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# Effects of Fertilization Methods on Chemical Properties, Enzyme Activity, and Fungal Community Structure of Black Soil in Northeast China

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**Abstract:** Understanding the influence of fertilizer on soil quality is vital to agricultural management, yet there are few studies, particularly in black soil. In this study, soils under various treatments, namely no fertilizer, bio-organic + humic acid, bio-organic + chemical, and chemical fertilizer, were sampled to identify their major physiochemical properties, and to investigate the fungal community structure using environmental sequencing techniques. Physiochemical properties and fungal community structure were examined at four important stages of the maize life cycle: seedling, jointing, heading period, and maturity. We found that chemical fertilizer in the mature stage increased the soil available phosphorous (AP) content. Organic matter content was greatly affected by bio-organic + chemical fertilizer during the mature stage. Bio-organic + humic acid significantly increased soil phosphatase activity in maturing maize, whilst chemical fertilizers reduced invertase activity. Taken together, our results clearly illustrated that bio-organic + humic and chemical fertilization indirectly alter fungal community structure via changing soil properties (especially AP). Chemical fertilizer markedly heightened the AP content, thereby decreasing specific fungal taxa, particularly *Guehomyces*. OM was of positive connection with bio-organic + humic acid and *Mortierella* abundance, respectively, through RDA analysis, which are in agreement with our result that bio-organic + humic acid fertilization to some extent increased *Mortierella* abundance. Additionally, bio-organic + humic acid decreased the abundance of *Fusarium* and *Humicola*, suggesting that bio-organic + humic acid possibly could help control crop disease. These results help to inform our fundamental understanding of the interactions between fertilizers, soil properties, and fungal communities.

**Keywords:** black soil (Mollisols); fertilization method; Illumina MiSeq sequencing; fungal diversity

## 1. Introduction

The USDA soil classification categorizes black land as Udoll black soil, which is also known as Mollisols [1]. There are just four large black soil regions in the world, one of which is located in

the northeast plains of China [2]. Black land offers a vital soil resource, with soils that are highly productive fertile soil containing a high (5–8%) organic carbon content [3]. Thus, the Northeast Black Soil Region of China has become the primary grain-producing area of the country. To enhance crop yields, substantial quantities of chemical fertilizers are commonly applied to croplands [4]. However, the excessive use of chemical fertilizers may alter soil properties, such as the reduction of extracellular enzyme activity, soil zinc content, soil pH content, or the accumulation of soil phosphorus content etc., which finally resulted in soil acidification, nitrogen leaching, and compaction [5]. In addition, chemical fertilizer not only influences soil properties but also has a negative impact on soil fungi community diversity and composition. Although previous investigations reported that chemical fertilizer could increase fungal biomass, it greatly decreased fungal community diversity and altered community composition, shifting dominant flora from bacteria to fungi [6]. Application of bio-organic fertilizers with addition of humic acid is a cost-effective agricultural practice to avoid soil degradation issues mentioned above [7]. Multiple long-term studies have demonstrated that bio-organic + humic acid fertilizers increase microbial biomass and alter community composition and diversity by introducing considerable external carbon (C) into the soil [8]. Additionally, bio-organic + humic acid addition can bolster the capacity of soil to hold water, enhance its cation exchange capacity, increase biological enzyme activity, improve the soil structure, and prevent soil acidification [9], implying that bio-organic + humic acid additions have the potential to reverse the degradation associated with the long-term use of chemical fertilizers [10].

Soil enzymes, one of the most valuable parts of the soil biochemical process, acts in a crucial role in the decomposition of organic matter and nutrient cycling [11]. A review by Lemanowicz et al. [11] elaborated that phosphatase activity is an index to evaluate the direction and intensity of soil phosphorus biotransformation. Additionally, urease activity could be used to characterize soil nitrogen status [12]. Apart from this, a fertilization by Wu et al. [13] indicted that invertase activity could reflect the utilization of soluble substances in soil by microorganisms and the accumulation, transformation of soil organic matter. In a word, it could be seen that the above enzyme activities do matter for healthier soil and current modernization of agriculture. Hence, we chose the above enzyme activities to explore if various fertilizers have a positive or negative influence on soil.

Fungal community structure and diversity play essential roles in maintaining soil function, such as the decomposition of plant residues both above and below ground [14]. Prior research has proved that the C-to-N ratio in fungal cells is much greater than that in bacterial cells, which requires the fungi to obtain more soil-derived C [15]. Additionally, Wu et al. [16] confirmed that fungi have a greater ability to acquire nitrogen (N) and phosphorus (P) than bacteria. Remarkably, the dominant soil fungal community, compared to the bacterial community, is more likely to affect soil fertility [15,17], while not much is known on how fertilization impacts fungal composition, structure, and diversity.

Black soil, which is highly productive, has become an important resource for main grain production. So, understanding the impact of fertilizers on soil quality is particularly important for a modern agricultural system, while this remains rarely documented in black soil. Herein, we utilized Illumina MiSeq technology and aimed to evaluate how particular types of fertilizer affected the soil fungi community structure and assessed the relationship between soil properties and fungi communities in northeastern China black soil.

## 2. Materials and Methods

### *2.1. Site Description*

An experimental field with an area of 4.5 m × 400 m was selected in Bayan County (45°54′28″–46°40′18″ N, 126°45′53″–127°42′16″ E), Harbin, Heilongjiang Province, China. This region has a mid-temperate continental monsoon climate. The annual mean temperature is −2.9 °C, with a monthly mean temperature reaching a maximum of 22.4 °C in July and a minimum of −20.9 °C in January. The cumulative average precipitation is 582.2 mm, with a minimum of 372.5 mm. The soil

is classified as typical black soil with a clay loam soil texture. The soil background is as follows: alkali-hydrolysis nitrogen (AN), 172.4 mg/kg, available phosphorus (AP), 58.5 mg/kg; available potassium (AK), 182.75 mg/kg; pH 5.8; organic matter (OM), 38.68 g/kg.

### 2.2. Fertilizer Preparation

Bio-organic and chemical fertilizers used in this experiment are commercially available and were purchased from the Harbin Tong Dazhou Agricultural Resources Co., Ltd. (Harbin, China). Before executing the experiment, the microbial community composition of the bio-organic fertilizer was detected preliminarily. *Bacillus megaterium* and *B. mucilaginosus* are the main active components of the bio-organic fertilizer with an effective viable count ≧ 0.2 billion/gram and OM content ≧ 25%. *B. megaterium* is a phosphate-decomposing bacterium, which can produce a variety of organic and inorganic acids, lower the environment pH, and transform insoluble phosphate into AP, which is easily absorbed by plants. *B. mucilaginosus* is capable of dissolving P and K, and fixing $N_2$, which reduces the overall amount of fertilizer required. In addition, *B. mucilaginosus* can also decompose minerals, such as feldspar, mica, and other aluminosilicates, converting insoluble K, P, and Si into available nutrients for plant activity and growth. Chemical fertilizer consists of ≧ 45% N: $P_2O_5$: $K_2O$ at a ratio of 12:22:11. These nutrients are mainly present as MAP (monoammonium phosphate), DAP (diammonium phosphate), ammonium sulfate, potassium sulfate, urea, and some small impurities, such as calcium sulfate, iron phosphate, aluminum, magnesium, and other salts, including unreacted potassium chloride. Humic acid (organic matter and humic acid), with a particle size of 2–4 (%), was mixed with black soil, resulting in an organic content of ≧ 55%. This mixture was combined with 45 kg $ha^{-1}$ of bio-organic fertilizer (as described above). To minimize the introduction of fungi between experimental treatments, all humic acid was sterilized (121 °C, 0.1 MPa for 1.5 h) prior to soil amendment.

### 2.3. Experimental Design

We divided the experimental field into three experimental belts of 1 m × 400 m. A minimum buffer area 0.75 m wide was established between belts to avoid interference. In the middle of each belt, four plots (1 m × 10 m), with a 5-m buffer between adjacent plots, were each treated with a different fertilization treatment: (1) no fertilizer; (2) 1950 kg $ha^{-1}$ of 30% bio-organic fertilizer and 70% humic acid (bio-organic + humic acid); (3) 45 kg $ha^{-1}$ of bio-organic fertilizer combined with 300 kg $ha^{-1}$ of chemical fertilizers (bio-organic + chemical); and (4) 375 kg $ha^{-1}$ chemical fertilizer was applied (chemical fertilizer). The four treatments contained the same amount of the main nutrient components (i.e., nitrogen (N), phosphorus ($P_2O_5$), potassium ($K_2O$)). Each treatment normally top-dressed urea 37.5 kg $ha^{-1}$ at the jointing period.

Maize was planted in mid-May and harvested in late September of 2017. At the four growth stages (seedling, jointing, heading period, and maturity), five individual soil cores of 5–7cm diameter and from 20-cm deep below the edge of roots were collected in each plot and mixed to yield a sample for that plot. A 2-mm mesh was used to sieve soil samples, and visible organic debris, stones, and plant residue were manually removed. In total, 1 g of each soil sample was added to a 50-mL tube and stored at −80 °C until DNA was extracted. The remaining soil was dried at room temperature for analysis of enzyme activity and chemical properties.

### 2.4. Soil Physicochemical Property Analysis

A 1:2.5 soil–water suspension (*w*/*v*) was used for measurements of soil pH. Total N (TN) content was determined by the semi-micro Kjeldahl method [18]. The total P (TP) and the available P (AP) were measured as described by Barrow and Shaw [19]. The available potassium (AK) and total K (TK) were quantified using a neutral ammonium acetate solution extraction and the flame photometric method [20]. Soil available N (AN) was assessed via the alkaline hydrolysis diffusion method [21]. Soil organic matter (OM) was determined using the $K_2Cr_2O_7$-capacitance method [22].

### *2.5. Analysis of Soil Enzyme Activities*

Urease activity was measured using the phenol sodium hypochlorite colorimetric approach. Invertase was measured with the 3,5-dinitrosalicylic acid colorimetric method. Acid phosphatase activity was measured using the disodium phenyl phosphate colorimetric method. All enzyme activities were measured according to Ge et al. [23].

### *2.6. Fungal Community Diversity Analysis*

To assess the fungal community diversity, 0.5 g of soil DNA was extracted (Follow the MoBio Power Soil DNA Isolation Kit (100), QIAGEN) and ITS nrRNA was amplified using the primer pair ITS1F (5′-CTTGGTCATTTAGAGGAAGTAA-3′) and ITS2R (5′-TGCGTTCTTCATCGATGC-3′) (Allwegene Company, Beijing, China). All PCRs were conducted in triplicate in a Mastercycler Gradient (Eppendorf, Germany), with 4 uL of 12.5-mM dNTP Mix, 5 mL of 10 × Ex Taq Buffer containing $Mg^{2+}$, 2 mL of the sample template DNA, 1.25 U of Ex Taq DNA polymerase, and 36.75 uL of dd $H_2O$ per reaction. PCR settings were 2 min at 94 °C; 30 cycles of 30 s at 94 °C, followed by 30 s at 57 °C and 30 s at 72 °C, with a final 10 min extension at 72 °C. The PCR products were isolated via the QIAquick Gel Extraction Kit (Qiagen, Dusseldorf, Germany), and were pooled in equimolar amounts. Sequencing was performed by the Allwegene Company on an Illumina MiSeq PE300 machine (Paired-end sequencing, on-machine sequencing reagent MiSeq® Reagent Kit v3 (600 cycle) (PE300), 300 bp length).

We used the Illumina Analysis Pipeline (version 2.6) to process and analyze the raw sequence data [24]. The raw data were filtered such that reads with a length < 200 bp, low-quality scores (≤20), ambiguous bases or nonprime sequences, or barcode tags that did not match exactly were removed. Unique barcodes were used to separate samples, and the Illumina Analysis Pipeline (version 2.6) was used for barcode trimming. Subsequently, QIIME 1 was used for data analysis [25]. Operational taxonomic units (OTUs) that had at least 97% similarity were clustered together. These were used to construct clustered rarefaction curves and derive diversity and richness index values [26]. Next, taxonomic group assignments were made using the Ribosomal Database Project (RDP) Classifier tool [24], and Fast Tree [27] was used for phylogenetic tree construction. For sampling effort correction, the lowest number of sequences for any sample (34,033) was used to randomly downsample sequences from other samples. All reads were accessioned into the GenBank short-read archive (SRP189595). In database of SRP189595, A, B, C, and D represent the maize growth period of seedling, jointing, heading period, and maturity; CK, T1, T2, and T3 indicate fertilization treatments of no fertilizer, bio-organic + humic acid, bio-organic + chemical, and chemical fertilizer, receptively.

### *2.7. Statistical Analysis*

We used QIIME to compute Good's coverage, Chao1 estimator of richness, Simpson diversity index, PD_whole_tree index, and the Shannon diversity index to assess soil fungal alpha diversity. One-way ANOVAs were used to compare alpha diversity, soil characteristics, and relative fungal taxa abundance within each sample at each time-point using SPSS (v16.0; SPSS, Inc., Chicago IL, USA). In addition, nonmetric multidimensional scaling (NMDS) ordination plots were used to compare the composition of fungal communities. Mantel tests were employed to compute the correlation between the soil microbial community and soil properties. Environmental factors related to soil microbial communities were assessed via a redundancy analysis (RDA) with CANOCO 4.5. These analyses were performed using the sample OTU results in the "vegan" R packages (v3.1.2; http://www.r-project.org/).

## 3. Results

### *3.1. Soil Chemical Properties*

Fertilization treatments significantly altered measured soil properties (Figure 1). It was not difficult to observe that organic matter (OM) content in the maize mature stage was greatly affected by

bio-organic + chemical fertilizer and chemical fertilizer ($p < 0.05$). In particular, bio-organic + chemical and chemical fertilizer in the maize mature stage exerted a significant impact on AP, which was enhanced by 173.8% and 209.9% relative to no fertilizer ($p < 0.05$), respectively. In addition, chemical fertilizer enhanced soil AN and AK compared with no fertilizer. Soil AK during the maize jointing and maturity stages increased by 8.6% and 59.8% ($p < 0.05$), respectively, and soil AN during the maturity stage increased by 19.4% ($p < 0.05$). Furthermore, the application of chemical fertilizer during maize jointing decreased pH of the soil from 5.78 to 5.47 ($p < 0.05$), whereas bio-organic + humic acid and bio-organic + chemical treatments kept the soil pH stable.

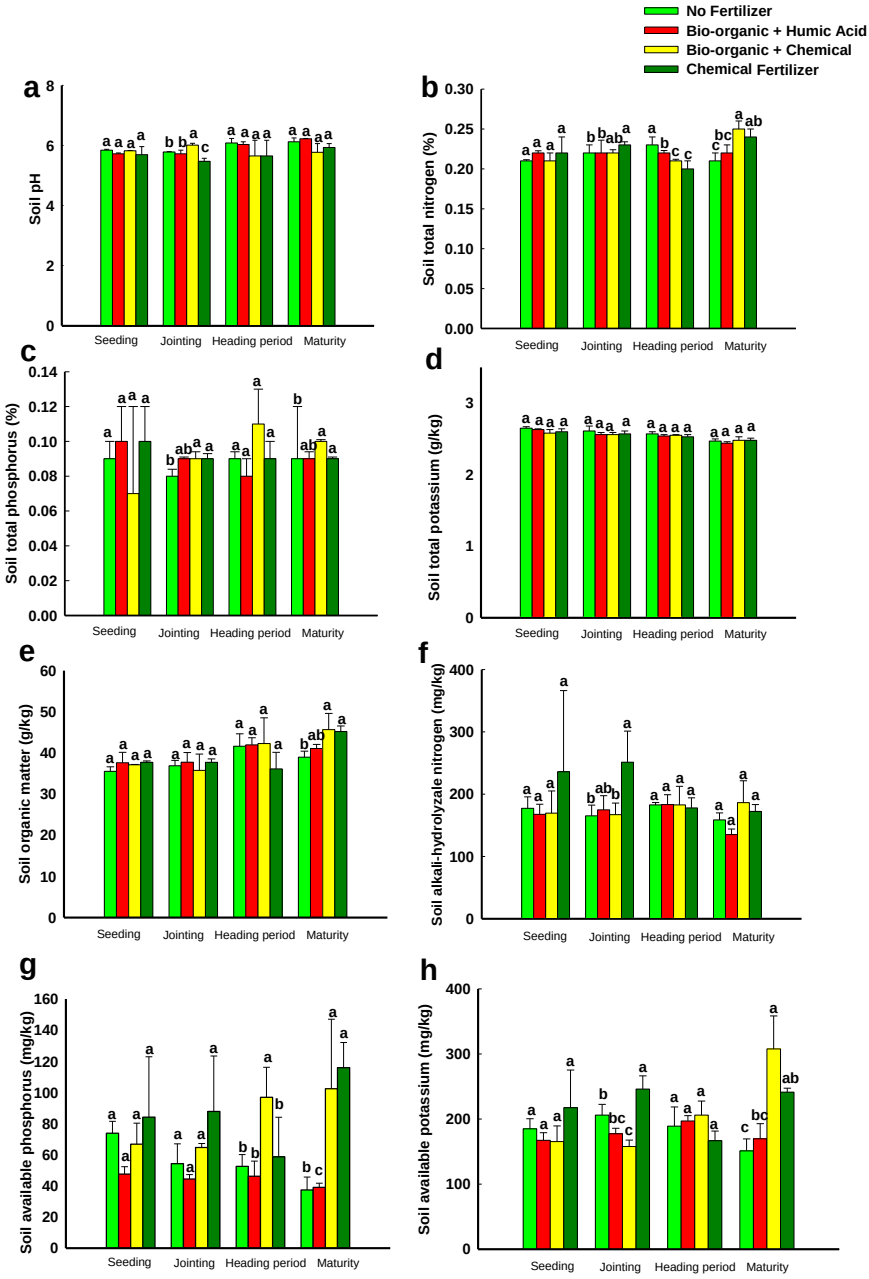


**Figure 1.** The impact of different fertilization methods on soil chemical properties at four maize growth stages: (**a**) soil pH; (**b**) soil total nitrogen; (**c**) soil total phosphorus; (**d**) soil total potassium; (**e**) soil organic matter; (**f**) soil alkali-hydrolysis nitrogen; (**g**) soil available phosphorus; and (**h**) soil available potassium. Values are mean and standard deviation (± SD, $n = 3$), different letters indicate significant difference at the 0.05 level.

### 3.2. Soil Enzyme Activity

The invertase activity treated with chemical fertilizer treatment in all maize growth stages was lower than that of no fertilizer (Figure 2, $p$ <0.05). Moreover, soil phosphatase levels were elevated in response to the bio-organic + chemical group especially at the maturity stage ($p < 0.05$).

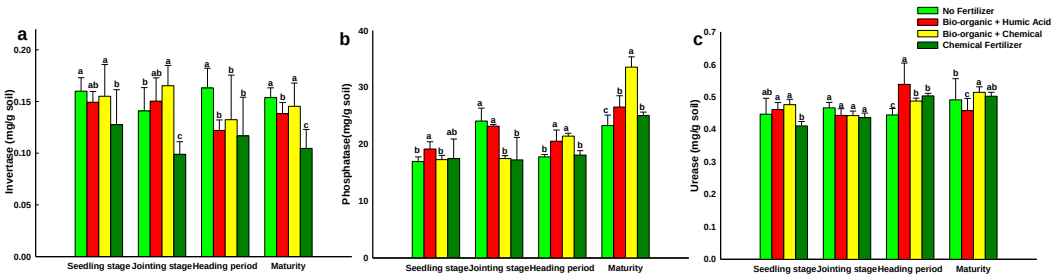


**Figure 2.** The impact of fertilization methods on soil enzyme activity at four maize growth stages: (**a**) Invertase, (**b**) Phosphatase, and (**c**) Urease. Values are mean and standard deviation (±SD, $n$ = 3), and different letters correspond to significantly different values as determined via one-way ANOVA ($p < 0.05$).

### 3.3. Fungal Taxonomic Classification and Relative Abundance

After filtering, we obtained 2,070,714 sequences from the illumina MiSeq sequencing run (Table 1), of which 34,033–47,208 were obtained for each soil sample (mean 43,140). Read lengths ranged from 200 to 260 bp. We assessed the fungal community diversity based on the relative abundance of OTUs. Across samples, the most abundant fungal phyla were Ascomycota (54.15–78.13%), Basidiomycota (11.65–32.69%), and Mortierellomycota (4.12–11.94%) (Figure 3; Table S1). In addition, the minor fungal phyla and their relative abundances were Chytridiomycota (0.4–5.59%) and Glomeromycota (0.06–1.58%) (Figure 3; Table S1). Despite some degree of fluctuation in the relative levels of these dominant fungal phyla after the application of different fertilization treatments, the difference between the four treatments was mostly not statistically significant. However, soil from the jointing stage that was treated with bio-organic + humic acid showed an increased relative abundance of Mortierellomycota and reduced relative abundance of Ascomycota ($p < 0.05$). Furthermore, chemical fertilizer reduced the relative abundance of Basidiomycota at the maize jointing and maturity stages compared with the bio-organic + humic acid treatment ($p < 0.05$; Table S1).

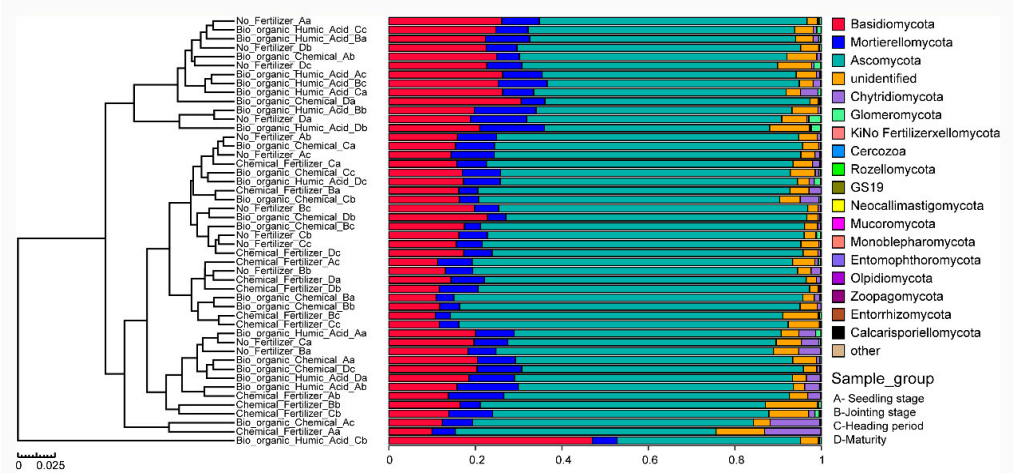


**Figure 3.** Phylogenetic relationships of fungal communities shown with the relative abundances of different fungal phyla. The letters a, b, and c indicate the three replicates.

**Table 1.** Illumina Mi-Seq sequenced fungal data and fungal community diversity indices (at 97% sequence similarity) based on the ITS nrRNA gene.

| Period of Growth | Sample | Quality Sequences | Fungal Sequences | Number of Species [a] | Chao 1 Richness [a] | Shannon's Diversity [a] | PD _Whole _Tree [a] | Simpson's Diversity [a] | Coverage [a] (%) |
|---|---|---|---|---|---|---|---|---|---|
| Seedling stage | No Fertilizer | 44,165 ± 842.0 | 43,841 ± 817.0 | 426 ± 32.0 a | 568 ± 32.0 ab | 4.91 ± 0.34 a | 103.48 ± 12.64 ab | 0.0113 ± 0.0150 a | 99.60 |
| | Bio-organic + Humic Acid | 42,243 ± 5929 | 41,950 ± 5852 | 490 ± 33.0 a | 666 ± 46.0 a | 5.51 ± 0.05 a | 118.05 ± 3.600 a | 0.0033 ± 0.0040 b | 99.53 |
| | Bio-organic + Chemical | 40,350 ± 3217 | 40,124 ± 3183 | 406 ± 16.0 a | 515 ± 20.0 b | 5.20 ± 0.17 a | 95.53 ± 4.430 b | 0.0033 ± 0.0400 ab | 99.66 |
| | Chemical Fertilizer | 42,251 ± 6977 | 41,961 ± 6914 | 407 ± 43.0 a | 511 ± 56.0 b | 5.09 ± 0.46 a | 98.01 ± 9.400 ab | 0.0216 ± 0.0310 ab | 99.67 |
| Jointing stage | No Fertilizer | 44,948 ± 2914 | 44,585 ± 2896 | 430 ± 17.0 a | 574 ± 45.0 a | 5.08 ± 0.15 a | 101.12 ± 4.080 a | 0.0001 ± 0.0001 ab | 99.60 |
| | Bio-organic + Humic Acid | 43,838 ± 3143 | 43,480 ± 3128 | 434 ± 19.0 a | 581 ± 35.0 a | 5.27 ± 0.14 a | 100.80 ± 3.810 ab | 0.0045 ± 0.0060 b | 99.58 |
| | Bio-organic + Chemical | 44,785 ± 1227 | 44,415 ± 1153 | 425 ± 3.00 a | 569 ± 26.0 a | 4.90 ± 0.13 a | 95.86 ± 1.520 ab | 0.0153 ± 0.0200 a | 99.60 |
| | Chemical Fertilizer | 41,566±2349 | 41,295 ± 2352 | 400 ± 36.0 a | 534 ± 58.0 a | 5.09 ± 0.28 a | 88.30 ± 7.630 b | 0.0065 ± 0.0090 ab | 99.62 |
| Heading period | No Fertilizer | 45,884 ± 546.0 | 45,564 ± 484.0 | 380 ± 28.0 b | 481 ± 45.0 b | 4.74 ± 0.69 a | 99.77 ± 11.03 b | 0.0706 ± 0.0920 a | 99.67 |
| | Bio-organic + Humic Acid | 42,116 ± 1548 | 41,801 ± 1569 | 501 ± 10.0 a | 657 ± 28.0 a | 5.33±0.38 a | 114.66 ± 2.570 ab | 0.0190 ± 0.0250 a | 99.52 |
| | Bio-organic + Chemical | 44,280 ± 1429 | 43,931 ± 1402 | 587 ± 36.0 a | 776 ± 67.0 a | 5.63 ± 0.27 a | 132.97 ± 7.740 a | 0.0123 ± 0.0160 a | 99.44 |
| | Chemical Fertilizer | 43,644 ± 1206 | 43,323 ± 1192 | 530 ± 68.0 a | 707 ± 76.0 a | 5.75 ± 0.49 a | 127.42 ± 14.92 a | 0.0109 ± 0.0140 a | 99.52 |
| Maturity | No Fertilizer | 44,248 ± 1426 | 43,911 ± 1445 | 562 ± 37.0 a | 747 ± 69.0 a | 5.52 ± 0.11 a | 128.34 ± 5.270 a | 0.0025 ± 0.0030 a | 99.43 |
| | Bio-organic + Humic Acid | 43,964 ± 1426 | 43,657 ± 2751 | 692 ± 96.0 a | 692 ± 96.0 a | 5.82 ± 0.07 a | 124.95 ± 11.35 a | 0.0014 ± 0.0020 a | 99.53 |
| | Bio-organic + Chemical | 42,428 ± 2002 | 41,579 ± 1480 | 674±133 a | 674 ± 133 a | 5.31 ± 0.25 a | 133.09 ± 20.21 a | 0.0088 ± 0.0120 a | 99.50 |
| | Chemical Fertilizer | 45,264 ± 4131 | 44,821 ± 4131 | 613±53.0 a | 613 ± 53.0 a | 5.31 ± 0.28 a | 107.93 ± 5.010 a | 0.0107 ± 0.0150 a | 99.58 |

[a] The data was calculated from 34,033 fungal sequences per soil sample. [b] Different letters within the same column indicate significant difference between the treatments in individual sampling time tested using a one-way analysis of variance (ANOVA) ($p < 0.05$).

Additional genus-level classification revealed > 400 fungal genera in our samples. The whole fungal community was different even among the samplings at the genus level (Figure S1) and the 20 dominant fungal genera showed differently under different fertilization methods at different maize growth stages (Figure S2). Among them, the most abundant and successfully identified genera were *Humicola* (8.38–28.48%), *Mortierella* (4.12–11.32%), *Fusarium* (9.35–20.81%), and *Guehomyces* (2.35–8.20%). Relative *Mortierella* abundance was usually not significantly different, except for soil samples that were collected during the jointing stage of maize. In this case, the bio-organic + humic acid treatment exhibited the highest *Mortierella* abundance of all treatments. Moreover, although the relative abundance of *Fusarium* was not significantly different, *Fusarium* abundance marginally fell for the bio-organic + humic acid application, especially in the heading stage of maize (Figure 4). Conversely, the relative *Guehomyces* and *Humicola* levels were significantly affected by the chemical and bio-organic + humic acid fertilizers, respectively. The relative *Humicola* levels from the soil samples collected in the maize seedlings decreased ($p < 0.05$), and the bio-organic + humic acid treatment exhibited the lowest abundance of the maize seedlings of all treatments. Likewise, the abundance of *Guehomyces* was the lowest during the maturing stage when treated with chemical fertilizer. In addition, some fungal genera were affected by the growth cycle of maize inconsistently. The relative abundances of two Ascomycota genera (*Coniochaeta* and *Chloridium*) and one Basidiomycota genus (*Mrakiella*) decreased or increased with maize development (Figure S3).

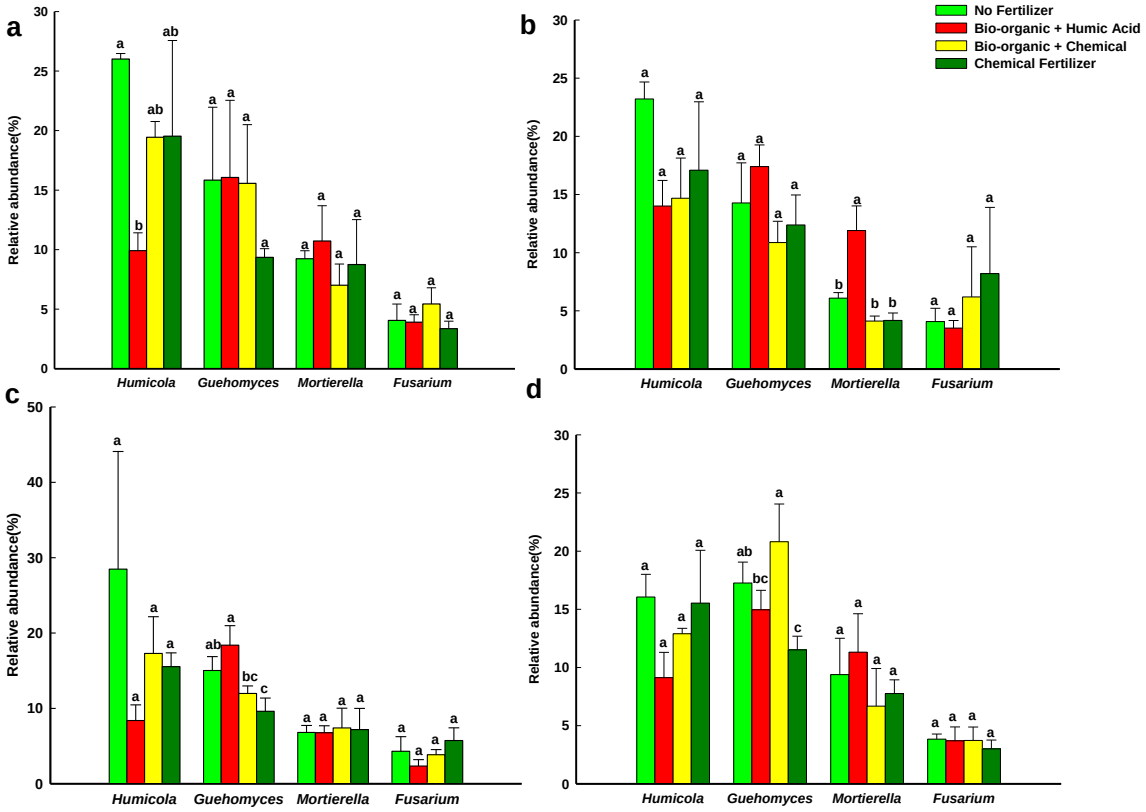


**Figure 4.** The impact of different fertilization methods on the relative abundances of the top four fungi genera during stage of (**a**) seedling, (**b**) jointing stage, (**c**) heading period, and (**d**) maturity. Values are mean and standard deviation (±SD, $n = 3$), different letters correspond to significantly different values as determined via one-way ANOVA ($p < 0.05$).

### *3.4. Fungal Community Diversity*

We assessed overall fungal community diversity across differently treated samples. In order to control for survey effort, we randomly downsampled sequences to the minimum depth found in any

sample (i.e., 34,033 sequences). Our analyses showed that fertilization methods exerted a minimal impact on the number of phylotypes and on fungal alpha-diversity indices, including Shannon and Simpson diversity (Table 1).

### 3.5. Fungal Community Structure

The NMDS results show that fungal community composition varied among fertilization methods (Figure 5). The fungal communities at the maize heading and maturity stage in the bio-organic + chemical and chemical fertilizer treatments were separated from those in the no fertilizer and bio-organic + humic acid treatments along the NMDS2 axis (dashed line 5–1), implying that different fertilization methods affected the community structure of black soil fungi. Simultaneously, the fungi community sampled at the first two sampling dates of bio-organic + chemical treatment was independent from those sampled during the latter two sampling dates (along the NMDS1). This difference illustrated that the soil fungal community also responded to the growth stage of maize. Overall, these findings suggested that the fungal community was not only affected by different fertilization methods but also by growth stage.

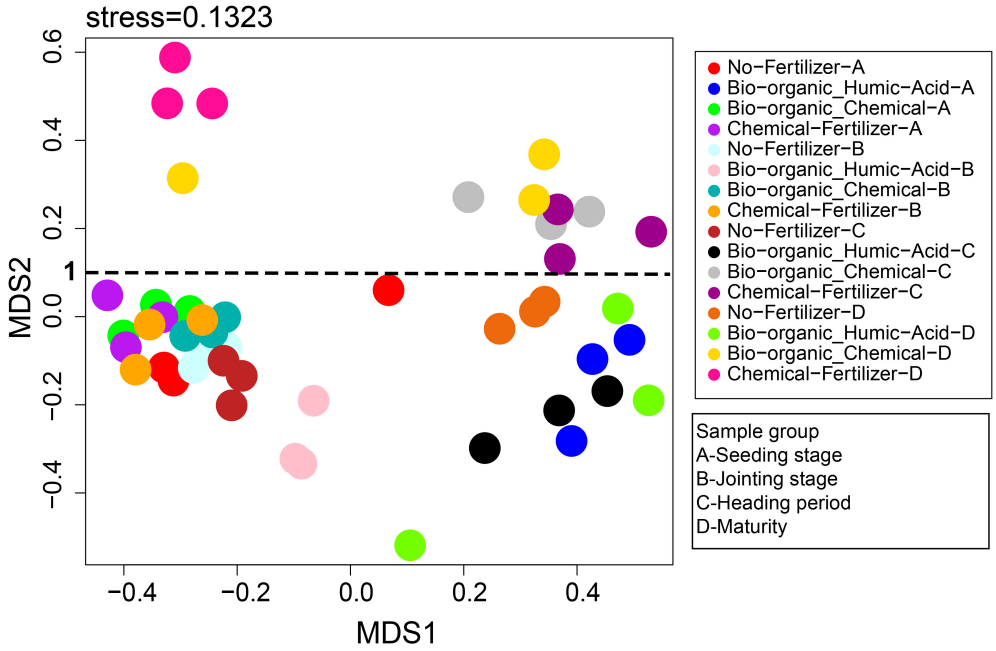


**Figure 5.** The nonmetric multidimensional scaling (NMDS) plot for soil fungal communities under different fertilization methods. Differently shaped and colored symbols correspond to different sampling dates and different fertilization methods, respectively. The fungal communities of the maize heading stage and maturity stage in bio-organic + chemical and chemical fertilizer treatments were separated from those in no fertilizer and bio-organic + humic acid treatment along the NMDS2 axis (dashed line–1).

### 3.6. The Relationship between Soil Properties and Fungal Community Composition

The fungal community structure in soil treated with bio-organic + humic acid and bio-organic + chemical was similar to the no fertilizer treatment but distinct from the chemical fertilizer treatment along RDA1 axes (Figure 6a). The Mantel test highlighted that AP, OM, and TN dictated the structure of the fungal communities, suggesting a strong link between soil fungal community structure with the alteration of soil properties (Table 2). Chemical fertilizer treatment was positively correlated with AP, while OM was positively correlated with bio-organic + humic acid treatment. Correlation analysis, also, showed that *Guehomyces* was negatively associated with AP and *Mortierella* was positively correlated with OM (Figure 6b), which was consistent with our results that chemical fertilizer markedly

heightened the AP content, thereby decreasing specific fungal taxa, particularly *Guehomyces*, or that bio-organic + humic acid fertilization was of positive connection with OM via RDA analysis and then to some extent increased *Mortierella* abundance.

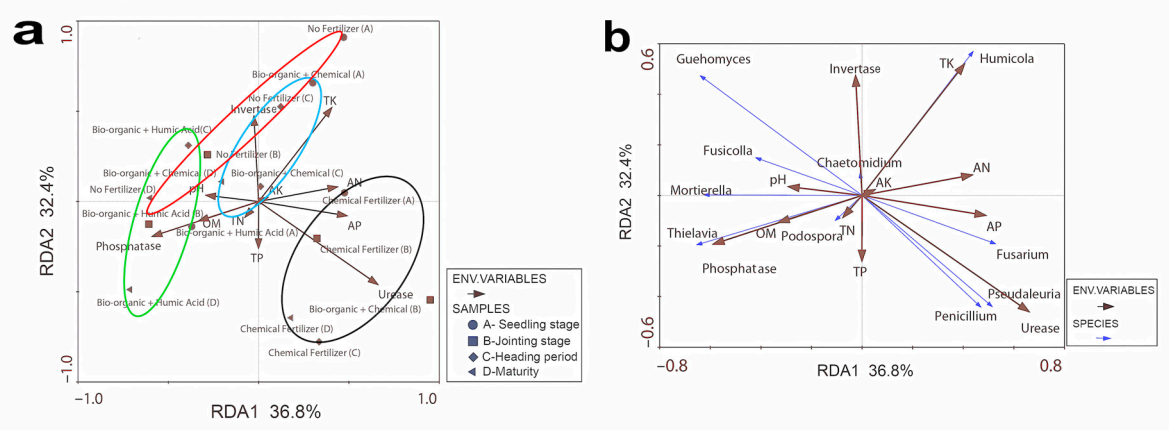


**Figure 6.** Redundancy analysis (RDA) of the change in the fungal community with environmental variables. (**a**) The relationship between different fertilizer treatments and environmental variables, and (**b**) The correlation between soil environmental variables and fungal community profiles.

**Table 2.** Correlation Analysis of the Soil Fungal Community and Environmental Factors.

| Environmental Factors | r Value | *p* Value |
|---|---|---|
| pH | −0.08865 | 0.75 |
| Organic matter (OM) | 0.2156 | **0.011** |
| Total nitrogen (TN) | 0.1816 | **0.021** |
| Total phosphorus (TP) | 0.122 | 0.112 |
| Total potassium (TK) | −0.1194 | 0.854 |
| Alkaline nitrogen (AN) | 0.1219 | 0.134 |
| Available phosphorus (AP) | 0.3166 | **0.001** |
| Available potassium (AK) | 0.1504 | 0.064 |
| Phosphatase ($P_2O_5$) | 0.139 | 0.056 |
| Urease ($NH^{3+}$-N) | 0.125 | 0.087 |
| Sucrase | −0.01976 | 0.553 |

The data were used to analyze the correlation between the fungal community structure and physical and chemical factors by integrating data from the four sampling periods. Values marked in bold indicate significance at $p < 0.05$ level.

## 4. Discussion

### *4.1. Impact of Different Fertilization Strategies on the Properties of Soil*

It was quite evident that different fertilization methods altered soil properties, such as P and N. Among them, chemical fertilizer significantly enhanced AP content; soil possesses strong adsorption for phosphorus, which can be released by chemical fertilizer [28]. Furthermore, bio-organic + chemical fertilizer contributed to soil OM and N content in two ways: one was the direct input as the bio-organic fertilizer itself contains OM, and the other is the indirect effect of increasing the OM and N content in the field by increasing crop yield and stubble residue [29].

### *4.2. Impact of Different Fertilization Treatments on Soil Enzymes*

We found that invertase activity was lessened by the application of chemical fertilizer. A previous study noted that pH and invertase activity were significantly positively correlated [30]. Therefrom, we deemed that chemical fertilizer resulted in a reduction of invertase activity via decreasing soil pH, which would be an important direction of future research. Additionally, Liu et al. (2017) pointed out

that as the amount of chemical fertilizer increased or there was too much chemical fertilizer, invertase activity showed a remarkably downward trend [31].

In our experiments, we also observed an increase in phosphatase activity following the application of bio-organic + humic acid, which was because the bio-organic + humic acid not only enhanced soil organic colloids but also provided extra nutrient for soil, thereby ameliorating soil fertility, promoting microorganism reproduction, and indirectly increasing soil phosphatase activity [32,33].

### *4.3. Impact of Fertilization Treatments on Fungal Diversity in Soil*

Our study discovered an interesting phenomenon: fungi species number and Chao 1 richness during the heading stage of maize were notably higher in soil treated with bio-organic + chemical and chemical fertilizer compared with no fertilizer ($p < 0.05$; Table 1). We proposed that this phenomenon may be caused by the addition of chemical fertilizers, which could result in an imbalance of soil nutrients and soil pH, thereby disrupting the normal growth and metabolism of some microorganisms [34]. Yet, fungi may use specialized organs, such as hyphae, to obtain large amounts of nutrients from crop roots or other nutrient sources for their own metabolism [35,36]. This study also could not exclude the influence of the growth period on the fungal community. Soil microbial biomass reached its maximum at the maize heading stage, which might be the reason for processing topdressing of crop in the jointing period (seen material: the addition of urea). Topdressing of crop further caused an increase in soil moisture and available nitrogen, which promoted the strengthening of root metabolism, increased secretions, and led microorganisms to use more nutrients for reproduction. Meanwhile, during the heading stage, the demand for crop nutrients in the soil decreased, thereby boosting soil microbial biomass [37].

### *4.4. Impact of Fertilization Treatments on Fungal Community Structure*

Different fertilization treatments inevitably changed soil conditions which affected the formation and structure of microbial communities [10]. For example, *Humicola* and *Fusarium* abundance decreased with the application of bio-organic + humic acid, which further supported the viewpoint of Song et al. (2018), who found that *Humicola* and *Fusarium* abundance was negatively correlated with bio-organic + humic acid [38]. *Humicola* and *Fusarium* abundance, major common crop diseases, were reduced, implying that bio-organic humic acid possibly could inhibit the spread of plant pathogens [39]. Another noteworthy result was that *Mortierella* abundance, known as antagonize pathogenic fungi, such as *Atheliales*, seemed to increase with the addition of bio-organic + humic acid, further conforming that the bio-organic + humic acid may obstruct the growth of pathogenic fungi [40]. Moreover, through NMDS and fungal relative abundance analysis, we found that the fungal community was not only impacted by fertilization methods but also the maize growth stage (Figure S3), which was consistent with previous studies [41].

### *4.5. The Relationship between Soil Properties and the Composition of Fungal Communities*

Chemical and bio-organic + humic acid fertilization were closely related with soil indexes (AP, OM), which indirectly led to alterations of the fungal community structure. Maina et al. (2009) found that *Guehomyces* abundance was significantly negatively correlated with AP [42]. Furthermore, a positive connection of AP with chemical fertilizer application was found by Cai et al. (2015) [28]. Based on our results, we came to an assumption that chemical fertilizer may heighten the AP content, thereby decreasing specific fungal taxa like *Guehomyces*. Simultaneously, the fungal community was influenced by bio-organic + humic acid application, which was possibly linked with OM via RDA analysis [43]. *Mortierella*, regarded as an indicator of rich OM and nutrients, was positively correlated with OM [44], which conformed to the results that bio-organic + humic acid fertilization to some extent increased *Mortierella* abundance.

### *4.6. Impact of Different Fertilization Treatment on Soil-Borne Plant Pathogens*

We concluded that the relative abundance of dominant *Fusarium* and *Humicola* genera was to a certain extent decreased with the application of bio-organic + humic acid. Several *Fusarium* species, including *F. oxysporum* Schltdl. (1824) and *F. equiseti* (Corda) Sacc. (1886), are the causal agents of root rot [45], and *Humicola* is the pathogen that induces root rot on other commercial crops [40]. Additionally, we found that the addition of bio-organic + humic acid not only decreased the abundance of pathogens from these fungal genera but also decreased the abundance of relatively minor fungal genera, such as *Nigrospora*. *Nigrospora* is a pathogen that causes crop root rot and is also the causative agent of wilt disease (data not shown; Figure S4) [7]. So, our study provided the hypothesis that bio-organic + humic acid may decrease the population of soil-borne plant pathogens and help to inhibit the prevalence of plant diseases. Further research, such as isolating pathogenic species and pathogen inhibition experiment, is needed to determine if the reductions of these genera would really reduce crop pathogens.

## 5. Conclusions

Our results clearly illustrate that bio-organic + humic and chemical fertilization indirectly alter fungal community structure in black soil via changing soil properties (especially AP). Chemical fertilizer markedly heightened the AP content, thereby decreasing specific fungal taxa like *Guehomyces*. Bio-organic + humic acid fertilization showed a positive connection with OM through RDA analysis, and then OM content was positively associated with *Mortierella* abundance, which was in line with the result that bio-organic + humic acid fertilization to some extent increases *Mortierella* abundance. In addition, we found that bio-organic + humic fertilization decreased the relative abundance of several potential crop pathogens, such as *Fusarium*, *Humicola*, and *Nigrospora*, providing further support for the idea that organic fertilizers might help to control crop disease. Taken together, these findings help to improve our fundamental understanding of the interactions between fertilizers, soil properties, and fungal communities. Additionally, our results may provide a scientific basis for black soil fertility cultivation by applying chemical fertilizer prudently.

**Supplementary Materials:** The following are available online at http://www.mdpi.com/1424-2818/12/12/476/s1, Figure S1: The relative abundance of fungal genera under different treatments and at different maize growth stages, Figure S2: Phylogenetic relationships of communities shown with the relative abundance of dominant fungal genera, Figure S3: Seasonal changes of the relative fungal genera abundances of (a) *Mrakiella*, (b) *Coniochaeta*, and (c) *Chloridium* at the four maize growth stages, Figure S4: Effect of different fertilization methods on the relative abundances of soil pathogen *Nigrospora* at the four maize growth stages, Table S1: Relative abundance (%) of fungal phyla of all soil samples.

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