3.2. Fungal community size and diversity analysis

The qPCR results for fungi are presented in Table 2. The copy numbers of the fungal ITS1 region of the ribosomal RNA gene were from 1.11×10^6 to 3.07×10^6 per g soil. The copy number for NPK treatment (3.07×10^6 per g soil) was slightly greater than for MNPK (2.30×10^6 per g soil), although this was not significant. In addition, Pearson's correlation analysis between soil properties and fungal ITS gene copy numbers (Table 3) showed that the copy numbers of ITS1 were significantly correlated with soil OM concentration ($r = 0.667$, $P < 0.05$).

The QIIME pipeline was used to process the raw sequence reads obtained from Illumina Miseq platform, and produced a total of 553,141 effective sequences with a length in the range of 220–300 bp (from the 12 soil samples, four treatments × triplication), including 470,650 high-quality sequences (fungal sequences) that represented >85% of the total. Sequence clustering showed that the number of OTUs in the MNPK treatment (533 ± 33) was significantly higher than for NPK (435 ± 65, $P < 0.05$; Table 2). Shannon–Wiener curves showed that fungal biodiversity was greater in the M and MNPK than the CK and NPK treatments (Fig. 1). Moreover, the numbers of community richness indices (Chao1 and ACE) in the MNPK (772.87 and 785.12, respectively) were higher than for the NPK treatment (689.39 and 672.28, respectively). Furthermore, the community diversity indices [Shannon and Simpson (expressed as the reciprocal of Simpson's index, 1/D)] in the MNPK (4.08 and 32.34, respectively) were increased compared with the NPK treatment (4.03 and 24.98, respectively). The highest values for Shannon and Simpson indices (1/D) in M (4.38 and 39.91, respectively), and followed by CK (4.24 and 35.33, respectively), and the Tukey's multiple comparison showed significant differences ($P < 0.05$) between the different fertilizer regimes (Table 2). However, Tukey's multiple comparison test did not show large differences in the changes in fungal diversity between MNPK and NPK treatments (Table 2). Furthermore, the relationship of α-diversity with soil properties is shown in Table 3. The values of the Simpson index were negatively correlated with soil pH ($r = -0.845$, $P < 0.01$), but positively correlated with AP, TP, TN and NN. The Shannon index was positively correlated with pH ($r = 0.810$, $P < 0.01$) but negatively with AP, TP and TN.

Based on weighted Fast UniFrac metrics, we used PCoA to investigate the variations in fungal communities caused by long-term fertilization. The first three principal coordinates (PCs) represented 27% (PC1), 20% (PC2) and 12% (PC3) of the total variation in fungal communities (Fig. 2). The PCoA plot showed that NPK was located in the upper part of PC3 and was separated far from MNPK, M and CK. PC2 showed MNPK and NPK clustered together, separated from CK and M.

Table 3
Pearson's correlation coefficients between soil properties and α-diversity indices, and fungal ITS gene copy numbers.

Variable	Chao 1	ACE	Simpson	Shannon	ITS gene copy numbers
AP	0.452	0.458	0.78[a]	−0.689[b]	−0.499
AK	0.248	0.195	−0.195	0.323	0.423
NN	0.555	0.528	0.584[b]	−0.432	−0.283
AN	0.272	0.186	0.123	−0.022	−0.086
TK	0.346	0.289	0.246	−0.055	0.124
TP	0.511	0.501	0.896[a]	−0.728[a]	−0.436
TN	0.527	0.528	0.717[a]	−0.598[b]	−0.250
OM	0.232	0.139	−0.422	0.553	0.667[b]
pH	−0.347	−0.348	−0.845[a]	0.810[a]	0.542

Abbreviations: APAvailable phosphorus; AKAvailable potassium; NNNitrate nitrogen; ANAmmonium nitrogen; OMorganic matter; TKTotal potassium; TPTotal phosphorus; TNTotal nitrogen.
[a] Correlation is significant at the 0.01.
[b] Correlation is significant at the 0.05.

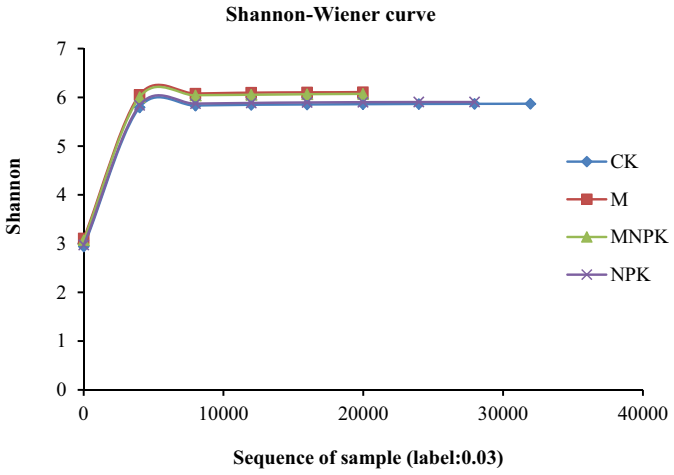

Fig. 1. Shannon-Wiener curves of samples. Abbreviations: CK, without fertilizer; M, single manure; NPK, inorganic fertilizer; MNPK, inorganic fertilizer with manure.

3.3. Composition and relative abundance of fungal communities

The 470,650 high-quality sequences were classed into five phyla across the entire data set. The phylum compositions of the four treatments are shown in Fig. 3A. The top three phyla were Ascomycota (63.77–78.70%), Zygomycota (8.33–14.80%) and Basidiomycota (4.03–13.47%), and 6.67–13.93% were unclassified. Ascomycota was the dominant phylum in all plots, with the percentage lowest for NPK (63.77%) and highest for MNPK (78.70%). The highest Zygomycota percentage was for NPK (14.80%). Furthermore, there were evident differences among the treatments at the class level of taxonomic classifications

Table 2
Estimated number of observed OTUs and diversity (mean ± standard deviation*) of soil fungal community for each treatment.

Treatment	Richness		Diversity		OTUs	ITS gene copy numbers (×10⁶ per g soil)
	Chao 1	ACE	1/Simpson	Shannon		
CK	619.14 ± 10.42^{a}	616.14 ± 19.59^{a}	35.33 ± 0.35^{ab}	4.24 ± 0.04^{ab}	404 ± 7^{a}	2.26 ± 0.05^{ab}
M	693.06 ± 76.94^{a}	671.59 ± 76.81^{a}	39.91 ± 5.91^{a}	4.38 ± 0.15^{b}	446 ± 35^{ab}	1.11 ± 0.09^{a}
MNPK	772.87 ± 59.04^{a}	785.12 ± 77.31^{a}	32.34 ± 7.58^{b}	4.08 ± 0.14^{a}	533 ± 33^{b}	2.30 ± 0.92^{b}
NPK	689.39 ± 109.03^{a}	672.28 ± 109.77^{a}	24.98 ± 1.38^{b}	4.04 ± 0.07^{a}	435 ± 65^{ab}	3.07 ± 0.42^{b}

OTUs: operational taxonomic units (97% similarity).
*Values followed by different letters are significantly different ($P < 0.05$) according to Tukey's multiple comparison.

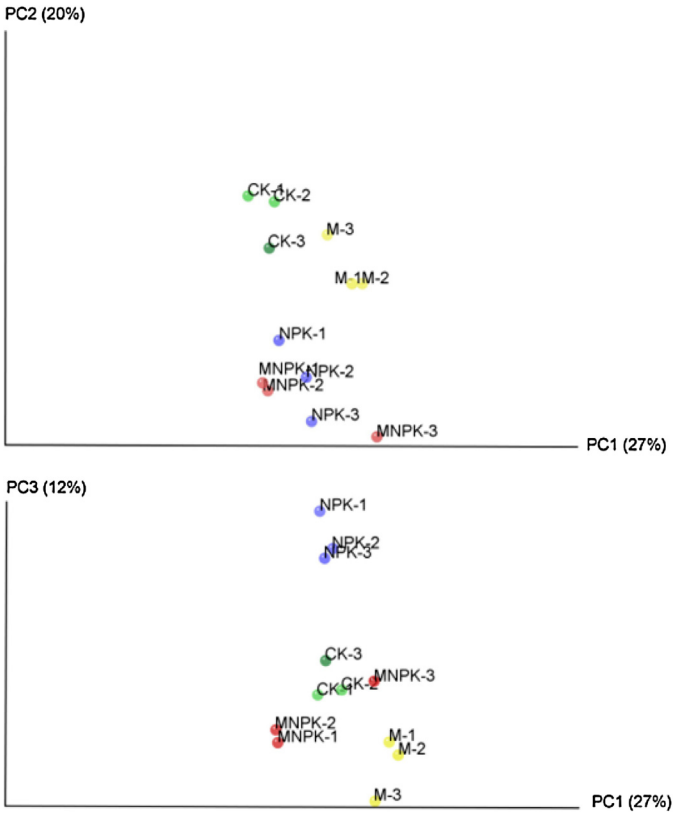

Fig. 2. PCoA plot based on weighted UniFrac distances of fungal communities sampled from each soil sample. Abbreviations: CK, without fertilizer; M, single manure; NPK, inorganic fertilizer; MNPK, inorganic fertilizer with manure.

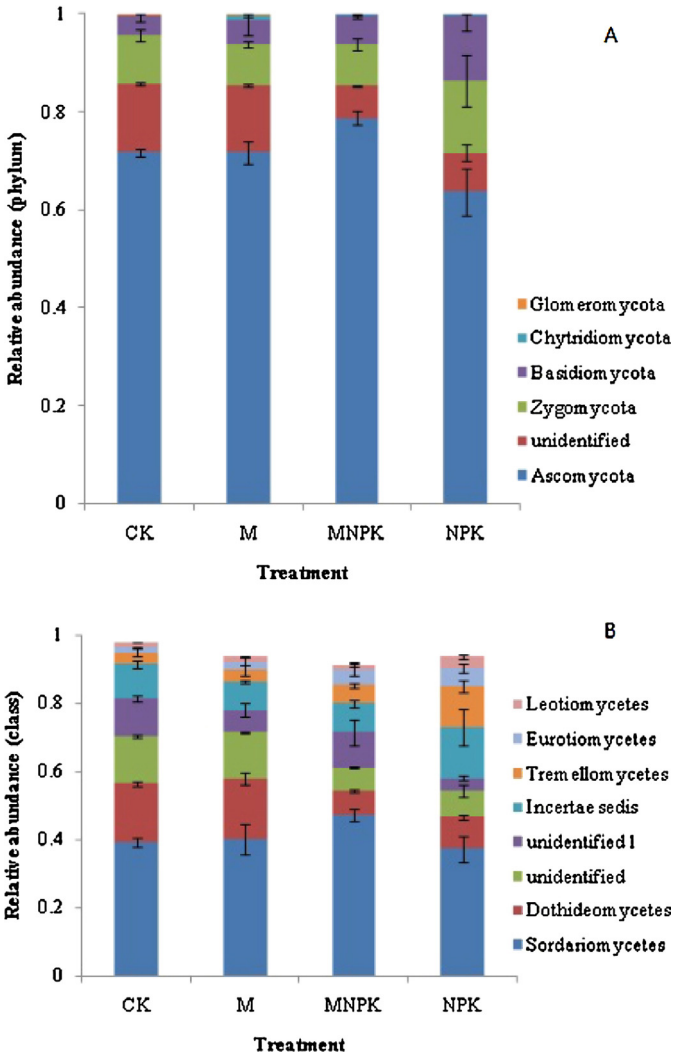

Fig. 3. Relative abundance of soil fungi under long-term organic and inorganic fertilization (A: at phylum level) and Relative abundance (>1%) of soil fungi under long-term organic and inorganic fertilization (B: at class level). Abbreviations: CK, without fertilizer; M, single manure; NPK, inorganic fertilizer; MNPK, inorganic fertilizer with manure. Note: unidentified1 belongs to Ascomycota; unidentified belogns to which phylum is not clear.

(Fig. 3B). Sordariomycetes, Dothideomycetes and Tremellomycetes were the three dominant classes in all treatments, accounting for 37.43–47.27%, 7.20–17.50% and 3.33–12.07% of the total high-quality sequences, respectively. Moreover, the four treatments exhibited remarkable differences in the relative abundance of the classes Sordariomycetes, Dothideomycetes, Eurotiomycetes and Leotiomycetes (Ascomycota). For example, the Sordariomycetes percentage for MNPK (47.27%) was more than for the NPK treatment (37.43%), while the relative abundance of Dothideomycetes for MNPK (7.20%) was less than for the NPK treatment (9.23%).

A thorough investigation at the genus level showed differences among the treatments. The genera were selected according to their relative abundance (1%) in one group of four treatments (Table 4). We analyzed the relative abundance changes among the four treatments for six genera chosen from Table 4. The sequence number of *Mortierella* was the highest for NPK (11.50%), but was the lowest for MNPK (6.80%). The percentage of *Fusarium* was significantly higher for NPK (9.20%) than MNPK (7.90%) and M treatments (7.10%). *Gibberella* was around 8.70% in NPK and was higher than for MNPK (5.50%) and M (3.70%). The relative abundance of *Schizothecium* in MNPK (11.40%) was greater than for NPK (4.40%) and M (7.0%); that for NPK significantly differed from MNPK. *Podospora* for MNPK (2.40%) surpassed that for NPK (0.90%); there were distinction between NPK and MNPK. While *Leptosphaerulina* was lower for MNPK (1.60%) than for NPK (2.90%).

3.4. Correlations between selected soil properties and fungal taxa

The RDA (Fig. 4) between soil properties and fungal taxa showed that the three most important contributors to the variation in fungal communities were soil concentrations of AP ($F = 3.7$, $P = 0.02$) and OM ($F = 2.0$, $P = 0.02$), and soil pH ($F = 1.9$, $P = 0.042$), which individually accounted for 30.7%, 15.2% and 12.0% of the variation, respectively. The order of influence was AP > OM > pH > TP > TN > TK > NN > AN > AK. Of the variation in fungal communities between samples, 87.9% was explained by all the environmental variables together. The first two constrained axes of the RDA explained 43.7% of the total variance: axis 1 explaining 28.7% and axis 2 explaining 15.0%. The fungal communities in CK, M, MNPK and NPK treatments were respectively grouped. The M and MNPK treatments were separated from NPK treatments along axis 2. In addition, Pearson's correlation analysis was used to evaluate relationships between relative abundances of fungi and environmental factors. The relationships between relative abundance of order level of Ascomycota and soil properties are shown in Table 5. The relative abundance of Dothideomycetes was negatively correlated with AP, NN, TP and TN ($r = -0.951$, -0.714, -0.89 and -0.813, respectively; all $P < 0.01$), but positively correlated with soil pH ($r = 0.882$, $P < 0.01$). The percentage of Leotiomycetes was negatively related to soil pH ($r = -0.705$, $P < 0.05$). In addition, the relative abundance of Sordariomycetes was positively related to NN and AN ($r = 0.606$ and 0.633, respectively; both $P < 0.05$).

Table 4
Relative abundance of phylogenetic genus in the different fertilization managements [according to its relative abundance (1%) in one group of four treatments].

Phylum	Class	Order	Family	Genus	Relative abundance (%)			
					CK	M	MNPK	NPK
Zygomycota	Incertaesedis	Mortierellales	Mortierellaceae	*Mortierella*	16.40^{b}	7.50^{a}	6.80^{a}	11.50^{b}
				unidentified	0.70^{a}	0.70^{a}	0.60^{a}	2.20^{b}
Ascomycota	Sordariomycetes	Hypocreales	Nectriaceae	*Fusarium*	7.10^{ab}	7.10^{ab}	7.90^{a}	9.20^{b}
				Gibberella	2.30^{a}	3.70^{a}	5.50^{b}	8.70^{b}
				Nectria	2.20^{b}	0.90^{a}	1.00^{a}	2.60^{ab}
				Neonectria	1.10^{b}	1.00^{ab}	0.80^{a}	1.60^{ab}
			Incertaesedis	*Myrothecium*	0.20^{a}	1.00^{a}	0.20^{a}	0.00^{a}
			Hypocreaceae	*Trichoderma*	0.10^{a}	0.10^{a}	1.00^{b}	0.10^{a}
		Sordariales	Lasiosphaeriaceae	*Schizothecium*	4.30^{a}	7.00^{a}	11.40^{b}	4.40^{a}
				Podospora	1.50^{a}	1.20^{a}	2.40^{b}	0.90^{a}
			Chaetomiaceae	*Chaetomium*	3.80^{b}	1.30^{a}	0.40^{a}	0.60^{a}
				Humicola	0.80^{a}	1.40^{a}	1.50^{a}	0.50^{a}
			unidentified	unidentified	0.80^{a}	1.10^{a}	1.20^{a}	0.90^{a}
		Incertaesedis	Plectosphaerellaceae	*Plectosphaerella*	0.60^{c}	3.90^{a}	1.00^{b}	1.40^{b}
				Verticillium	0.30^{b}	1.20^{a}	1.90^{a}	1.80^{a}
		Xylariales	unidentified	unidentified	1.00^{c}	0.40^{b}	0.10^{a}	0.10^{a}
		unidentified	unidentified	unidentified	1.00^{a}	0.70^{a}	1.30^{a}	1.00^{a}
	Dothideomycetes	Pleosporales	unidentified	unidentified	2.00^{a}	1.60^{a}	1.10^{a}	1.80^{a}
			Incertaesedis	*Leptosphaerulina*	3.40^{b}	3.80^{b}	1.60^{ab}	2.90^{b}
			Pleosporaceae	*Alternaria*	1.50^{ab}	3.70^{b}	0.80^{a}	0.80^{a}
			Corynesporascaceae	*Corynespora*	1.20^{b}	0.60^{a}	1.40^{b}	1.90^{b}
		Capnodiales	Mycosphaerellaceae	unidentified	0.10^{a}	1.30^{b}	0.10^{a}	0.30^{a}
		unidentified	unidentified	unidentified	0.70^{a}	1.00^{a}	0.40^{a}	0.80^{a}
	Leotiomycetes	Helotiales	Helotiaceae	*Crocicreas*	1.40^{b}	0.00^{a}	0.00^{a}	0.10^{a}
			Incertaesedis	*Tetracladium*	0.50^{a}	0.70^{a}	0.80^{a}	1.00^{a}
			unidentified	unidentified	0.30^{a}	0.30^{a}	0.50^{ab}	1.70^{b}
	Eurotiomycetes	Eurotiales	Trichocomaceae	*Penicillium*	0.40^{a}	0.70^{a}	1.90^{b}	2.30^{b}
			unidentified	unidentified	0.80^{a}	1.10^{ab}	2.20^{b}	1.90^{b}
		Chaetothyriales	Herpotrichiellaceae	unidentified	0.10^{a}	0.60^{ab}	0.20^{a}	1.00^{b}
	Incertaesedis	Incertaesedis	Incertaesedis	*Remersonia*	0.10^{a}	1.10^{b}	1.20^{b}	0.00^{a}
				Retroconis	1.60^{a}	2.00^{a}	5.30^{b}	4.20^{b}
	unidentified	unidentified	unidentified	unidentified	13.30^{b}	6.20^{ab}	8.10^{b}	4.00^{a}
Basidiomycota	Tremellomycetes	Cystofilobasidiales	Cystofilobasidiaceae	*Guehomyces*	0.80^{a}	1.20^{a}	3.70^{b}	6.90^{c}
		unidentified	unidentified	unidentified	1.80^{c}	1.30^{ab}	0.80^{a}	1.70^{bc}
unidentified	unidentified	unidentified	unidentified	unidentified	12.80^{a}	16.40^{a}	9.80^{b}	7.30^{b}

*Values followed by different letters are significantly different ($P < 0.05$) according to Tukey's multiple comparison.
Abbreviations: CK, without fertilizer; M, single manure; NPK, inorganic fertilizer; MNPK, inorganic fertilizer with manure.

4. Discussion

The fungal ITS gene abundances showed that fungal growth was high at almost all pH values – in close accord with previous studies showing that fungal species typically have a wide pH optimum, often covering 5–9 pH units without significant inhibition of their growth (Rousk et al., 2010). The copy number of the NPK treatment was slightly higher than MNPK, showing that inorganic fertilizer plus manure amendment decreased the fungal community size. Moreover, manure also decreased fungal gene abundance. Previously reported amendment with biochar decreased fungal gene abundance (Chen et al., 2013) and manure added into chemical fertilizer distinctly increased fungal populations (Zhang et al., 2012). Rousk et al. (2009) found that, within the pH range of 4.0–8.3, lower soil pH benefited fungal growth, consistent with the fungal abundance of NPK being higher than that of MNPK. In addition, fungal abundance was positively correlated with soil OM. This indicated that, in Black soils, fungal abundance was mainly determined by soil nutrition, which is consistent with the saprophytic status of most fungi. Liu et al. (2015a) reported that fungal abundance in Black soil was positively related with soil total carbon content.

Compared with inorganic fertilizer, soil fungal diversity was weakly improved by the mix of inorganic fertilizer and manure, consistent with research of Marschner et al. (2003) in loess soil using PCR-DGGE. Luo et al. (2015) found that inorganic and organic fertilizer both significantly increased fungal diversity, especially for the latter. However, He et al. (2008), using a culture-dependent approach, found that long-term organic fertilizers, especially manure, could decrease fungal diversity. The differences in these results are likely due to different methods and soil types. Furthermore, Pearson's correlation coefficients between soil properties and α-diversity indices showed that soil fungal diversity was primarily determined by soil AP, TP and TN contents and soil pH, in line with results of Rousk et al. (2010). The PCoA showed that the manure in MNPK enhanced the diversity of soil total fungi, reducing the difference of fungal communities between MNPK and CK.

The Ascomycota, Zygomycota and Basidiomycota were the top three occurring phyla. Fungal community structure was also previously determined by pyrosequencing methods (Buee et al., 2009; Brown and Jumpponen, 2014), and Ascomycota and Basidiomycota were the two most abundant in forest soil, which is roughly similar to our study. In Amazon farmland, the most abundant phylum was Zygomycota rather than Ascomycota (Fracetto et al., 2013). Bradley et al. (2006) found N fertilization caused significant changes in the composition of soil fungal communities. In the present study, soil fungal community relative abundance strongly differed between different fertilizer regimes, for example, Ascomycota in inorganic fertilizer plots (63.77%)

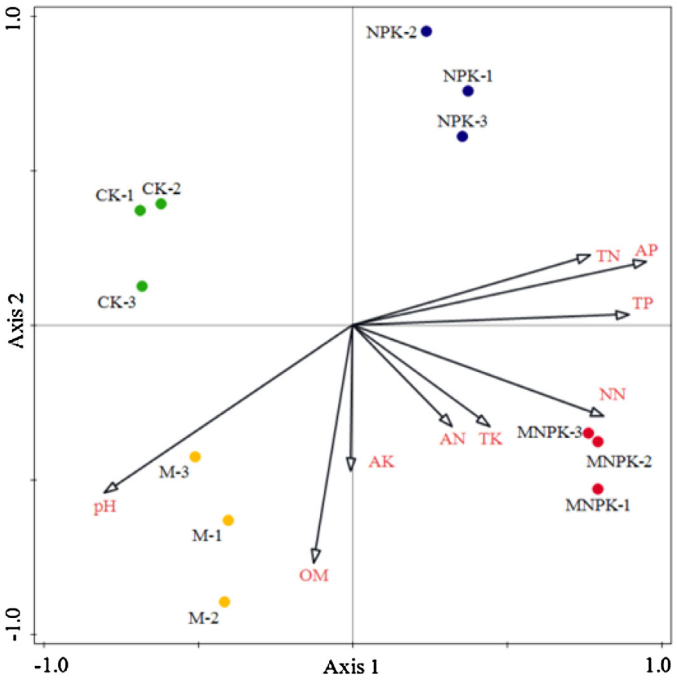

Fig. 4. Result from RDA to explore the relationship between soil fungal community and soil physiochemical characteristics. Abbreviations: AP, Available phosphorus; AK, Available potassium; NN, Nitrate nitrogen; AN, Ammonium nitrogen; OM, organic matter; TK, Total potassium; TP, Total phosphorus; TN, Total nitrogen.CK, without fertilizer; M, single manure; NPK, inorganic fertilizer; MNPK, inorganic fertilizer with manure.

distinctly differed from the inorganic fertilizer plus manure (78.70%), and Zygomycota in inorganic fertilizer plus manure (8.43%) was clearly lower than in inorganic fertilizer plots (14.8%).

Tremellomycetes and Dothideomycetes were the two dominant fungal classes in forest Black soils (Liu et al., 2015a), whereas Sordariomycetes and Dothideomycetes were the dominant class in farmland Black soils in the present study. Sordariomycetes responded to N addition, with a nearly linear increase (Mueller et al., 2015). In our study, Sordariomycetes was sensitive to all fertilization regimes, especially the incorporation of inorganic fertilizer and manure. A deeper analysis, at genus level, showed that relative abundance differed among the treatments (Table 4). *Mortierella*, *Fusarium* and *Schizothecium* were the three dominant fungal genera in MNPK, M and CK treatments. However, *Mortierella*, *Fusarium* and *Gibberella* were the three dominant fungal genera in NPK treatment. *Fusarium* wilt in soil is usually caused by *Fusarium* spp. and reduces plant growth (Ling et al., 2010; Ma et al., 2015). The number of *Fusarium* sequences in NPK was obviously higher than in MNPK and M treatments, showing that manure amendment decreased the relative abundance of *Fusarium* and may have counteracted the negative influence of inorganic fertilizer. Moreover, *Fusarium* is known to function in denitrification (Mueller et al., 2015), and can reduce the N content of soil. However, nutrient cycling rates are likely due not only to species richness, but also to interacting factors. In addition, *Gibberella* spp. are considered as secondary invaders, potentially pathogenic, or participants in synergistic enhancement of pathogenicity (Lenc et al., 2011). Manure combined with inorganic fertilizer reduced the number of *Gibberella* and possibly lessened the probability of morbidity. Furthermore, *Leptosphaerulina* spp. are also fungal pathogens (Mitkowski and Browning, 2004; Liu et al., 2015b), and the inorganic fertilizer with manure amendment resulted in lower numbers of *Leptosphaerulina*. Moreover, prior studies demonstrated that species of *Podospora* are antifungal agents (Che et al., 2002). The relative abundance of *Podospora* in MNPK was clearly the highest, and was the lowest in the NPK treatment. The manure in MNPK increased the abundance of beneficial fungi and amended the adverse effect of inorganic fertilizer. Application of organic amendment has been proposed as a strategy for the management of diseases caused by soil-borne pathogens (Bonanomi et al., 2010). Bonanomi et al. (2007) reported that the ability of organic amendments to suppress disease varied largely for different pathogens. Despite limited direct links between community measures and crop disease, our findings have potential implications for disease incidence in soil. Thus, we predicted that the improved structure of soil fungal communities due to application of manure was good for crop health. In addition, the RDA results revealed that the structure of fungal communities was closely correlated with the concentrations of soil AP ($P = 0.02$) and OM ($P = 0.02$), and influenced by soil pH ($P = 0.042$) which is in agreement with previous research by Liu et al. (2015a).

5. Conclusion

We analyzed the effect of fertilization regime on fungal communities in Black soil of northeast China. Inorganic fertilizer increased soybean yield and decreased soil pH, while manure amendment increased both yield and soil pH. Soil fungal diversity was increased by manure amendment and decreased by inorganic fertilizer, and was weakly improved by the combination of inorganic fertilizer and manure compared with inorganic fertilizer. Compared to inorganic fertilizer, fungal structure for the inorganic fertilizer amended with manure was more strongly related to that of unfertilized soil. Concurrently, the structure of soil fungal communities improved by manure was good for crop health.

The diversity of fungal communities was closely related to the soil contents of AP, TP and TN and to soil pH. Furthermore, soil AP

Table 5
Pearson's correlation coefficients between soil properties and Ascomycota (class, relative abundance >%).

Variable	Dothideomycetes	Eurotiomycetes	Incertaesedis	Leotiomycetes	Sordariomycetes
AP	−0.951[a]	0.826[a]	0.776[a]	0.559	0.336
AK	0.122	0.03	0.255	−0.03	0.076
NN	−0.714[a]	0.429	0.661[b]	0.086	0.606[b]
AN	−0.307	0.135	0.429	0.035	0.633[b]
TN	−0.322	0.409	0.412	0.147	0.146
TP	−0.89[a]	0.546	0.548	0.208	0.463
TN	−0.813[a]	0.698[b]	0.567	0.456	0.215
OM	0.294	−0.092	0.182	−0.328	0.175
pH	0.882[a]	−0.72[a]	−0.487	−0.705[b]	−0.099

Abbreviations: AP, Available phosphorus; AK, Available potassium; NN, Nitrate nitrogen; AN, Ammonium nitrogen; OM, organic matter; TK, Total potassium; TP, Total phosphorus; TN, Total nitrogen.

[a] Correlation is significant at the 0.01.

[b] Correlation is significant at the 0.05.

and OM contents were the dominant factors (compared to soil pH) in determining the soil fungal community structure. This study indicated that fertilization regimes significantly changed the structure of soil fungal communities most likely by altering the soil properties and fertility status.

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