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Green Manure Return Strategies to Improve Soil Properties and Spring Maize Productivity under Nitrogen Reduction in the North China Plain

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Abstract: In order to study the effect of green manure return for stabilized spring maize (*Zea mays* L.) grain yield (GY) we reduced nitrogen fertilizer input by regulation and examined effects on soil nutrients, enzyme activity, and fungal communities. This two-year field experiment was conducted in the North China Plain. The field experiment was undertaken with a split-plot design; the primary plots were winter fallow (WF) and green manure (GM), and the split-plots were five N application rates of 0 (N0), 189 (N189), 216 (N216), 243 (N243), and 270 (N270) kg ha^{-1}. The results showed that, spring maize GY under GM treatments (GY_{GM}) were significantly increased by 5.38–11.68% more than WF treatment (GY_{WF}), and GY_{WF} and GY_{GM} significantly increased by 35.9–91.5% and 80.1–135.5% across all N treatments. By linear-platform model analysis, spring maize under GM treatments obtained higher GY, reaching 1270.5–14,312.2 kg ha^{-1} with optimized N application rate at 238–265 kg ha^{-1}, which resulted in a GY higher than WF (11,820.0 and 13,654.2 kg ha^{-1}) and N reduced 11.2% (238 vs. 268 kg ha^{-1}). GM treatment significantly increased soil organic carbon by 3.90–12.23% more than WF over all N application rates, and total nitrogen and available nitrogen were significantly increased by 3.79–15.76% and 4.87–17.29%, with total phosphorus and available phosphorus for GM higher than WF by 6.1–13.6% and 9.6–5.3%, respectively. However, there were lesser effects of GM on total potassium and available potassium. Compared to WF, soil catalase, sucrose, urease, and alkaline phosphatase activity were significantly increased by 6.2–16.4%, 5.8–48.1%, 3.3–21.5% and 11.5–82.3%, respectively, over all N application rates under GM over two years. GM increased *Zygomycota* and *Basidiomycota* relative abundances significantly, and reduced *Thielavia*, *unclassified fungi*, and *Podospora* relative abundances by 35.35%, 52.92% and 52.77% more than WF treatment, respectively. In summary, due to the GM return into fields, increased soil nutrients were available, which were positively affected by soil enzyme activity and fungal communities, and reduced nutrient requirements, and so the farmers could obtain a spring maize grain yield higher than 14,000 kg ha^{-1} with a reduced 11.2% N application rate from 268 kg ha^{-1} to 238 kg ha^{-1} by sowing winter green manure for a long time period in the North China Plain.

Keywords: spring maize; green manure return; nitrogen reduction; grain yield; soil enzymes; soil nutrients; fungal communities

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1. Introduction

The North China Plain is the main maize production region, where almost 35–40% of China's maize is produced every year through winter a wheat-summer maize rotation system and a single spring maize system [1]. Nitrogen (N) is one of the most important nutrients for maize growth and yield formation, contributing 30–50% to grain yield increase [2]. Farmers apply large amounts of N fertilizers in order to gain more cereal grain yields in China [3–5]. However, numerous studies have focused on the negative effects of excessive amounts of N fertilizers, Meng et al. reported that excessive N application

results in later harvesting or plant lodging [6], but more seriously, caused negative environmental impacts, such as soil acidification, sutrophication of surface water, nitrate leaching, greenhouse gas emission, etc., leading to the degradation of miscellaneous ecosystem functions [7–13]. Therefore, one of the most urgent issues in agriculture is to study how to coordinate crop production and ensure sustainable ecosystems. To date, numerous studies focused on rational N management measures to improve maize yields and achieve sustainable N utilization [14], such as, the steady-state N balance (SSNB) management system [15], the nutrient expert (NE) system [16], N inhibitors application [17], etc. In recent years, sowing green manure and returning it to the field could achieve fertilizer savings, emission reduction and nutrient loss through endogenous driving [18]. The application of green manure has been widely accepted as a sustainable management practice in agriculture due to its environmentally friendly properties [19–21]. On a global scale, green manure intercropping with main crops saved 26% of N fertilizer, increased surface soil organic carbon by 320 kg ha^{-1} a^{-1}, compensates about 8% of the greenhouse effects, and also reduced N leaching loss by 40−50%, especially in winter when green manure becomes more significant [22]. Green manure increased available soil N, organic matter, available potassium content [23], and can replace 11–32% nitrogen in spring wheat systems in Northwest China. Furthermore, it reduces CH_4 emissions by 3.5%, increases carbon sequestration potential by 21.8% by intercropping with maize, and maintains equal crop yields [24–26].

The decomposition of green manure and the subsequent release of nutrients depends largely on the physical, chemical, and biological aspects of soil [27]. Additionally, green manure had no significant effect on catalase activity, but significantly increased soil sucrase, urease and phosphatase activities by intercropping with spring maize in Northwest China [28]. Soil microbial communities and soil enzyme activity respond much more sensitively to changes in soil management practices compared to total soil organic matter [29]. Previous studies concerning green manure have indicated that the introduction of green manure directly changed the soil carbon and nitrogen input, which is enough to change soil microbial community structures, functions, and related enzyme activity [30–32]. A rich soil microbiome can improve the service functions of terrestrial ecosystems, enhance crop production, and increase plant resistance to global climate changes as well as biological stress and resilience through the improvement of nutrient utilization [33,34]. Soil total phospholipid fatty acids (PLFAs) and bacterial PLFAs were significantly increased with green manure plant years, however, the ratio of fungal to bacterial biomarkers was decreased significantly [23]. The research indicated green manure increased soil microbial biomass C, as well as respiration. Micro-organisms degrade organic matter through the production of diverse extracellular enzymes [27,35]. Maize intercropping with different green manure increased the richness of bacteria and archaea and Chao Index but had no significant effect on fungi [28]. The redundant analysis (RDA) showed that soil available N, organic matter, and available potassium were positively correlated with dominant flora of bacteria and fungi and were negatively correlated with dominant flora of bacteria and fungi with green manure [23].

Water shortage is an urgent problem for crops growth, especially in the North China Plain, where is the underground water extraction limit region in China [36]. In some provinces, winter wheat seeding areas were reduced to recharge underground water, however, ways to compensate for the winter crops' ecological effects also need to be studied. In recent years, sowing green manure over winter reduced water consumption in the North China Plain. How the effects of green manure return on soil properties, and reduced N application on spring maize grian yield formation occurred were limited. Thus, a two-year field experiment was conducted, and the main objectives were to (1) explore how green manure and nitrogen application affected spring maize grain yield, soil nutrients, enzyme activity and fungal communities and (2) understand the amount of N fertilizer green manure can replace to achieve green and stainable production for spring maize in the North China Plain.

2. Materials and Methods

2.1. Site Description and Initial Soil Properties

Site, Climate and Soil

A 2-year field experiment was established in 2019 and 2020, at the High-quality Agricultural Products Development Demonstration Center (117°49′ E, 39°42′ N, 8 m elevation), Tianjin, China. The region has a semiarid continental monsoon climate, with annual mean sunshine hours of 2230 h, a temperature of 11.2 °C, and 240 frost-free days, precipitation of 642 mm, with about 60% of which occurs mainly from June to September. Dry farmland for crop production mostly depends on precipitation in this region. The soil properties of the soil plow layer (0–20 cm) are shown in Table 1.

Table 1. The soil properties of the soil plow layer (0–20 cm).

PH	SOC (g kg^{-1})	TN (g kg^{-1})	TP (g kg^{-1})	TK (g kg^{-1})	AN (g kg^{-1})	AP (g kg^{-1})	AK (g kg^{-1})
8.05	13.40	1.21	0.93	24.50	118.10	111.50	353.28

SOC, soil organic carbon; TN, total nitrogen; TP: total phosphorus; TK: total potassium; AN, available nitrogen; AP: available phosphorus; AK: available K.

2.2. Experimental Design and Field Management

The field experiment was conducted in a split plot design with three replications over 2 years. Each subplot was 8 m × 6 m, and the subplots were separated by 1 m width pathways to avoid cross-contamination and treatment effects. Two planting patterns were utilized: (i) winter fallow–maize (WF) (control, spring maize only), and (ii) green manure (GM)–maize (winter green manure followed by spring maize). During the whole spring maize growing period, a N application rate of 270 kg ha^{-1} 270 (N270, 100%) was conducted by farmers, and another four N application rates were conducted at 0 (N0), 189 (N189, 70%), 216 (N216, 80%) and 243 (N243, 90%) kg ha^{-1}, respectively. The total N was applied at the planting and jointing stages at 70−30% by basal fertilizer and top dressing. Meanwhile, P_2O_5 was applied at 90 kg ha^{-1} and K_2O was applied at 120 kg ha^{-1} at the planting stage. The basal fertilizer was applied by contained biochar-based fertilizer (N-P_2O_5-K_2O: 24%-10%-10%), super-phosphate (P_2O_5: 12%), and potassium sulfate (K_2O: 50%), and the top dressing was by urea (N: 46%).

The winter green manure rape seeds (*Brassica campestris* L., Longyou 12) were sown at 25 kg ha^{-1} via a planter in 25–28 September after spring maize harvest, and no chemical fertilizers were applied during the whole growth period. The green manure rape fresh plants were chopped into pieces and incorporated into the field via a farming rotary cultivator at the full bloom stage in 1–5 May. The spring maize (*Zea mays* L., Zhengdan 958) was sown 18 May, and harvested 22–25 September, respectively. The density of maize seedlings was 67,500 plants ha^{-1}, and the field management was carried out in a similar manner to the common field.

2.3. Measurement Methods

2.3.1. Maize Grain Yield (GY)

At the maize maturity stage, 20 ears from adjacent plants in the middle two rows of each plot were harvested manually and threshed. Then the dry weight and moisture content were measured, and the grain yield was calculated at a moisture content of 14%.

2.3.2. Soil Sampling

At maize jointing, tasseling, filling, and maturity stages, soil from five sites in each plot were sampled (0–20 cm) by a soil auger, and the visible and plant debris were removed, and then thoroughly mixed to form pooled soil samples. The moist pooled samples at maturity stages were dried at room temperature and sieved at 1 mm for total nitrogen (TN), total phosphorus (TP) and total potassium (TK) measurements, at 0.25 mm for available nitrogen

(AN), available p phosphorus (AP) and available potassium (AK) measurement. The moist pooled samples at four-stages were sieved at 0.5 mm for enzyme activity determination. The soil samples at the maturity stage were used to determined fungal communities, and so the moist, pooled soil samples were stored in an ultra-low temperature freezer at −80 °C.

2.3.3. Soil Nutrients

A total of eight soil nutrients were measured, C content (SOC) was measured by the $K_2Cr_2O_7$-H_2SO_4 wet oxidation method [37], TN and AN determined by Kjeldahl and alkali N-proliferation method [38], TP and AP were measured by Mo–Sb colorimetry and $NaHCO_3$ extraction method [39], while TK and AK were measured by a flame photometer method [40].

2.3.4. Soil Enzyme Activity

Additionally, the four soil enzyme activities were analyzed by using relevant kits (Beijing Solarbio science & technology Co., Ltd., Beijing, China), and catalase activity was measured by $KMNO_4$ titration [41,42], sucrase activity was determined by 3,5-dinitrosalicylic acid colorimetry using sucrose as the substrate [43,44], urease activity was measured by indophenol blue colorimetry [45], alkaline phosphatase activity was determined by $C_6H_5Na_2O_4P$ colorimetry using p-nitrophenyl phosphate as the substrate [46,47].

2.3.5. DNA Extraction, PCR-Amplification and Illumina Miseq Sequencing

The DNA of each soil sample was extracted by using the CTAB method according to the manufacturer's instructions, and the total DNA quality and quantity were assessed by agarose gel electrophoresis and an ultraviolet spectrophotometer at LC-Bio Technology Co., Ltd., Hang Zhou, Zhejiang Province, China [48–50].

The primers used for PCR amplification and Illumina sequencing were from the ITS 1 region.

The ITS 1 region was amplified with the primers ITS 1 F (5′-GAACCWGCGGARGGATCA-3′) and ITS 1 R (5′-GCTGCGTTCTTCATCGATGC-3′) [51–53]. The 5′ ends of the primers were tagged with specific barcodes for sample and sequencing universal primers. PCRs were performed in a reaction mixture with a total volume of 25 µL, containing 25 ng of template DNA, 12.5 µL of PCR premix, 2.5 µL of each primer, and PCR-grade water to adjust the volume. The PCR program consisted of an initial denaturation at 98 °C for 30 s, followed by 32 cycles of 98 °C for 10 s, 54 °C for 30 s, and 72 °C for 45 s, and a final extension at 72 °C for 10 min. The PCR products were confirmed through 2% agarose gel electrophoresis. After this, the PCR products were purified by AMPure XT beads (Beckman Coulter Genomics, Danvers, MA, USA) and quantified by Qubit (Invitrogen Corporation, Waltham, MA, USA). The amplicon pools were prepared for sequencing, and the size as well as the quantity of the amplicon library were assessed on an Agilent 2100 Bioanalyzer (Agilent Technologies Co., Santa Clara, CA, USA) and with the Library Quantification Kit for Illumina (Kapa Biosciences, Woburn, MA, USA), respectively. The amplicon libraries were sequenced on a NovaSeq PE250 platform according to the manufacturer's recommendations at LC-Bio Technology Co., Ltd., Hang Zhou, China.

The sequences of barcodes and primers were removed, and reads were paired [54–57]. Paired-end reads were merged using Pear (v0.9.6). Quality filtering on the raw reads was performed under specific filtering conditions to obtain high-quality clean tags according to fqtrim (v0.94). The chimeric sequences were removed by Vsearch software (v2.3.4). After dereplication by using DADA2, we obtained a feature table and a feature sequence. Alpha diversity and beta diversity were calculated by QIIME2, in which the same number of sequences were extracted randomly through reducing the number of sequences to the minimum of some samples, and the relative abundance (X fungi count/total count) is used in fungi taxonomy. Alpha diversity and beta diversity were analyzed by the QIIME2 process, and pictures were drawn by R (v3.5.2). The sequence alignment of species annotation was performed by a QIIME2 plugin feature classifier, and the alignment database was RDP and united.

2.4. Statistical Analysis

Differences in the spring maize yield, soil enzyme activity, soil nutrients, and fungal average relative abundance among the treatments were evaluated by using a two-way analysis of variance (ANOVA) in the SPSS 19.0 software (SPSS Inc., Chicago, IL, USA). Differences between the mean values were evaluated by using Duncan's multiple-range test with a probability of $p < 0.05$.

3. Results

3.1. Mazie Grain Yield

Compared with the maize grain yield (GY) of WF treatment (GY_{WF}), the GY of GM treatment (GY_{GM}) was significantly increased by 5.38% and 11.68% in two years over all N application rates (Figure 1 and Table 2). However, GY_{GM} was higher than GY_{WF} under N243 levels in 2020 ($p < 0.05$), and in 2021 GY_{GM} were significantly higher than GY_{WF} under N0~N243 levels. With N application rate increased, GYs were increased significantly by 35.9–60.7% and 84.4–135.5% more than N0 in 2020 and 2021 under WF treatment, that for GM treatment were 56.1–91.5% and 80.1–107.1% ($p < 0.01$), respectively. Meanwhile, the highest GYs were always at N243 levels, under WF and GM treatments were 11,775–13,682 kg ha^{-1} and 13,105–14,985 kg ha^{-1} in two years, which were significantly higher than N189 and N216 levels.

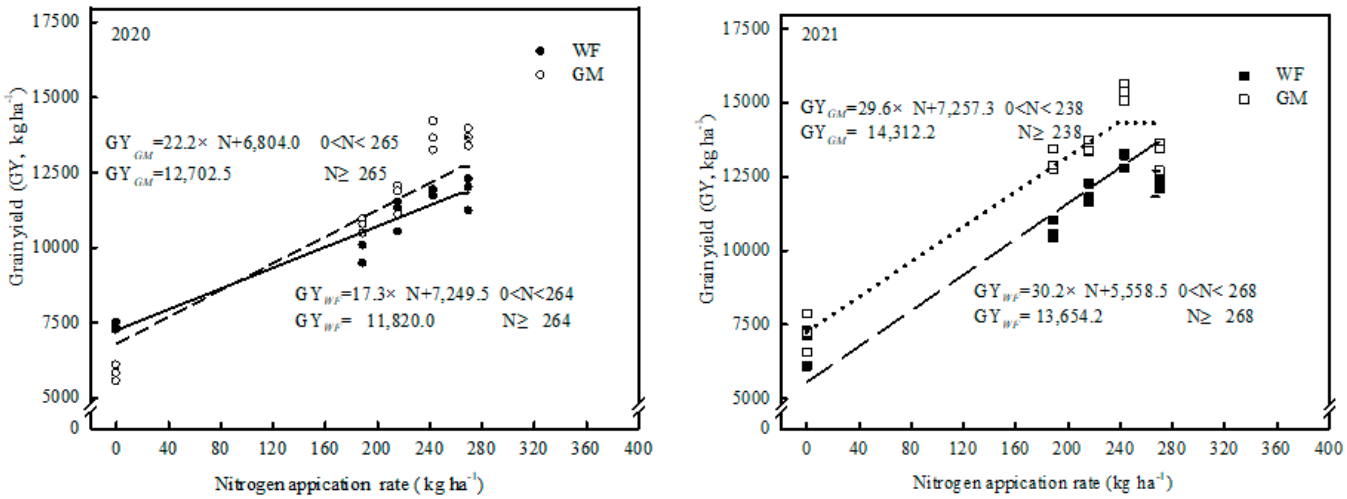


Figure 1. Spring maize grain yield (GY) response to N application under winter fallow (WF) and green manure return (GM) treatments in the North China Plain.

Table 2. The ANOVA for GY under WF and GM treatments.

Years	Sources	Type III Sum of Squares	Degrees of Freedom	Mean Squares	*F* Values	Sig.
2020	GM	2,326,867.5	1	2,326,867.5	14.5	0.001
	N	1.117×10^8	4	27,915,217.5	174.1	0.000
	GM × N	2,724,420.0	4	681,105.0	4.25	0.012
	Error	3,207,300.0	20	160,365.0		
	Sum	3.547×10^9	30			
2021	GM	12,613,973.6	1	12,613,973.6	60.995	0.000
	N	2.257×10^8	4	56,425,058.1	272.846	0.000
	GM × N	6,315,293.9	4	1,578,823.5	7.634	0.001
	Error	4,136,039.3	20	206,802.0		
	Sum	4.392×10^9	30			

The linear + platform function was also used to fit the N application rate and maize GY, which showed that the GY was increased linearly by 17.3–30.2 kg ha^{-1} and 22.2–29.2 kg ha^{-1}

at first, when the N application rate increased by 1 kg ha^{-1}, and then the obtained platform yields were 11,820.0 and 13,654.2 kg ha^{-1}, and 12,702.5 and 14,312.2 kg ha^{-1} under WF and GM treatments in two years, when the N application rate reached the optimized levels of 264 and 268 kg ha^{-1}, and 265 and 238 kg ha^{-1}, respectively. That indicated that in the early period of green manure return, there were no significant effects on maize GY and N application rate reduction (264 vs. 265 kg ha^{-1}), but GYs were increased significantly (14,312.2 vs. 13,654.2 kg ha^{-1}) with N application rate reduced by 11.2% (238 vs. 268 kg ha^{-1}).

3.2. Soil Nutrients

Soil nutrients were varied drastically by green manure return and N application rate (Table 3). The SOC for GM treatments were significantly increased by 12.23% and 3.90% more than WF, respectively, over all N application rate in 2020 and 2021. however, SOC_{GM} was significantly higher than SOC_{WF} under all N levels in 2020, and only at the N243 level in 2021 ($p < 0.05$). With N application rate increased, SOC always displayed an increased tendency, and there was a significant performance difference if N application rate was higher than N243 under WF and GM treatment over two years.

Table 3. The responses of soil chemicals to the application of nitrogen under green manure return strategies in the North China Plain.

		N	SOC (g kg^{-1})	TN (g kg^{-1})	TP (g kg^{-1})	TK (g kg^{-1})	AN (mg kg^{-1})	AP (mg kg^{-1})	AK (mg kg^{-1})
2020	WF	N0	14.00 ± 0.57b	1.05 ± 0.01b	0.72 ± 0.06c	19.95 ± 0.49b	59.16 ± 2.58d	77.00 ± 2.55d	306.24 ± 11.63b
		N189	15.15 ± 0.31b	1.14 ± 0.04ab	0.81 ± 0.02bc	20.12 ± 0.21ab	67.34 ± 2.09c	87.20 ± 0.71c	311.01 ± 4.33ab
		N216	16.05 ± 0.30a	1.14 ± 0.05ab	0.85 ± 0.05ab	20.82 ± 0.82ab	70.98 ± 1.93bc	92.8 ± 1.27bc	317.65 ± 10.2ab
		N243	15.72 ± 0.44a	1.23 ± 0.04a	0.89 ± 0.01ab	21.03 ± 0.28a	81.90 ± 1.93a	96.25 ± 3.18b	325.93 ± 4.97ab
		N270	15.93 ± 0.79a	1.22 ± 0.06a	0.93 ± 0.01a	20.46 ± 0.03ab	75.76 ± 6.76ab	104.65 ± 2.33a	332.38 ± 24.78a
	GM	N0	16.25 ± 0.88b	1.20 ± 0.01c	0.84 ± 0.05b	19.95 ± 0.68b	69.16 ± 0.65c	81.00 ± 7.50c	310.28 ± 3.19b
		N189	16.37 ± 0.12b	1.25 ± 0.03c	0.86 ± 0.04ab	20.33 ± 0.65b	74.39 ± 0.97bc	97.10 ± 2.40b	324.49 ± 9.9ab
		N216	17.59 ± 0.97ab	1.27 ± 0.03bc	0.92 ± 0.07ab	20.42 ± 0.86ab	82.81 ± 5.14ab	104.60 ± 4.10ab	326.88 ± 0.19ab
		N243	18.78 ± 1.11a	1.35 ± 0.03a	0.96 ± 0.02a	21.72 ± 0.65a	90.09 ± 5.79a	116.25 ± 1.20a	345.82 ± 1.11a
		N270	17.25 ± 0.33ab	1.32 ± 0.03 ab	0.88 ± 0ab	20.87 ± 0.65ab	83.73 ± 2.58ab	104.05 ± 5.44ab	342.55 ± 19.42a
	G		34.66 **	63.14 **	6.78 *	1.00 ^{ns}	25.43 **	34.30 **	1.00 ^{ns}
	N		5.43 *	15.10 **	7.38 **	5.00 **	18.15 **	41.55 **	5.00 **
	G × N		1.28 ^{ns}	0.28 ^{ns}	4.68 *	0.48 ^{ns}	0.214 ^{ns}	2.57 ^{ns}	1.00 ^{ns}
2021	WF	N0	12.48 ± 0.07c	0.91 ± 0.02b	0.76 ± 0.04c	20.07 ± 0.46a	57.33 ± 2.08c	71.05 ± 1.20b	262.35 ± 16.57b
		N189	14.89 ± 0.36b	1.14 ± 0.02a	0.78 ± 0.02bc	19.56 ± 0.83a	61.74 ± 2.08bc	78.15 ± 1.06ab	273.32 ± 5.49ab
		N216	14.94 ± 0.58ab	1.15 ± 0.05a	0.83 ± 0.01ab	20.22 ± 0.61a	65.42 ± 3.12b	88.50 ± 3.11a	283.25 ± 1.92a
		N243	15.35 ± 0.54ab	1.15 ± 0.02a	0.84 ± 0.01ab	19.76 ± 0.42a	77.18 ± 3.12a	91.95 ± 2.47a	290.5 ± 4.31a
		N270	15.76 ± 0.13a	1.21 ± 0.01a	0.86 ± 0.04a	19.51 ± 0.56a	73.47 ± 0.04a	93.8 ± 4.38a	288.38 ± 9.34a
	GM	N0	13.21 ± 0.08c	1.08 ± 0.01b	0.82 ± 0.05c	19.83 ± 0.54a	58.37 ± 2.28c	70.65 ± 5.30c	276.81 ± 2.25b
		N189	15.12 ± 0.7b	1.11 ± 0.07b	0.86 ± 0.01c	20.37 ± 0.85a	62.62 ± 5.40b	84.00 ± 1.27b	291.66 ± 14.12ab
		N216	15.64 ± 0.73ab	1.23 ± 0.03a	0.88 ± 0.05bc	19.84 ± 1.09a	77.18 ± 1.04ab	94.90 ± 1.56a	292.63 ± 8.9ab
		N243	16.54 ± 0.33a	1.24 ± 0.07a	1.07 ± 0.02a	19.72 ± 0.28a	82.77 ± 0.63a	98.35 ± 0.07a	303.83 ± 16.43a
		N270	15.89 ± 0.44ab	1.23 ± 0.06a	0.99 ± 0.10ab	19.85 ± 0.16a	72.77 ± 5.20b	97.15 ± 3.61a	294.02 ± 3.36ab
	G		13.99 **	14.02 **	44.78 **	0.17 ^{ns}	5.16 *	9.27 *	7.94 *
	N		41.40 **	16.89 **	14.08 **	0.35 ^{ns}	27.28 **	34.69 **	4.95 *
	G × N		2.00 ^{ns}	2.73 ^{ns}	4.23 *	0.87 ^{ns}	2.24 ^{ns}	0.90 ^{ns}	0.24 ^{ns}

SOC, soil organic carbon; TN, total nitrogen; TP: total phosphorus; TK: total potassium; AN, available nitrogen; AP: available phosphorus; AK: available K. Different letters following values in the same column are significantly different between N treatments at the $p < 0.05$ *, difference significant at $p < 0.05$; **, Difference significant at $p < 0.01$; ns, no significant difference.

Compared with WF treatments, the TN and AN for GM treatments were significantly increased by 3.79–15.76% and 4.87–17.29%, respectively, over all N application rates in two years. With N application rate increased, TN and AN occurred in the order of N243 > N270 > N216 > N189 > N0, were increased significantly by 9.3–31.9% and 7.7–38.4% than N0 under WF treatment in two years, those for GM treatment were 3.2–15.3% and 7.2–41.8% ($p < 0.01$), respectively.

The TP and AP for GM treatments in 2020 and 2021 were significantly higher than WF by 6.1% and 13.6%, and 9.6% and 5.3%, respectively, over all N application rates. The TP and AP were increased with N application rate increased, which were increased significantly by 2.74–28.3% and 10.0–35.9%, and 1.2–31.2% and 18.9–43.5% more than N0

under WF and GM treatment in two years, respectively. The highest was always at N243 or N270 levels which were significantly higher than N189.

In addition, there were no significant differences between WF and GM on TK over all N levels in two years, and only TK at the N243 level was significantly higher than N0 and N189 in 2020. However, the AK under GM treatments was significantly increased by 4.36% more than WF in 2021, and with N application the rate increased with AK highest with N243, which was higher than N0, although there were no significant differences among N levels.

3.3. Soil Enzyme Activity

The soil enzyme activities during spring maize growth stages always expressed a significant difference between WF and GM treatments, and among different N application rates, however, they were not significantly affected by interaction between GM and N levels, over two years (Table 4).

Table 4. The ANOVA for soil catalase, sucrose, urease, and alkaline phosphatase activity at different growth stages under WF and GM treatments in 2020 and 2021.

Soil Enzyme Activity	Years	Sources	V6		VT		R1		R6	
			F Values	Sig.	*F* Values	Sig.	*F* Values	Sig.	*F* Values	Sig.
Catalase	2020	GM	20.53	0.001	9.99	0.007	27.77	0	8.95	0.011
		N	29.83	0.000	5.43	0.006	3.82	0.030	3.91	0.025
		GM × N	4.67	0.026	0.621	0.687	0.89	0.526	0.88	0.523
	2021	GM	32.89	0	7.69	0.021	15.48	0.002	8.18	0.016
		N	2.68	0.087	11.41	0.001	8.56	0.002	6.77	0.004
		GM × N	0.49	0.773	0.25	0.932	0.36	0.863	0.62	0.69
Sucrose	2020	GM	215.31	0.000	289.26	0	80.41	0	67.50	0.000
		N	34.19	0.000	112.46	0	5.11	0.011	17.34	0.000
		GM × N	0.63	0.650	16.02	0	1.23	0.359	1.81	0.191
	2021	GM	18.25	0	227.40	0	118.99	0	196.42	0
		N	0.46	0.799	57.09	0	18.63	0	19.45	0
		GM × N	0.60	0.699	5.54	0.007	4.74	0.011	3.05	0.057
Urease	2020	GM	4.93	0.040	31.50	0	26.66	0	13.08	0.002
		N	6.34	0.002	20.37	0	2.97	0.038	1.06	0.407
		GM × N	0.61	0.663	0.938	0.485	1.20	0.345	0.54	0.744
	2021	GM	36.68	0	17.88	0.001	5.88	0.036	21.37	0
		N	10.92	0.001	9.212	0.001	20.47	0	29.39	0
		GM × N	0.99	0.471	0.34	0.88	1.12	0.408	17.23	0
Alkaline Phosphatase	2020	GM	93.49	0	101.49	0	68.86	0	187.46	0
		N	2.13	0.169	6.78	0.003	1.89	0.161	8.82	0.001
		GM × N	0.88	0.515	2.40	0.094	1.02	0.444	0.86	0.532
	2021	GM	108.80	0	46.36	0	69.69	0	105.44	0
		N	13.29	0	13.33	0	2.78	0.068	20.62	0
		GM × N	4.75	0.008	4.02	0.022	0.54	0.741	3.27	0.043

V6, spring maize jointing stage; VT, spring maize tasseling stage; R1, spring maize filling stage; R6, spring maize maturity stage.

Specifically, compared to WF, the soil catalase activities (s-CA) were significantly increased by 9.3–16.4%, 6.2–9.3%, 10.2–12.6% and 4.9–8.9%, respectively, at four growth stages over all N application rates under GM over two years, which always significantly decreased with maize growth (Table 4 and Figure 2). During spring maize total growth duration, and s-CA were significantly increased, by 7.3–23.4% and 8.1–21.9% with N application, the rate increased more than N0 under WF treatments in 2020 and 2021, that for GF were 7.4–26.1% and 7.6–20.5%. Meanwhile, s-CA at N243 levels were the highest and had a significant difference to N189, no significant difference to N216 or N270 under WF and GM treatments over two years.

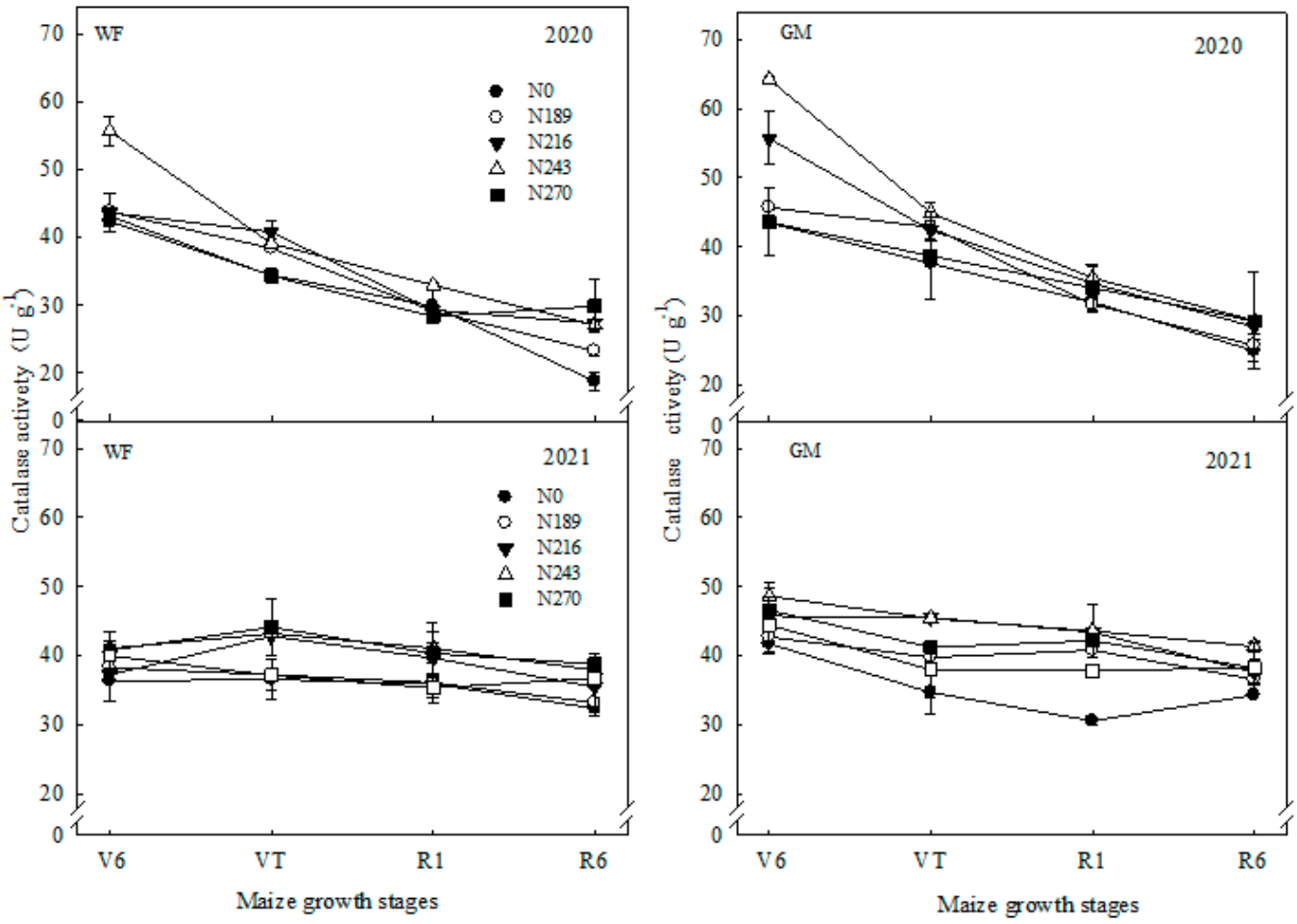


Figure 2. The soil catalase activity response to N application under WF and GM treatments in the North China Plain. V6, spring maize jointing stage; VT, spring maize tasseling stage; R1, spring maize filling stage; R6, spring maize maturity stage.

The soil sucrase activity (s-SA) performance increased initially and then decreased at the VT stage, which was significantly higher by 27.4–43.2%, 11.3–120.2%, 15.2–51.7% and 8.7–160.0% than V6, R1 and R6 stages under WF and GM treatments over two years (Table 4 and Figure 3). Additionally, s-SAs for WF were significantly decreased compared to GM by 31.4% and 5.8%, 33.8% and 25.0%, 48.1% and 24.2%, and 25.8% and 29.3%, respectively, at four growth stages over all N application rates in the years 2020 and 2021 (Table 3 and Figure 2). During the spring maize total growth duration, with N application rate increased, s-SA were significantly increased by 16.4–45.9% and 23.8–38.4% compared to N0 under WF treatments over two years, and for GM were 15.0–27.1% and 7.2–32.1%. However, the s-SA at N189 levels were significantly lower than at N216–N270, with a reduced range from 8.9% to 25.4% and 4.3% to 11.8%, from 9.3% to 10.5% and 12.6% to 22.4%, respectively, under WF and GM treatments in 2020 and 2021.

The soil urease activity (s-UA) was extremely reduced in 2021 compared to 2020 (Table 4 and Figure 4). With spring maize growth, the s-UA performance increased initially and then decreased, and in 2020 the highest s-UA at the R1 stage was significantly increased by 28.6–42.4% and 30.5–47.7% compared to the V6, VT and R6 stages under WF and GM treatments. Meanwhile, in 2021 the highest s-UA at the VT stage was significantly increased by 6.1–57.4% and 13.8–42.1% compared to the V6, R1 and R6 stages under WF and GM treatments (Figure 4). In addition, s-UA for WF was significantly decreased compared to GM by 3.3% and 14.7%, 5.6% and 12.9%, 7.2%and 5.3%, and 3.0% and 21.5%, respectively, at four growth stages over all N application rates in two years (Table 3 and Figure 2). Over spring maize total growth duration, s-APA was increased with increased N application

rate, and under WF treatment were significantly increased by 10.3–26.1% and 5.1–20.0% in 2020 and 2021 compared with N0, and those for GM were 1.9–13.7% ($p < 0.05$) and 3.9–21.8% ($p < 0.05$). However, only in 2021, were the s-UA at N243–N270 levels under WF significantly higher than N189 and N216 by 17.5–30.5% and 12.9–25.4%, while under GM treatments N243 was significantly raised by 31.9%,16.3% and 13.0% compared to N189, N243 and N270, respectively.

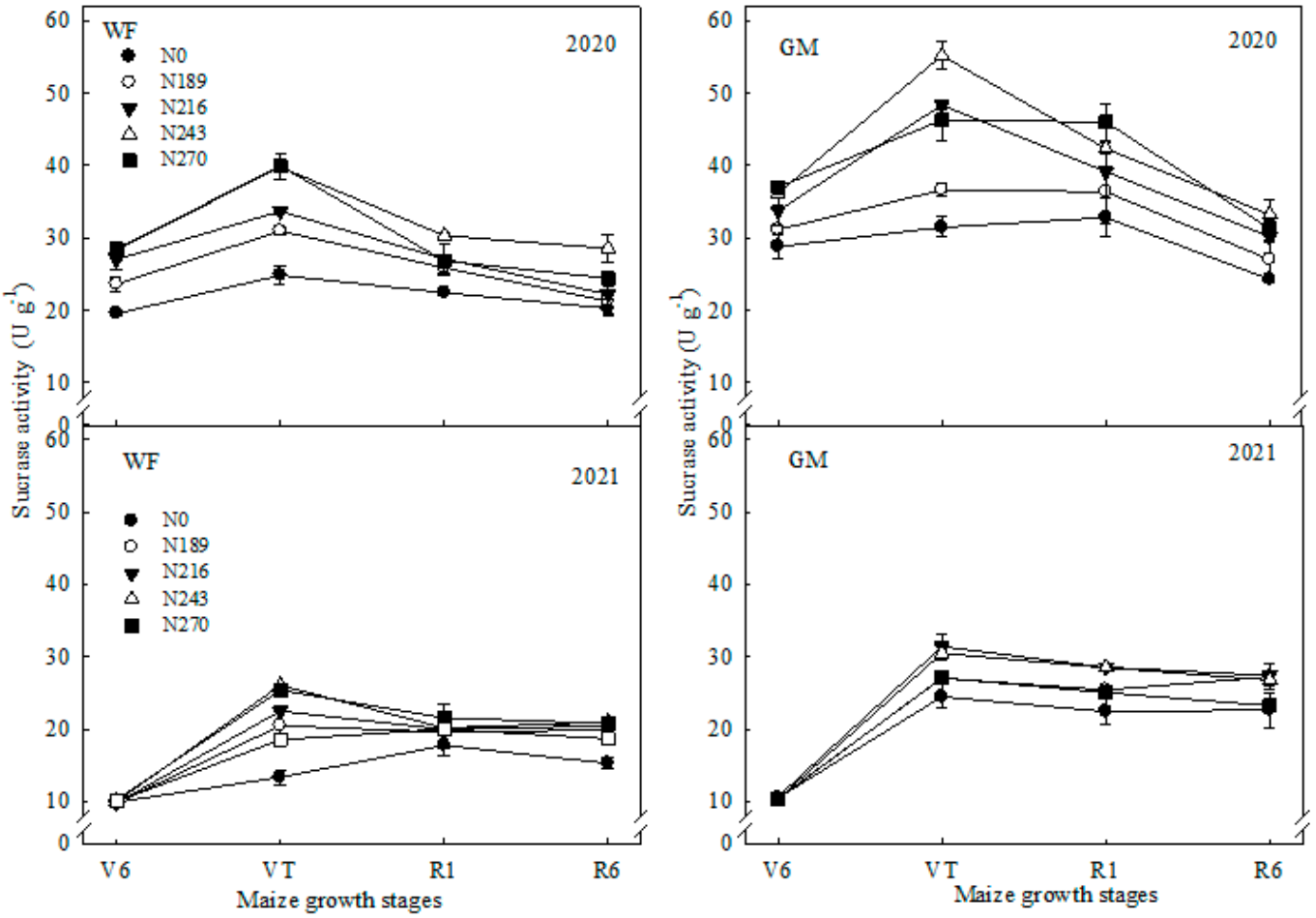


Figure 3. The soil sucrase activity response to N application under WF and GM treatments in the North China Plain. V6, spring maize jointing stage; VT, spring maize tasseling stage; R1, spring maize filling stage; R6, spring maize maturity stage.

There were significant differences in soil alkaline phosphatase activity (s-APA) between the two study years (Table 4 and Figure 5). The s-APA performance increased initially and then decreased, with the highest values obtained at the R1 stage where significant rises of 17.6–77.3% and 5.0–29.4%, and 5.7–14.1% and 0.9–27.2% compared to the other three stages under WF and GM treatments over two years (Figure 5). Additionally, s-APA at V6, VT, R1 and R6 stages under GM treatments were significantly increased by 82.3% and 18.3%, 22.0% and 11.5%, 17.42%and 16.3%, and 29.6% and 19.8%, respectively, compared to WF over all N application rates over two years (Table 3 and Figure 2). With N application rate increased, s-APA were increased by 5.6–9.1% ($p < 0.05$) and 17.9–53.8% ($p < 0.05$), and 1.9–2.8% ($p > 0.05$) and 21.4–60.2% ($p < 0.05$) compared to N0 under WF and GM treatments over two years. Additionally, there were only significant differences between N243 and N189 under WF and GM treatments over two years.

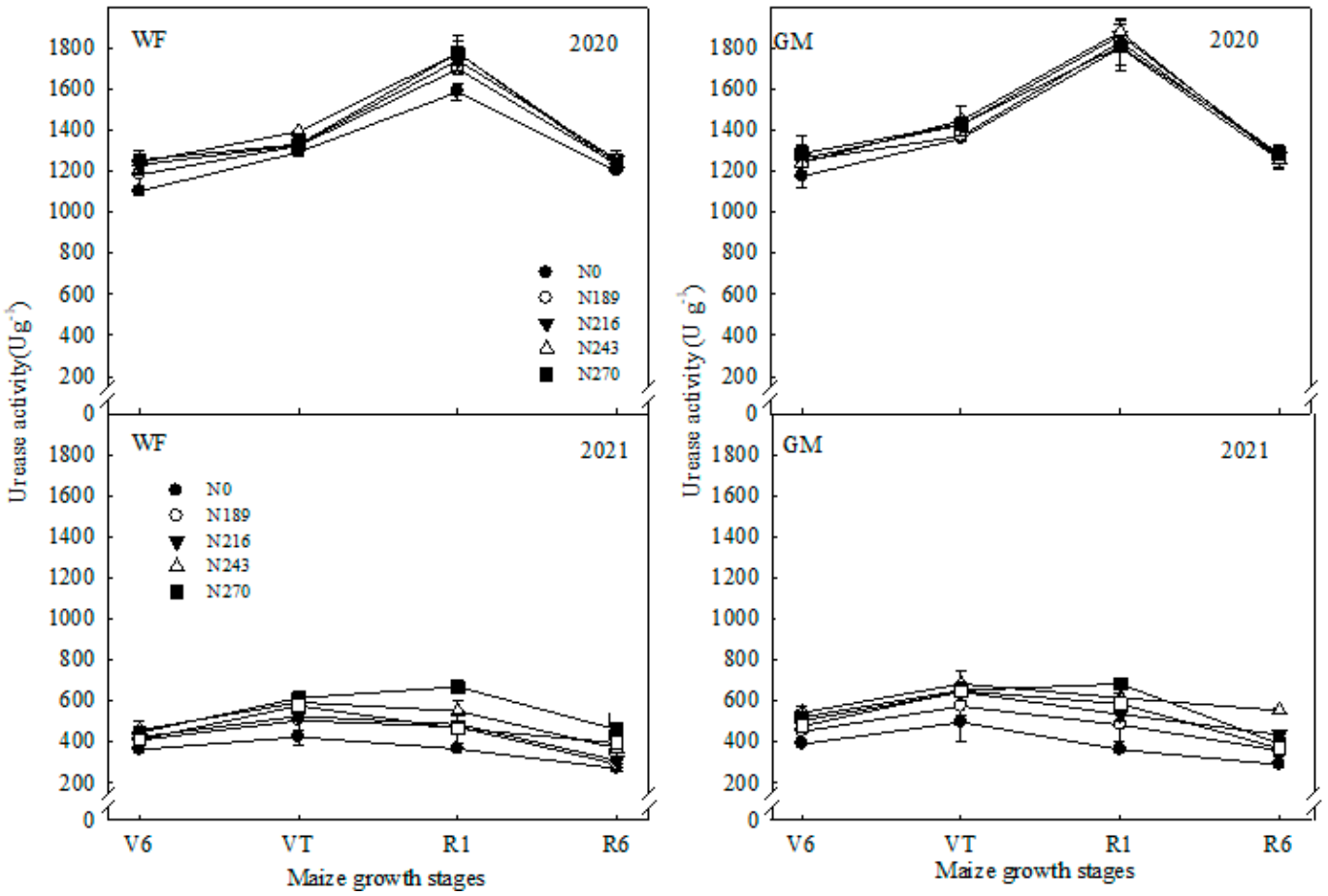


Figure 4. The soil urease activity response to N application under WF and GM treatments in the North China Plain. V6, spring maize jointing stage; VT, spring maize tasseling stage; R1, spring maize filling stage; R6, spring maize maturity stage.

The dominant fungal phyla at the phylum level were *Ascomycota* (85.06% average relative abundance), *unclassified fungi* (6.29%), *Zygomycota* (5.50%), and *Basidiomycota* (1.39%), across all the N application rates under WF and GM treatments (Figure 6A), as well as at the genus level were *Thielavia* (29.35% average relative abundance), unclassified *fungi* (8.66%), *Podospora* (7.39%), *Gibberella* (5.37%), and *Mortierella* (5.04%) (Figure 6B).

The WF and GM treatments and N application rates, and the interactions significantly affected the relative abundances of some dominant fungi at the phylum and genus levels, which were related to soil nutrients such as SOC and AP (Figure 7). By cluster analysis, compared to WF, GM return without N (GMN0) decreased the unclassified fungal relative abundance at the phylum and genus levels. Regardless of N application, fungal communities forming a distinctive group were strongly altered by GM, which was significantly different to that of the WF treatment (Bray–Curtis distance analysis, Figure 7). At the phylum level, *Ascomycota* was the dominant fungal order in all of the N applications rate treatments, but GM reduced their abundance (all abundances are relative abundances in this MS) (Figure 7A). Compared to WF, GM treatments also decreased the abundance of *Chytridiomycota*, but increased the abundance of *Basidiomycota* and *Zygomycota* over all N application rates. At the gene level, the averages of *Thielavia*, *unclassified fungi*, and *Podospora* under GM treatment were significantly reduced by 35.35%, 52.92%, and 52.77% compared to WF treatment, respectively (Figure 7B). For *Mortierella*, the amount under GM treatment was 47.37% higher than that of the WF treatment ($p < 0.05$).

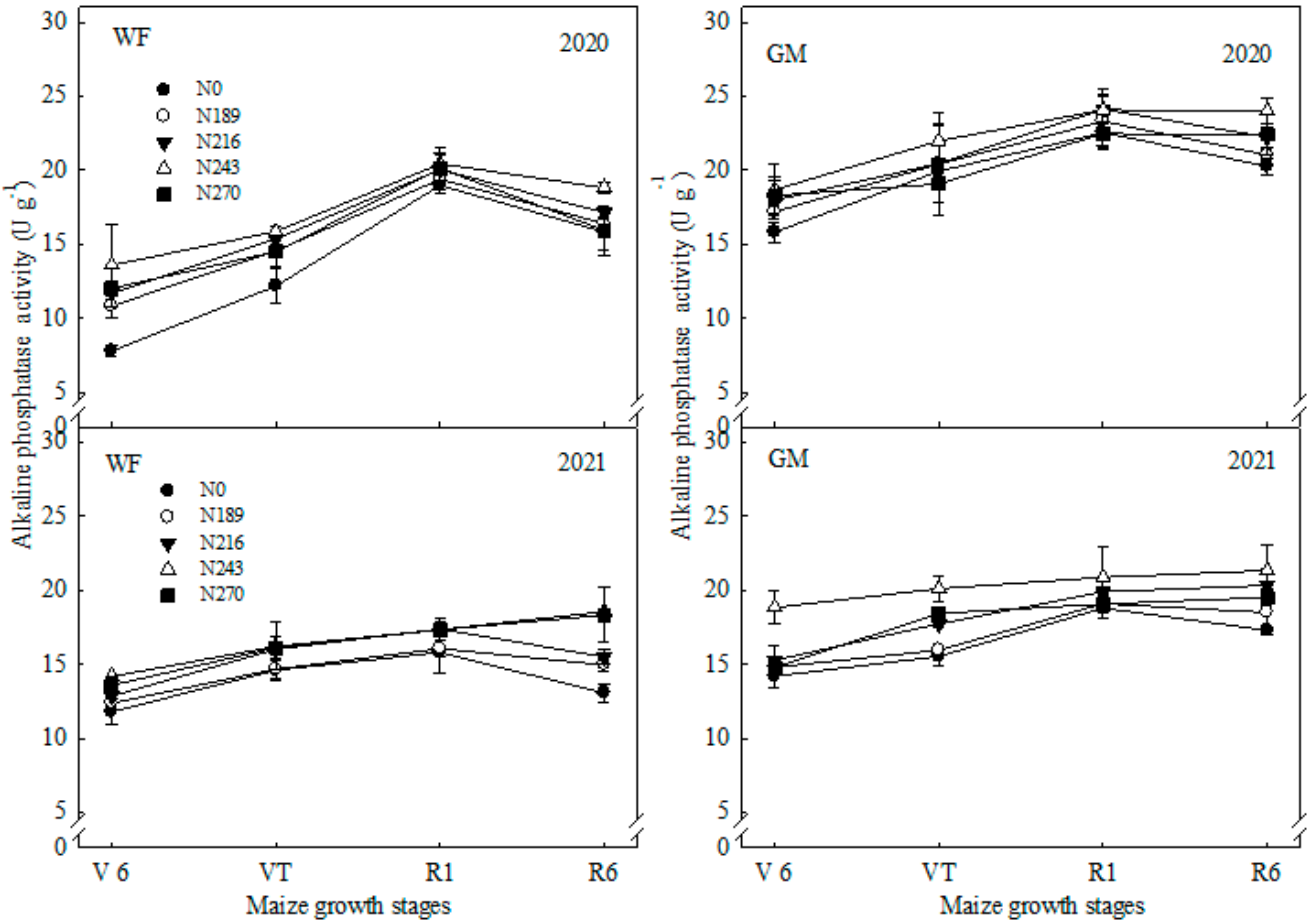


Figure 5. The soil alkaline phosphatase activity response to N application under WF and GM treatments in the North China Plain. V6, spring maize jointing stage; VT, spring maize tasseling stage; R1, spring maize filling stage; R6, spring maize maturity stage.

3.4. Link between Soil Microbial Community Structures and Environmental Factors

Using RDA analyses (RDAs) to determine the total variation in soil fungal community structures and their responses to the WF and GM treatments, and N application rates, we showed that soil fungal structures were significantly correlated with environmental factors across all treatments in the study (Figure 8). For soil fungi, the first and second RDA axes explained 49.82% and 17.97% of fungal community variation, respectively. In particular, the s-CA, s-SA and s-APA, as well as the SOC and TN levels, were significantly associated with fungal community variation. Generally, the contribution of soil enzyme activity to fungal community variation were significantly larger than that of soil nutrients. Furthermore, soil enzyme activities were positively correlated with the soil nutrients. Under WF treatment, the N application rate significantly affected the fungal community at the genus level, meanwhile under GM treatments with reduction of the N application rate, fungal community structures were significantly different from those of N270 (Figure 8).

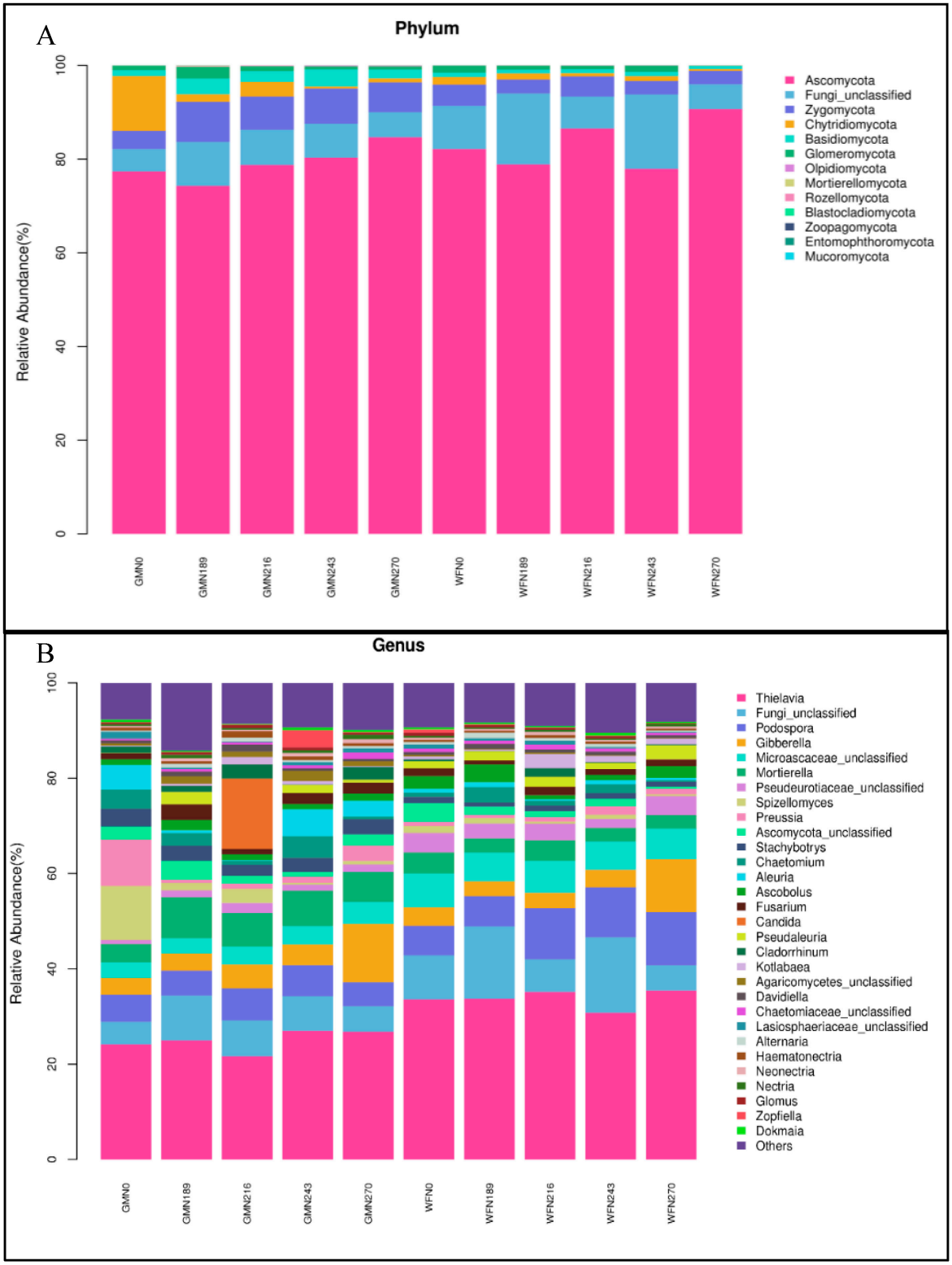


Figure 6. The soil fungal community's response to N application under WF and GM treatments in the North China Plain. (**A**), Phylum level; (**B**), Genus level.

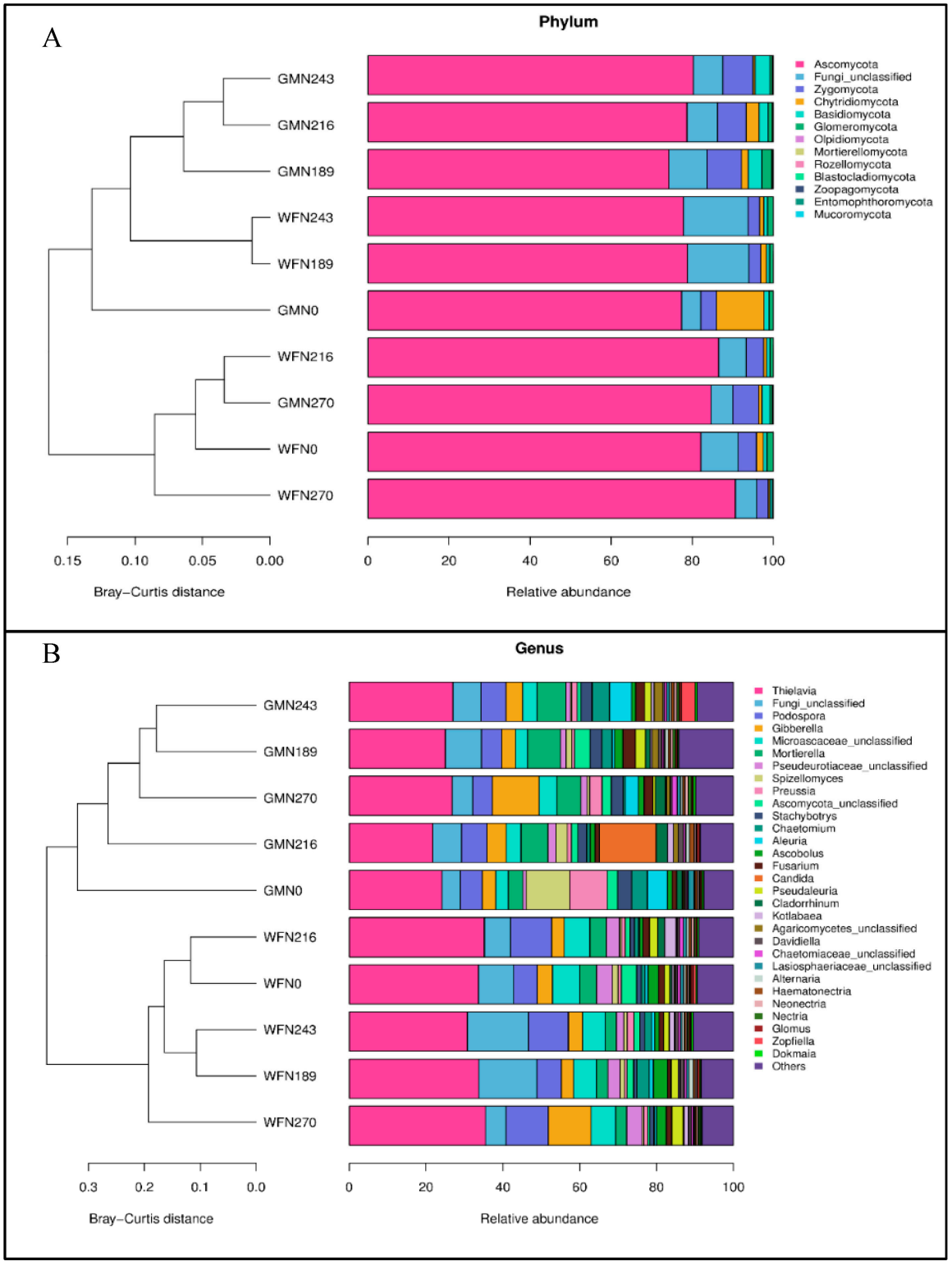


Figure 7. The soil fungal phylum and genus abundance group clusters response to N application under WF and GM treatments in the North China Plain. (**A**), Phylum level; (**B**), Genus level.

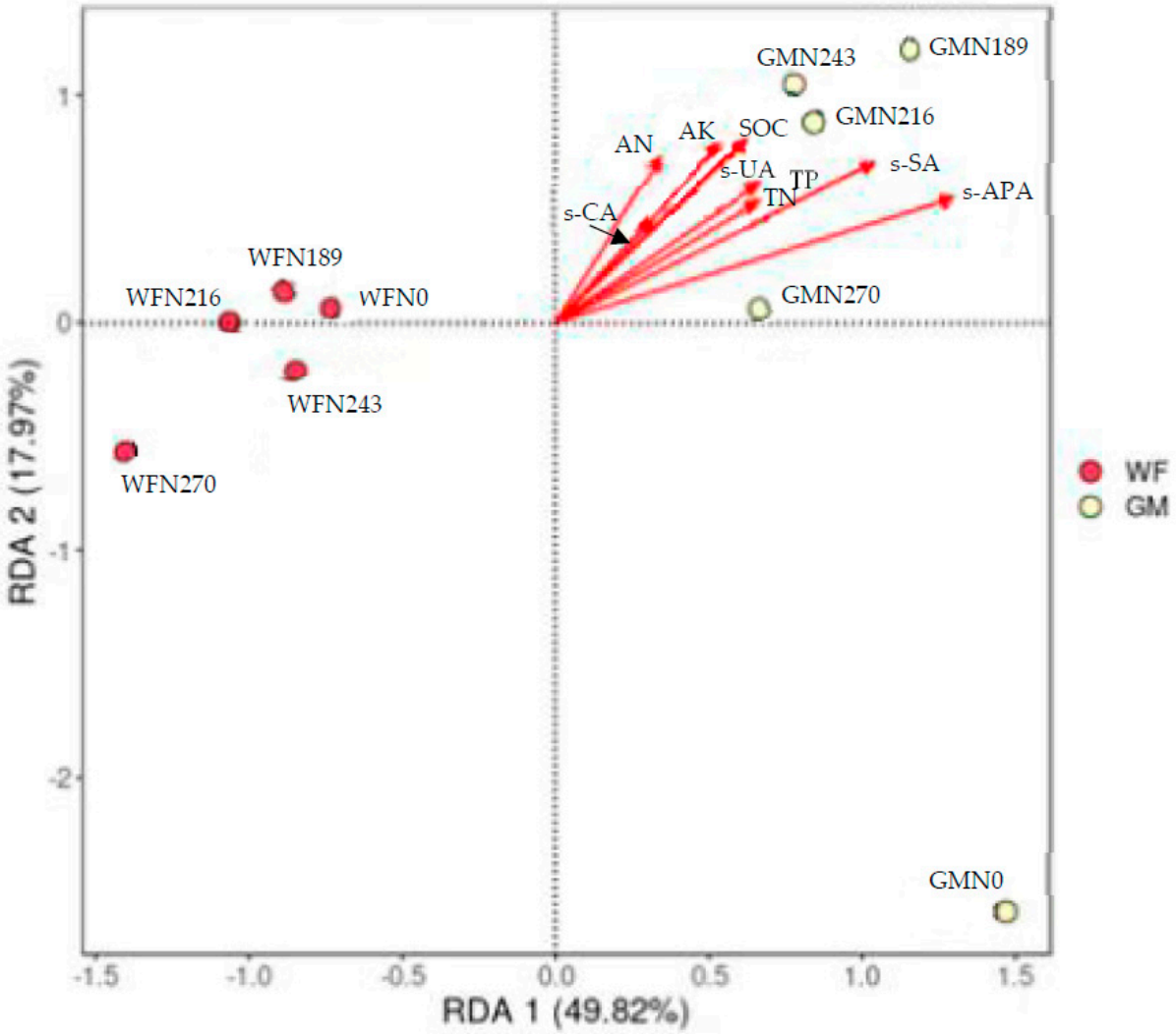


Figure 8. RDA analysis of soil microbial community structures and environmental factors. The red arrow line length represents the affect strength of environmental factors on soil microbial community structures SOC, soil organic carbon; TN, total nitrogen; TP: total phosphorus; AN, available nitrogen; AK: available potassium; s-CA, soil catalase activities; s-SA, soil sucrase activity; s-UA, soil uease activity; s-APA, soil alkaline phosphatase activity.

4. Discussion

Numerous studies have shown that crops grain yied (GY) could be improved by about 30–50% attributed to N application [2,58], especially in North China Plain [59–61]. The present results indicate that N treatments always increased spring maize GY by 35.9–91.5% and 80.1–135.5% in two years, respectively (Figure 1). Meanwhile, the GY with N application reduced 10% to N243, averaged over two years were the highest at 11,775–13,682 kg ha^{-1} and 13,105–14,985 kg ha^{-1}, respectively. In a recent study, spring maize GY under GM treatments (GY_{GM}) were significantly increased by 5.38–11.68% compared to WF treatment (GY_{WF}), which is supported by previous studies which reported that long-term use of astragalus smicus as green manure, can simultaneously improve double-rice GY and its stability [62], photo yield [23], and spring wheat GY in the Northwest China [25]. Generally, a linear-platform model has always been used for identifying the optimal N application for maximum crop yield [63]. In this study, we estimated an optimal N application rate of 264–268 kg ha^{-1} and 238–265 kg ha^{-1} for spring maize under WF and GM treatments, respectively. GY increased more under GM compared to WF (17.3–30.2 kg ha^{-1} vs. 22.2–29.2 kg ha^{-1}), and maximum GY was

1820.0–13,654.2 kg ha^{-1} and 1270.5–14,312.2 kg ha^{-1} (Figure 1), which indicated that GM return little affected maize GY and N application rate reduction in the first year, but GY was increased significantly (14,312.2 vs. 13,654.2 kg ha^{-1}) with N application rate reduced by 11.2% (238 vs. 268 kg ha^{-1}) over two years. This agreed with previous studies reported that long-term use of astragalus smicus can change 0–40% N and K fertilizer in the early rice season and 0–20% N and K fertilizer in the late rice season [62], and replace N 11–32% in spring wheat systems [25], and even in different GM return years, increased total yield differently [23].

In recent years, GM return to the field could achieve fertilizer savings and nutrient loss through endogenous driving [18]. Winter GM affected nitrogen transformation processes by regulating soil N; SOC and TN increased by 4.36% and 6.47% compared to single fertilizer application [62–64], and even increased soil AN and AK contents [23], and increased C sequestration potential by 21.8% in maize-GM intercropping systemd [24,25]. In this study, GM treatment significantly increased SOC by 3.90–12.23% compared to the WF overall N application rate in two years, and TN and AN were significantly increased by 3.79–15.76% and 4.87–17.29%, with TP and AP for GM higher than WF by 6.1–13.6% and 9.6–5.3%, respectively. However, there were less effects of GM on TK and AK. The different results with previous studies for TK and AK may be due to the research area, with South China having paddy soil and North China having dry land, and crop varieties such as rice and spring maize. With N application rate increased, SOC, TN and AN, TP and AP, and AK always played an increased tendency, which showed a significant difference if the N application rate was higher than N243, and under GM treatment was higher than WF at the same N levels over two years. The results were supported by recent studies showing that optimized N management can be associated with soil nutrient improvement [65].

Soil enzyme activities have been recognized as indicators of soil fertility and soil quality, and are responsible for SOC transformation and nutrient cycling [66,67]. The soil catalase activity (s-CA) and sucrase activity (s-SA) is related to the C cycle [68,69], soil urease activity (s-UA) plays an important role in the N cycle [70,71]. Soil alkaline phosphatase activity (s-APA) is secreted by microorganisms that mineralize unstable forms of organophosphate, which also become an important source for the mineralizing of soil organic phosphorus [72,73]. In the previous studies, GM had no significant effect on s-CA, but significantly increased s-SA, s-UA and s-APA in spring maize–GM intercropping [28]. In this study we showed that, compared to WF, s-CA, s-SA, s-UA and s-APA were significantly increased by 6.2–16.4%, 5.8–48.1%, 3.3–21.5% and 11.5–82.3%, respectively, over all N application rates under GM over two years. The soil enzymes' activity always increased initially and then decreased with spring maize growth stages and reached the highest values at the VT or R1 stages. With N application rates increased, the soil enzymes' activity increased significantly, and the N216 and N243 levels were the most suitable. These results were confirmed with previous studies that soil enzymes' activities decompose SOC to acquire energy for microbial growth [74], GM return increases substrate availability and supports microbial growth and leads to enhanced enzyme activity compared to that of inorganic fertilizers or no fertilizers [74,75].

Microbial diversity plays a crucial role in influencing ecosystem stability, productivity, and resilience towards stress [76], with soil microbial diversity increasing with long periods of C input [77]. Previous studies indicated that GM changed the soil C and N input, which were enough to change soil microbial community structures and functions [30–32]. Our results indicate that the fungal alpha diversity hardly changed with GM and N application, and the fungal community diversities significantly increased with GM at each N level, especially at N189, which is inconsistent with the results reported by Royta et al. [78]. In this study, GM and N application increased beneficial fungal microbes' relative abundance, but decreased harmful microbes' relative abundance, especially *Zygomycota* and *Basidiomycota* relative abundances which were significantly increased at the phylum level (Figure 7A). The results agree with previous studies, which have indicated that *Zygomycota* are oligotrophic microbes (K strategy) and dominate residue decompositions of highly

recalcitrant fractions [79,80], and *Basidiomycota* can quickly metabolize organic substrates deposited in the rhizosphere with the soil C cycle [81]. In a recent study, the averages of *Thielavia*, *unclassified fungi*, and *Podospora* under GM treatment were significantly reduced by 35.35%, 52.92% and 52.77% compared to WF treatment, respectively, while *Mortierella* were significantly increased by 47.37%. at the gene level (Figure 7B). To date, numerous studies indicated that, *Mortierella* fungal microbes stimulate the insoluble phosphorus in soil and promote the absorption of mineral elements [82]. GM can significantly inhibit a large colony of pathogenic *fungi* [83], but another report suggested that GM had no significant effect on *fungi* and increased bacteria and archaea richness [28]. The GM can significantly increase the number of soil microbial populations and change the soil microbial compositions of biological groups [84].

The redundant analysis (RDA) showed that the changes in the soil fungal community were explained to the greatest extent by soil enzyme activity, SOC, and TN, supporting similar conclusions of other studies [23,85]. In this study, soil fungal structures were significantly correlated with environmental factors across all treatments by the RDAs (Figure 8). For soil fungi, the first and second RDA axes explained 49.82% and 17.97% of fungal community variation, respectively. Nutrients release in soil from decaying SOC under GM increased soil enzyme activity [86], which was confirmed in a recent study showing that the s-CA, s-SA and s-APA as well as the SOC and TN levels, were significantly associated with fungal community variation. GM changes fungal communities, thus increasing enzyme activity [19,87], which could be attributable to the fact that GM and N provides an energy- and food-rich living environment for fungi [88,89], and in our study the N application rate significantly affected the fungal community at the genus level under WF treatments. Meanwhile, fungal community structures were significantly reduced with N application rate compared to N270 under GM treatments (Figure 8).

5. Conclusions

Our results demonstrated that, with green manure returned to the field, soil organic carbon, total nitrogen, available nitrogen, total phosphorus, available phosphorus were significantly increased, potentially due to the green manure providing nutrient requirements for soil enzymatic (catalase, sucrose, urease, and alkaline phosphatase) activity, and soil fungal communities, which can improve soil nutrient performance feedback. This can allow farmers to obtain spring maize grain yields higher than 14,000 kg ha^{-1} with reduced (11.2%) N application rates from 268 kg ha^{-1} to 238 kg ha^{-1} by consistently sowing winter green manure in the North China Plain.

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