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Response of Soil Nitrogen-Cycling Genes to the Coupling Effects of Arbuscular Mycorrhizal Fungi Inoculation and Biochar Application in Maize Rhizosphere

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Abstract: Nitrogen (N) is the primary element that limits crop growth, and improving the nitrogen uptake in crops is a key challenge in sustainable agricultural production. Arbuscular mycorrhizal fungi (AMF), as important symbiotic microbes associated with most plants, can facilitate nitrogen uptake by plants and reduce greenhouse gas emissions, meaning they can play an important role in the development of sustainable agriculture. However, the effects of biochar application on mediating AMF N absorption are not clear, especially regarding the functional genes related to the N cycle in soil. In this study, we conducted a pot experiment with two P application rates (−P and +P) to study the effects of biochar and AMF on the community of soil microorganisms and N-cycle genes using metagenomic methods. The N uptake of both the shoots and roots of maize was measured. It was observed that the N uptake in the maize shoots and roots was significantly increased when they were exposed to a combination of AMF and biochar. Under both the −P and +P application rates, the root weights of the AMF and biochar combined (AMBC) treatments increased significantly by 58.3% and 43.2%, respectively, compared with the control (CN) treatments. Furthermore, there were significant increases in the root lengths, of 78.43% and 53.09%, respectively, as well as increases in the superficial areas of 60.0% and 41.9%, respectively. The combination treatment significantly changed the soil microbe community structure and increased the abundances of *Geobacter* and *Pseudomonas*. In addition, the abundances of the N-cycle genes of each process were enhanced. Under the −P condition, the total abundances of the N-cycle genes increased significantly by 1.97–2.19 times in the AMBC treatment compared with the CN treatment. Overall, the results suggest that biochar and AMF can promote plant root growth and lead to changes in the soil microorganism structure, resulting in an increase in the abundances of N-cycle genes which, in turn, increase the N uptake in the shoots and roots of maize. This study provides a biological pathway to improve the efficiency of N utilization in soil and prevent environmental pollution in sustainable agricultural production.

Keywords: biochar; AMF; maize; N-cycle microbes; sustainable agriculture

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1. Introduction

Nitrogen (N) is a crucial element for the growth of plants and microbes, and its cycle is an important biogeochemical process in soil ecosystems that not only affects plant growth but also the development of sustainable agricultural production. Plants acquire nutrients not just through intricate molecular pathways but also by modifying rhizosphere microbial community composition, such as by recruiting arbuscular mycorrhizal fungi (AMF) and N-fixing bacteria through the secretion of root exudates to enhance nutrient accessibility [1]. AMF can set up a symbiotic relationship with more than 80% of terrestrial plants, meaning AMF play an important role in facilitating the plant uptake of nutrients, especially P and N [2,3]. Some studies suggested that beyond the mycorrhizal pathway affecting nutrient

uptake, AM fungal hyphae can release compounds and attract diverse microorganisms from the bulk soil to the rhizosphere, facilitating organic matter decomposition and enhancing N absorption by plants [4]. Hestrin et al. (2019) found that the synergies between AMF and soil microbial communities increased the ability of plants to acquire N from organic matter [5,6]. Recent studies have indicated that AMF not only enhance organic matter decomposition to help plants uptake N but that they are also capable of upregulating the expression of the *nifH* gene in soil microorganisms, contributing to N fixation and influencing the soil N cycle [7]. The *nifH* gene plays a pivotal role in the process of soil N fixation [8–10]. The process of N fixation involves microorganisms that possess the *nifH*, *nifD*, and *nifK* genes that convert N_2 back into NH_4^+, which then enters the soil N cycle. Some research has found that AMF not only upregulate the abundance of the *nifH* gene to aid in soil N fixation but also downregulate the abundance of the *nirK* and *nosZ* genes to decrease the denitrification (DE) process [11,12]. Gui et al. (2021) found that AMF can downregulate the abundances of the *nirK* and *nosZ* genes to reduce the N_2O flux to enhance the soil N content, which reduces greenhouse gas emissions and could slow the trend of global warming [11]. The DE process primarily converts useful plant nitrates into unabsorbable N oxides, NO, and N_2 through a range of genes. However, this symbiotic relationship between AMF and plants is regulated by the soil's physical and chemical properties, further affecting plant nutrient uptake. For example, the soil's moisture and available P content are critical regulators of the AMF–plant symbiotic relationship [13,14]. Previous research has consistently shown that low levels of available P content in soil can enhance AMF colonization, thereby increasing the uptake of macro- and micronutrients by plants [15]. A recent study revealed that plants have the ability to detect soil inorganic phosphate (Pi) content through specific proteins, wherein AMF colonization in the roots is promoted under low-Pi conditions and is inhibited when Pi is abundant [16]. These findings imply that the symbiotic relationship between AMF and plants is influenced by the soil's available P contents and their associated conditions, which could influence plants to absorb N elements and associated genes related to the N cycle of soil.

Biochar is a carbon-rich soil amendment produced through the pyrolysis of organic matter, and its application to soil has been demonstrated to impact the soil structure and nutrient-cycling processes [17]. Some studies have shown that the application of biochar can significantly increase the colonization rate of AMF in plants [18]. Hammer et al. (2014) also presents evidence showcasing the potential of biochar as a physical growth matrix and nutrient source that promote the growth of AMF in soil [19]. Additionally, biochar has the potential to enhance microbial habitats and to act as a carbon source, thereby impacting microbial communities [20,21]. Several studies have consistently demonstrated that the application of biochar significantly increases the soil microorganism diversity and upregulates the expressions of genes involved in regulating the soil N cycle, such as the *amoA* gene. This gene is crucial for converting ammonium (NH_4^+) into nitrate (NO_3^-) during the nitrification (NI) process [22–24]. However, a recent study found that applying biochar resulted in a decreased microbial composition and functional-gene presence under high Pi input, likely due to a significant alteration in the soil's carbon-to-phosphorus (C:P) ratio [25]. Nonetheless, it is evident that biochar enhances the AMF colonization of roots, impacting soil microorganisms [26]. Various studies have indicated the distinct effects of biochar and AMF on soil microorganisms, but these effects could depend on the soil's available P conditions. The impact of AMF on the N uptake by host plants is closely related to the composition, structure, and function of rhizosphere microorganisms. When revealing the promotion of plant N uptake via AMF, the relationship between soil microorganisms and environmental factors should be considered. Therefore, comprehending the interactive effects of AMF inoculation and biochar application on soil microbial structure and related N-cycling genes under different soil Pi conditions is necessary to understand how biochar mediates AMF to help plants absorb the N nutrient, which is influenced by soil properties.

The objectives of this study were to examine the combined effects of biochar and AMF application on (1) the N uptake and root morphology of maize, (2) the structure of

the soil microorganisms, and (3) the abundance of the functional genes regulating each process of N conversion in the N cycle of the soil microorganisms under two P application conditions. Through the elucidation of the interactions between AMF inoculation, biochar application, and the two P conditions in microbial N cycling within the rhizosphere soil of maize, this study provides insights into sustainable agricultural practices that contribute to enhancements in N-use efficiency to optimize agricultural production systems.

2. Material and Methods

2.1. Soil Characteristics

The soil used in our study was recognized as Mollisol under the classification of the United States Department of Agriculture (USDA). It was obtained from the tillage surface layer (0–20 cm) at the Acheng experimental site of the Northeast Agricultural University in Heilongjiang Province, People's Republic of China. Before use, the soil was air-dried, and any surface impurities, such as stones, plant roots, and straw, were removed. The soil was then sieved through a 2 mm sieve. The physicochemical characteristics of the soil were as follows: total N: 1.23 g/kg; available N: 108.5 mg/kg; organic matter: 33.4 g/kg; available P: 25.6 mg/kg; available potassium (K): 149.2 mg/kg; pH: 6.4 (1:2.5 *w/v* water).

2.2. Materials of Maize, AM Fungus, and Biochar

The plant tested in this experiment was maize, and the variety was *Zea mays L. cv Xianyu 335*. The AM fungus used in this experiment was *Glomus etunicatum* [BGC NM03F, 1511C001BGCAM0041] purchased from the Institute of Plant Nutrition and Resources, the Beijing Academy of Agriculture and Forestry Sciences, China. Rice straw biochar was selected as the soil conditioner in this experiment, and it was purchased from Nanjing Sanju Biomaterials Co., Ltd., Nanjing, China. The basic characteristics of the biochar were as follows: carbon (C) content: 636.7 g/kg; N content: 4.21 g/kg; P content: 0.55 g/kg; pH value: 7.38 (1:1 H_2O); cation exchange capacity (CEC): 34.21 cmol/kg; ash content: 11.70 g/kg; specific surface area: 224.24 m^2/g; average pore volume: 0.11 cm^3/g; average pore diameter: 2.66 nm.

2.3. Experiment Design

A pot experiment was designed to study the impact of the combination of biochar and AMF on maize growth and N uptake. The experiment included 8 treatments under 2 P (P_2O_5) application rates, which were a P addition of 30 mg/kg (+P) and without the P addition (−P), as follows: CN-P and CN+P: without AM fungus inoculation and biochar application; AM-P and AM+P: AM fungus inoculation alone; BC-P and BC+P: biochar application alone; AMBC-P and AMBC+P: both AM fungus inoculation and biochar application. All treatments were replicated 3 times, resulting in a total of 24 pots, each with a height of 25 cm and a diameter of 26 cm. Each pot was filled with 10 kg of air-dried soil. The application rate for both the N and potassium (K_2O) was 100 mg/kg of soil, the biochar was added at a rate of 20 g/kg soil, and the AM fungus inoculum, including spores ($\geq$70/g), hyphae, and infected plant root fragments, was mixed into the soil at a rate of 20 g/kg. NH_4NO_3 was employed as the N fertilizer, $Ca(H_2PO_4)_2$ as the P fertilizer, and K_2SO_4 as the potassium fertilizer. These fertilizers were applied as basal fertilizers to the soil before the maize sowing. In each pot, 3 maize seeds were sown, and only 1 plant was retained per pot after reaching the third-leaf stage. The soil moisture content in each pot was maintained at 70% of the maximum field capacity to ensure optimal soil water conditions. The experiment was conducted from 11 June to 19 August 2021.

2.4. Samples Collection

After the maize was harvested, the aboveground parts and roots were separated. The roots were carefully cleaned to remove any adhering soil particles and thoroughly rinsed with distilled water. For measuring the dry weights of the plants, samples were initially heated at 105 °C for 30 min and were then dried at 80 °C until a constant weight was

achieved. The dried plant samples were subsequently cooled and weighed to determine their dry weights. Then, the samples were ground to a fine powder and stored in a desiccator at room temperature for measuring the N concentrations. The N concentrations were later determined through digestion using a mixture of H_2SO_4 and H_2O_2. The N content was determined using the Kjeldahl method [27]. To determine the amount of N taken up by the plants, the concentration of N in the plant tissue was multiplied by the dry biomass of the plant sample [28].

To collect the rhizosphere soil, the roots were gently shaken to dislodge the surrounding soil. Any remaining soil adhering to the roots was removed. A portion of the soil samples was transferred into 10 mL centrifugal tubes and promptly stored at −80 °C for the subsequent genomic DNA analysis. The other soil samples were air-dried at room temperature for further analysis of their physicochemical characteristics.

2.5. Soil Microbial DNA Analysis

Genomic DNA was extracted from the soil samples using the E.Z.N.A.® Soil DNA Kit (Omega Bio-tek, Norcross, GA, USA) according to the manufacturer's protocol. The concentration and purity of the extracted DNA were evaluated using a TBS-380 spectrophotometer and NanoDrop2000 spectrophotometer, respectively. Agarose gel electrophoresis was performed to verify the quality of the extracted DNA. Subsequently, the DNA was randomly fragmented to an average size of approximately 400 bp using Covaris M220 (Gene Company Limited, Hong Kong, China) for the construction of paired-end libraries. The NEXTFLEX Rapid DNA-Seq kit (Bioo Scientific, Austin, TX, USA) was utilized for ligating adapters containing sequencing primer hybridization sites to the blunt ends of the DNA fragments during library preparation. The paired-end library was sequenced on an Illumina Novaseq 6000 sequencer (Illumina Inc., San Diego, CA, USA) at Majorbio Bio-Pharm Technology Co., Ltd., using NovaSeq Reagent Kits (ZhongBeiLinGe, Beijing, China) following the manufacturer's instructions. The sequence data from this study have been deposited in the NCBI Short Read Archive database under the accession number PRJNA804252.

2.6. Statistical Analysis and Data Processing

The R software (version 4.3.1) was used to conduct the statistical analysis. A two-factor ANOVA was used to assess the differences among the treatments. The significant differences were determined using Duncan's multiple range test ($p < 0.05$). The "ggplot2 package" in R was used for the visual analysis. Nonmetric multidimensional scaling (NMDS) and a permutational multivariate analysis of variance (PERMANOVA) were used to determine the microbiome assembly (R, v4.2.1, vegan package). The original readings of the shotgun sequencing were uploaded to MG-RAST for the reading quality control process [29]. Pretreatment of the uploaded readings included the removal of manual readings, host-specific sequences, and other ambiguous base pairs. The BLAST algorithm and M5NR database were used for the gene annotation.

3. Results

3.1. N Uptake and Root Morphology of Maize

Significant differences were observed in the maize shoot N uptake under both the −P and +P application rates (Figure 1). Both the AM fungus inoculation and biochar application significantly influenced the shoot N uptake in the maize under both P conditions, with a notable interaction between these treatments. In the AMBC+P treatment, the shoot N uptake in the maize increased significantly by 2.32 times compared with that of the CN+P treatment. Similarly, under the −P application condition, the AMBC-P treatment notably increased the shoot N uptake by 2.35 times compared with the CN-P treatment ($p < 0.05$).

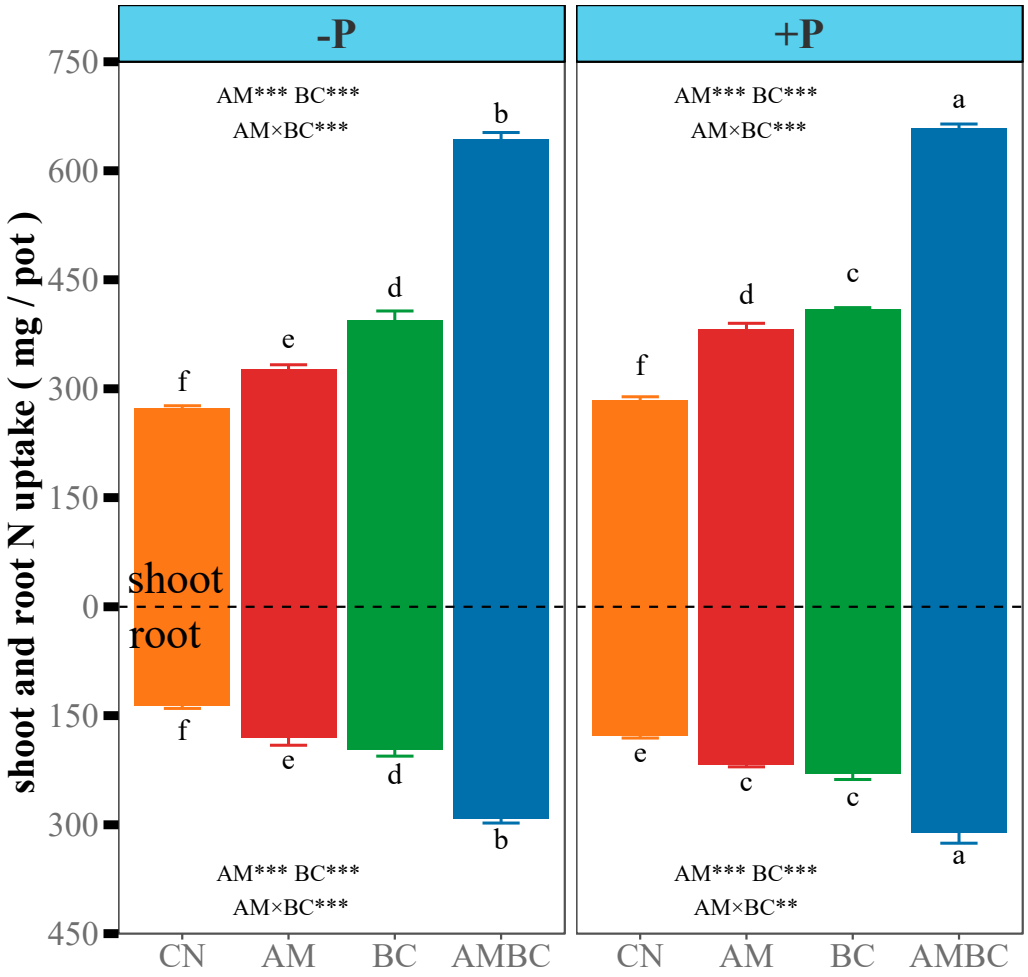

Figure 1. The coupled effects of AMF and biochar on N uptake in the shoot and root of maize under two P application rates. CN: without AMF inoculation and biochar application; AM: AMF inoculation alone; BC: biochar application alone; AMBC: both AMF inoculation and biochar application; −P: without P addition; +P: P addition of 30 mg/kg; AM: AM fungi; BC: biochar; AM × BC: interaction between AM fungi and biochar. NS means not significant, ** means significant difference at 0.01 level, *** means significant difference at 0.001 level. Different lowercase letters in the same column indicate significant differences in different treatments ($p \leq 0.05$).

Similar patterns were observed in the root N uptake. Both the AM fungus inoculation and biochar application significantly affected the root N uptake in the maize under both P conditions, showing a substantial interaction between the two treatments. In the AMBC+P treatment, the root N uptake in the maize increased significantly by 1.75 times compared with that of the CN+P treatment. Under the −P condition, the AMBC-P treatment significantly increased the root N uptake by 2.18 times compared with the CN-P treatment.

The root morphology of maize can impact the N uptake efficiency from the soil. Our findings indicated significant differences among the various treatments under both P conditions, as revealed by the root morphology (Table 1). Under both the −P and +P conditions, the root weights of the AMBC treatments increased significantly by 58.31% and 43.19%, respectively, compared with the NC treatments, and there were significant increases of 78.43% and 53.09% in the root lengths, respectively, 59.99% and 41.86% in the superficial areas, respectively, and 98.34% and 77.37% in the root volumes, respectively. The ANOVA results indicate that both the AMF and biochar significantly influenced the root morphology and demonstrated an interaction between them.

Table 1. Effect of biochar and arbuscular mycorrhizal fungi (AMF) inoculation on root characteristics of maize under two P application rates.

Treatment		Root Weight	Root Length	Root Superficial Area	Root Volume
		g/Plant	cm	cm^2	cm^3
−P	NC	21.66 ± 0.38 e	3896 ± 414 e	17,260 ± 1262 e	1148 ± 77 e
	AM	24.01 ± 0.72 d	5759 ± 414 c	22,392 ± 1297 c	1869 ± 127 bc
	BC	23.83 ± 0.55 d	4647 ± 332 d	19,925 ± 1067 d	1420 ± 172 de
	AMBC	34.29 ± 0.46 b	6952 ± 279 ab	27,442 ± 1157 ab	2277 ± 167 a
+P	NC	24.77 ± 0.49 d	4722 ± 217 d	19,904 ± 625 d	1445 ± 150 de
	AM	27.47 ± 0.69 c	6501 ± 232 b	25,761 ± 880 b	1915 ± 78 b
	BC	27.83 ± 1.01 c	4775 ± 220 d	19,699 ± 610 d	1552 ± 57 cd
	AMBC	35.47 ± 0.87 a	7229 ± 274 a	28,236 ± 1334 a	2563 ± 435 a
ANOVA (p value)					
P		***	**	**	*
AM		***	***	***	***
BC		***	***	***	***
P × AM		*	NS	NS	NS
P × BC		NS	*	**	NS
AM × BC		**	*	*	*
P × AM × BC		*	NS	NS	NS

Different lowercase letters in the same column indicate significant differences in different treatments ($p \leq 0.05$). P: phosphorus (P) application level; AM: AM fungi; BC: biochar; P × AM: interaction between the P application level and AM fungi; P × BC: interaction between the P application level and biochar; AM × BC: interaction between AM fungi and biochar; P × AM × BC: interaction among the above three factors. NS means not significant, * means significant difference at 0.05 level, ** means significant difference at 0.01 level, *** means significant difference at 0.001 level.

3.2. Soil Microbial Network Analysis

Initially, we established separate symbiotic networks for microbial communities under the two P conditions to investigate the influence of AMF and biochar on the soil microbial communities (Figure 2a,b). Based on co-occurrence network analysis, significant species segregation was observed among the different treatments under both P levels. Under the −P condition (Figure 2a), the CN-P treatment significantly influenced the microorganisms within module 2, while the AM-P treatment significantly impacted the microorganisms within module 3. Although both the BC-P and AMBC-P treatments markedly influenced the microorganisms within module 1, there was obvious segregation between the influenced microorganisms of the two treatments. Under the +P condition (Figure 2b), the BC+P treatment exhibited a substantial impact on the microorganisms within module 3, the CN+P treatment exhibited a considerable effect on the microorganisms within module 2, and the AMBC+P treatment exhibited a significant influence on the microorganisms within module 1. These modules represent groups of microbes that frequently co-occur and may play similar ecological roles or interact closely within the soil ecosystem.

The microbial composition, at the genus level, of different modules exhibited significant differences under the different P conditions (Figure 2c,d). Under the −P condition (Figure 2c), *Geobacter*, *Burkholderia*, and *Anaeromyxobacter* were the dominant genera in module 1, *Streptomyces*, *Mycobacterium*, and *Conexibacter* were the dominant genera in module 2, and *Sinorhizobium*, *Rhizobium*, and *Agrobacterium* were the dominant genera in module 3. Under the +P condition (Figure 2d), *Bradyrhizobium*, *Geobacter*, and *Burkholderia* were the dominant genera in module 1, *Candidatus*, *Solibacter*, *Rhodopseudomonas*, and *Gemmatimonas* were the dominant genera in module 2, and *Nitrobacter*, *Symbiobacterium*, and *Nitrococcus* were the dominant genera in module 3.

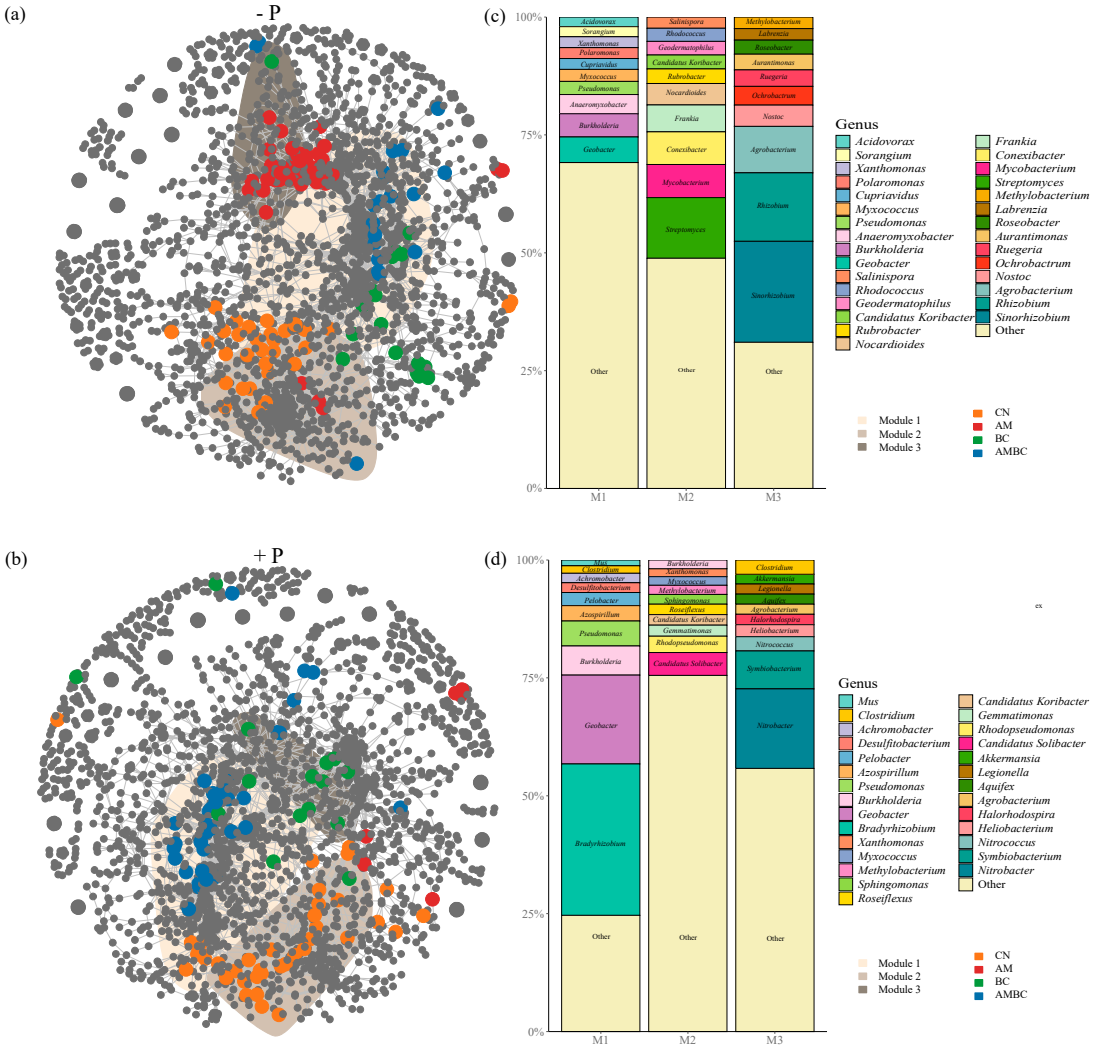

Figure 2. The coupled effects of AMF and biochar on microbial structure in the rhizosphere of maize (**a**,**b**); the microbial composition at genus level of different modules (**c**,**d**) under two P application rates. Other numbers in column stand for the sum of low-abundance microbes at genus level; CN: without AMF inoculation and biochar application; AM: AMF inoculation alone; BC: biochar application alone; AMBC: both AMF inoculation and biochar application; −P: without P addition; +P: P addition of 30 mg/kg; M1: module 1; M2: module 2; M3: module 3.

3.3. Abundances and Structures of N-Cycle Genes in Each Process

In all the samples, 136,520 genes related to N cycling were detected. The NMDS analysis conducted for N-cycling genes revealed stress values consistently below 0.06 under both P conditions, indicating the excellent fit of the model (Figure 3a). Under +P conditions, although there was no significant difference in the structures of the N-cycling genes, there was clear clustering among the same treatments. Under the −P condition, not only was there evident clustering, but there was also a significant difference between the AMBC treatment and the other treatments. The PERMANOVA provided statistical evidence supporting the hypothesis that, under the −P condition, the combined AM fungus inoculation and biochar application has a significant impact on the composition and structure of soil N-cycling genes ($p < 0.05$).

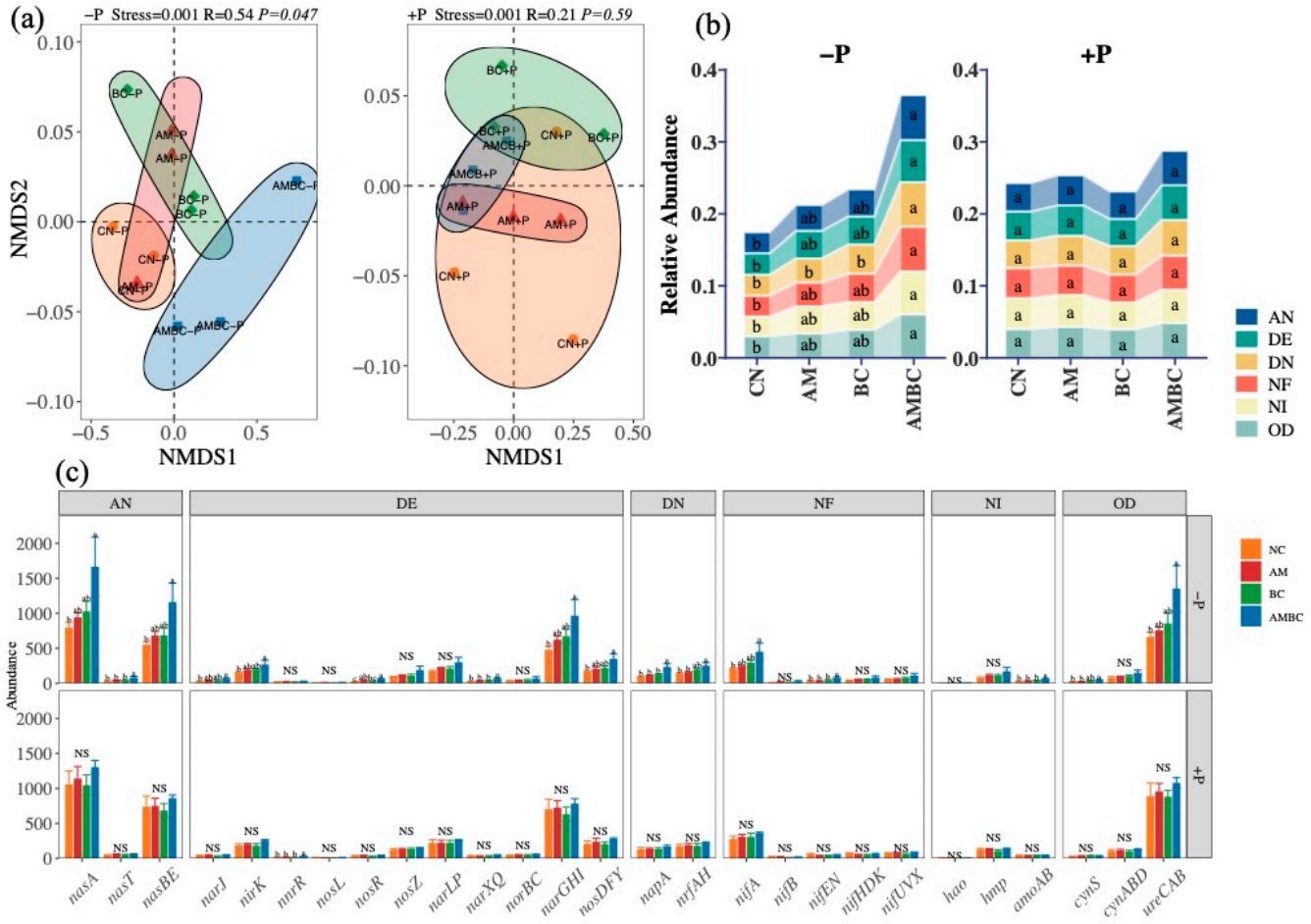

Figure 3. The coupled effects of AMF and biochar on the structures of N-cycle genes analyzed through NMDS; (**a**), the relative abundance of six N-cycle process genes and (**b**) the abundance of individual genes involving six N-cycle processes (**c**) under two levels of P application. CN: without AMF inoculation and biochar application; AM: AMF inoculation alone; BC: biochar application alone; AMBC: both AMF inoculation and biochar application; −P: without P addition; +P: P addition of 30 mg/kg; NI: nitrification process gene; AN: assimilation of nitrate process gene; DN: dissimilation of nitrate process gene; DE: denitrification process gene; NF: N fixation process gene; OD: organic decomposition process gene. Different lowercase letters in the same column indicate significant differences in different treatments ($p \leq 0.05$); NS means not significant.

All the N-cycle genes were classified according to six processes based on their functions: the assimilation of nitrate (AN), the dissimilation of nitrate (DN), denitrification (DE), organic decomposition (OD), N_2 fixation (NF), and nitrification (NI). Distinct differences were noted among the treatments regarding the gene expressions related to the six N-cycling processes (Figure 3b). The treatment involving simultaneous AM fungus and biochar application upregulated gene expression across the six N-cycling processes compared to the other treatments under both P conditions. Under the −P condition, the AMBC-P treatment demonstrated substantial increases in gene abundances compared to the CN-P treatment: the AN-process genes increased by 2.11 times; the DE-process genes increased by 1.97 times; the DN-process genes increased by 2.13 times; the NF-process genes increased by 2.14 times; the NI-process genes increased by 2.19 times; and the OD-process genes increased by 2.03 times ($p < 0.05$). Under the +P condition, the AMBC+P treatment exhibited a 1.21-fold increase in DE-process gene abundance compared with the CN+P treatment. The other N-cycle genes showed a similar trend under the −P condition.

In addition to the significant difference in the total number of genes of the six N-cycle processes, the expressions of the individual genes in each process of the N cycle also showed significant differences between the different treatments (Figure 3c). Under the −P condition, the AMBC-P treatment exhibited significant upregulation in all the genes related to each N-cycling process compared with the CN-P treatment. The results showed that the *nasBE* and *nasA* genes associated with the AN process in the AMBC-P treatment were upregulated by 2.1 and 2.08 times, respectively, compared with the CN-P treatment. Similarly, in the AMBC-P treatment, the *narGHI* and *nirK* genes involved in the DE process were upregulated by factors of 2.02 and 1.74, respectively, compared with the CN-P treatment. Furthermore, in the AMBC-P treatment, the *napA* and *nrfAH* genes linked to the DN process were upregulated by factors of 2.62 and 1.81, respectively, compared with the CN-P treatment. In the AMBC-P treatment, the *nifA* gene associated with the NF process exhibited a 2.15-fold increase, while the *amoAB* genes within the NI process were upregulated by factors of 2.06 and 2.03 compared with the CN-P treatment. However, under the +P condition, the AMBC+P treatment displayed significant upregulation solely in the *nnrR* gene related to the DE process, with an increase in its expression by a factor of 2.31 compared with the CN+P treatment. No noteworthy differences were observed in the other genes linked to the N-cycling process.

3.4. Analysis of the Composition of N-Cycle Genes in Soil Microbe

Through an analysis of the taxonomic assignment of the N-cycling genes to the six processes, it was discovered that a total of 355 genera from 21 phyla possess these genes. The N-cycling genes were mainly concentrated in Proteobacteria, Actinobacteria, and Nitrospirae (Figure 4a). The genes related to the OD, DN, and NF processes predominantly originated from Proteobacteria, whereas the genes associated with the AN and NI processes were mainly derived from Actinobacteria. The Actinobacteria and Proteobacteria shared similar proportions in their contributions of DE-process genes. Nitrospirae microorganisms primarily expressed genes related to the DE and OD processes. The N-cycling genes within Proteobacteria, Actinobacteria, and Nitrospirae predominantly originated from the top 10 genera in terms of abundance. The Nitrospirae only included *Nitrospira*. The Proteobacteria mainly included *Bradyrhizobium*, *Geobacter*, *Pseudomonas*, *Sphingomonas*, *Anaeromyxobacter*, and *Dechloromonas*, while the Actinobacteria mainly included *Nocardioides*, *Arthrobacter*, and *Streptomycin*. The *Nocardioides* microorganisms primarily expressed genes related to the DE, AN, and NF processes. The *Bradyrhizobium* microorganisms expressed all the N-cycling genes except for the NI-process genes. The *Pseudomonas* microorganisms primarily expressed genes related to the DE, OD, and NF processes. The *Arthrobacter* microorganisms, similar to *Bradyrhizobium*, expressed all the N-cycling genes except for the AN-process genes. The *Geobacter* and *Anaeromyxobacter* microorganisms primarily expressed genes related to the NF process. The *Nitrospira* microorganisms from Nitrospirae also mainly expressed genes related to the DE and OD processes. The *Streptomycin*, *Sphingomonas*, and *Dechloromonas* microorganisms primarily expressed genes related to the OD process.

Statistical analysis of the abundances of the top 10 genera (soil N-cycling microorganisms) which exhibited the highest abundances of N-cycling genes demonstrated a consistent pattern with the overall abundances of the genes involved in all the N-cycling processes (Figure 4b). Irrespective of the P condition, the combined AM fungus and biochar treatment consistently exhibited higher abundances of soil N-cycling microorganisms compared with the other treatments. Specifically, under the −P condition, the AMBC-P treatment demonstrated a significant 1.99-fold increase in the abundances of soil N-cycling microorganisms compared with the CN-P treatment.

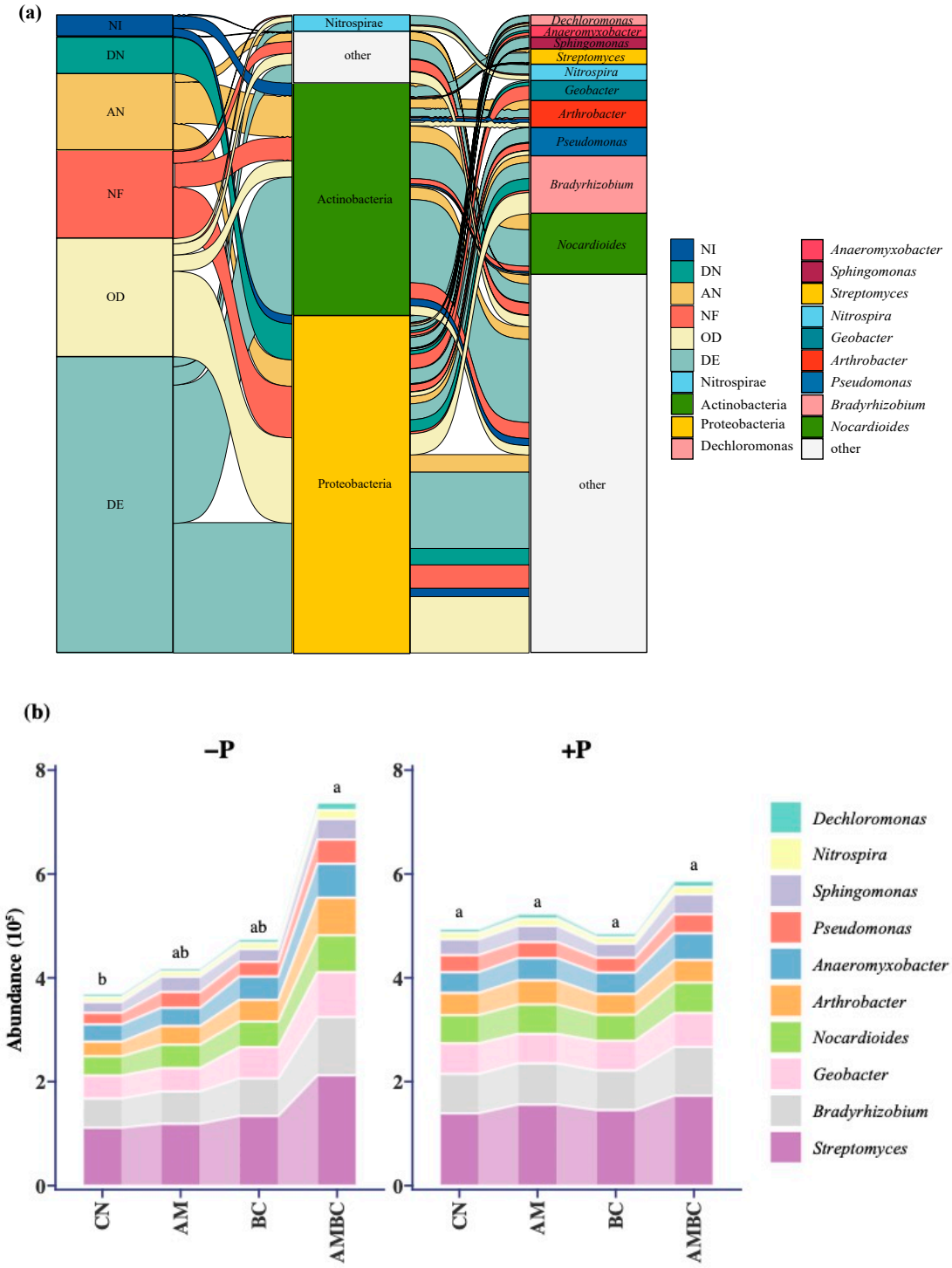

Figure 4. The taxonomic analysis of six N-cycle processes' genes (**a**) and the abundance of N-cycle microorganisms (**b**) under two P application rates. Other numbers in column stand for the sum of low-abundance microbes at phylum and genus level; CN: without AMF inoculation and biochar application; AM: AMF inoculation alone; BC: biochar application alone; AMBC: both AMF inoculation and biochar application; −P: without P addition; +P: P addition of 30 mg/kg. Different lower-case letters in the same column indicate significant differences in different treatments ($p \leq 0.05$). NI: nitrification process gene; AN: assimilation of nitrate process gene; DN: dissimilation of nitrate process gene; DE: denitrification process gene; NF: N fixation process gene; OD: organic decomposition process gene.

3.5. Correlation Analysis of Soil N Content and N-Cycling Genes

The Mantel test was employed to examine the correlations between different N-cycling genes and variables, such as the soil N-cycling microorganisms, AN, AP, and TN, C:P ratio, shoot N uptake, and root N uptake (Figure 5). The results of the Mantel test demonstrated significant positive correlations between, on the one hand, all the N-cycle genes and N-cycle microorganisms, shoot N uptake, the root N uptake, and the soil AN and, on the other, TN. Genes related to the DN process exhibited a significant positive correlation with the C:P ratio and soil AP. The other N-cycle genes did not show significant correlations.

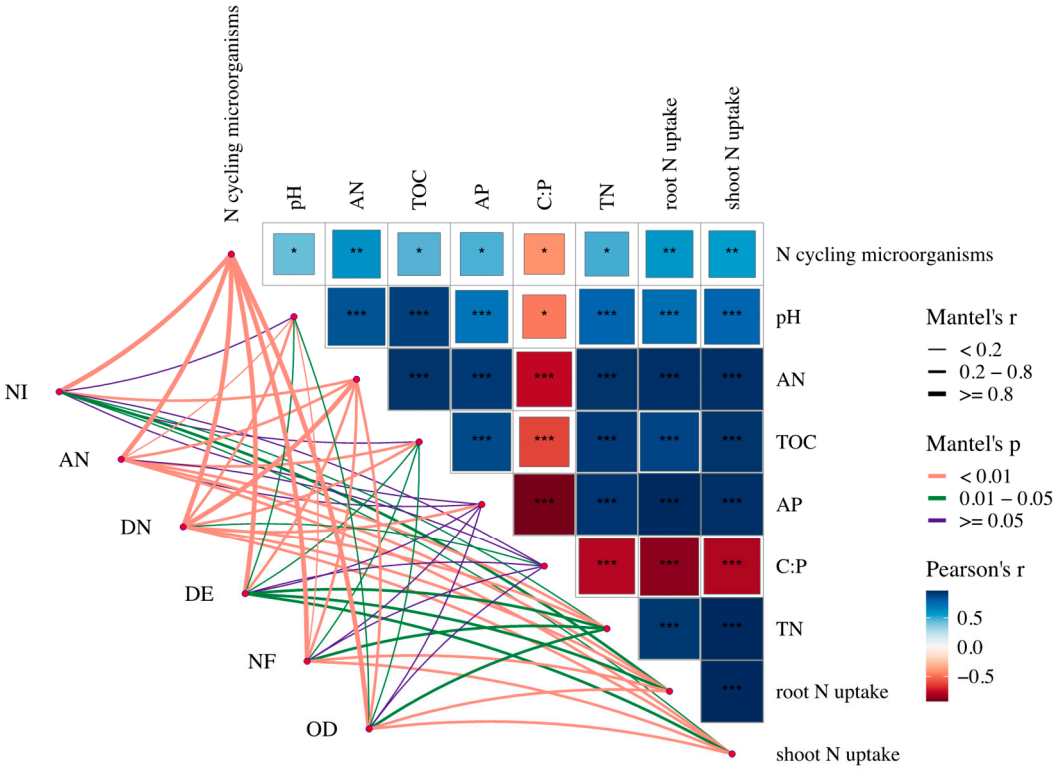

Figure 5. Correlations between physicochemical factors and the abundance of N-cycling process genes with different functions. NI: nitrification process gene; AN: assimilation of nitrate process gene; DN: dissimilation of nitrate process gene; DE: denitrification process gene; NF: N fixation process gene; OD: organic decomposition process gene. NS means not significant, * means significant difference at 0.05 level, ** means significant difference at 0.01 level, *** means significant difference at 0.001 level.

4. Discussion

4.1. Coupling Effects of AMF and Biochar on N Uptake and Root Morphology of Maize

The combination of biochar and AMF can have a significant positive impact on the N uptake of maize roots and shoots. Our study found that the application of AMF and biochar alone, as well as in combination, accelerated the maize root and shoot N uptake under both P conditions. The N uptake in the maize roots and shoots under the AMBC treatments was the highest under both P conditions, indicating that the combination of AMF and biochar has a stronger effect on the soil nutrient contents and plant growth than the individual treatments. Our research results are similar to those of previous studies that found that biochar, as a novel soil amendment, significantly improves soil conditions to enhance the colonization rate of AMF to increase the plant nutrient acquisition pathways and capabilities [30,31].

In addition, the porosity and large surface area of biochar can increase the porosity and permeability of the soil, providing a more convenient growth environment for the roots [32]. This improvement makes it easier for the roots to penetrate the soil and thereby

absorb water and nutrients more efficiently to increase the plant N uptake. In our study, we found that the combined AM fungus inoculation and biochar application treatment significantly increased the root weights and volumes of the maize better than the individual treatments. This finding is consistent with those of several previous studies which found that the combination of AMF and biochar can promote root growth and help plants access nutrients [33,34]. Biochar itself is rich in carbon and provides a variety of inorganic and organic nutrients, making it an important source needed by plants and a contributor to the growth and development of plant roots [35]. The growth and development of the roots can provide more surface area and rhizosphere space, help the AMF absorb water and nutrients in the soil, and provide more nutritional support for plants [5]. Similarly, we found that the biochar could mediate the AMF to facilitate the growth of the maize roots. Therefore, the N uptake in the AM-BC treatments was higher than that of a single inoculation or the application of biochar alone because the absorption areas of the roots were enlarged.

4.2. Coupling Effects of AMF and Biochar on Soil Microbial Community

Soil microorganisms participate in N-cycling and transformation, including mineralization, nitrification, reduction, and N fixation [36]. The activity and composition of the microbial communities can affect the content and availability of the different forms of N in the soil [10]. Our study demonstrates the potential of the combined treatment of AMF and biochar in shaping the soil microbial community, especially in −P treatments, indicated by its distinct clustering and its association with a specific module in the co-occurrence network analysis. The rhizosphere microbiota is in contact with the hypha surfaces of AMF, is sensitive to hypha exudation, and influences the N uptake of plants [37]. The co-occurrence network analysis revealed a strong association between the combined treatment and the specific microbial modules, indicating a significant impact on the soil microbial community. Under control test conditions, it was found that less than 21% of the absorbed N was derived from AMF and their extracellular hyphae [38]. We also found that individual AM fungus inoculation or biochar application significantly affected the soil microbial community, but the combined treatment had significantly different effects compared with the single individual treatments. Root exudates can recruit specific microbes by producing phenolic acids in the rhizosphere and providing substrates for specific taxa under N enrichment [7]. Recent research has revealed that AMF can secrete organic substances in the hypersphere, providing substrates for specific taxa under P stress and altering the microbial community structure in the mycorrhizal network [4]. Biochar, which offers a habitat and nutrients for AMF, can alter the structure of the soil microbial community by supporting microorganisms attracted by AMF [26,39]. An intriguing finding of our research is that *Geobacter*, *Burkholderia*, and *Pseudomonas* were the main microbes affected by the AMBC treatment. This finding suggests that the synergistic effects of AMF and biochar result in distinct microbial interactions and potentially modulate the functional dynamics of the soil microbial community. The experiment primarily investigates the effects of biochar and AMF on the soil microbial community in a greenhouse. In field experiments, consideration should also be given to other uncontrollable natural factors that may impact the result of experiments.

4.3. Coupling Effects of AMF and Biochar on the Abundance and Structure of N-Cycle Genes

Soil microbes that express N-cycling genes promote N cycling, transformation, and utilization in the soil [40]. Exploring the abundances of N genes contributes to unraveling the molecular mechanisms of N cycling, enhancing N use efficiency and maintaining soil nutrient stability. Through NMDS analysis, we found that the N-cycle genes were clustered among the different treatments, indicating that AMF and biochar could change the structures of soil N-cycle genes. Previous studies also found that AMF or biochar can alter the structure and regulate the abundance of N-cycle functional genes [11,23,41]. In our study, we observed that both the combined and individual uses of AMF and biochar enhanced the abundances of the N-cycle genes. However, it was only with the combined

application of AMF and biochar that a significant increase in N-cycle genes was observed under the −P condition.

The soil N-cycle is a complex process involving six steps. When studying N-cycling genes, exploring not only the primary functional genes but also the equally crucial regulatory and transport genes is vital. In our study, while the AM fungus inoculation and biochar application treatments showed no significant differences in certain functional genes, they notably influenced specific regulatory or transport genes, thereby impacting the soil N-cycle.

In the denitrification process, when comparing the AMBC-P treatment with the CN-P treatment, there were no significant differences observed in the abundances of the *nosZ* gene responsible for converting nitrous oxide to nitric oxide. However, notably, the AMBC-P treatment showed a significant upregulation in the abundances of the *nosR* and *nosDFY* genes compared with the CN-P treatment. The *nosR* gene acts as a transcriptional activator for the nitrous oxide reductase gene *nosZ*, while the *nosDFY* genes facilitate substrate transport for the *nosZ* gene [42,43]. Moreover, the joint application of AM fungus inoculation with biochar significantly influenced various steps in the soil N denitrification process. Compared with the CN-P treatment, the AMNC-P treatment exhibited a significant upregulation in the abundances of the *narXQ*, *narJ*, *narGHI*, and *nirK* genes. This upregulation facilitated the soil's conversion of nitrate to nitrite and, subsequently, nitric oxide. The *narXQ* gene sensor protein can sense nitrate and nitrite availability, transmitting the signal to the *narLP* gene, which can activate the expression of the *narGHJI* gene. The *narJ* gene encodes a transport protein that assists the *narGHI* gene's conversion of nitrate to nitrite [44]. The *nirK* gene converts the nitrite produced into nitric oxide. Previous studies indicate that the expression of *nirK* may be regulated by *nnrR* [45]. In our study, under the −P condition, the AMBC-P treatment did not significantly upregulate the *nnrR* gene abundance compared with the CN-P treatment. However, under the +P conditions, the AMBC+P treatment significantly upregulated the *nnrR* gene abundance compared with the CN+P treatment. Interestingly, the AMBC+P treatment did not significantly upregulate the *nirK* gene abundance compared with the CN+P treatment. Additionally, we did not detect the presence of the *nirS* gene, which is consistent with the findings of previous studies in which the coexistence of the *nirK* and *nirS* genes was not observed among the N-cycling genes [46]. However, recent research has challenged this notion, suggesting that the coexistence of the *nirK* gene and the existence of the *nirS* gene may not be mutually exclusive [47].

While AM fungus inoculation and biochar application may encourage denitrification processes, potentially reducing the soil N content, they also stimulate N fixation and organic decomposition processes, ultimately increasing the soil N content. In our study, the AMBC-P treatment significantly upregulated the *nifA*, *nifEN*, *ureCAB*, and *synS* genes compared with the CN-P treatment. In the N fixation process, the *nifA* gene can activate other genes that are directly involved in N fixation. The *nifEN* gene is involved in the assembly and binding of iron and molybdenum atoms. In the process of organic decomposition, the *ureCAB* gene, which can further decompose urea into NH_4^+ [48], and the cyanide hydratase *cynS* catalyzes the reaction between cyanate and bicarbonate to generate NH_4^+ with the assistance of the transport proteins *cynABD* [49].

In our study, the AMBC-P treatment significantly upregulated the expression of all the genes compared with the CN-P treatment in the processes of nitrate assimilation and dissimilation. In the N fixation, organic decomposition, and nitrate assimilation and dissimilation processes, the ammonia produced via nitrification requires conversion into nitrate, a more accessible form for plant absorption. The *amoAB* gene oxidizes NH_4^+ to hydroxylamine, while the *hao* gene catalyzes the conversion of hydroxylamine to nitric oxide (NO) [50,51]. Subsequently, the NO is reduced to nitrate (NO_3^-) by *hmp*, making it more readily absorbable by plants [52]. Notably, in our study, we did not detect the presence of the *nxrAB* gene. Previous research has indicated that *hao* catalyzes the oxidation of hydroxylamine to nitrite (NO_2^-) and the further oxidation of nitrite to nitrate by *nxrAB* [53].

This finding may support recent research that suggests that the oxidation product of hydroxylamine by the *hao* gene is NO rather than nitrite [40]. These findings suggest that in conditions with no P application, the application of AMF and biochar together may increase the soil nitrate levels through the N conversion process, consequently enhancing the plant uptake.

4.4. Taxonomic Analysis of Composition of N-Cycle Genes and Mantel Test

Based on the taxonomic analysis of the composition of the N-cycle genes, we found that the six N-cycling genes exhibit distinct distributions among the top 10 genera. Although most of the top 10 genera belong to the Proteobacteria and Actinobacteria phyla, the composition of the N-cycle genes differs among these genera, indicating varying levels of gene presence and expression. These differences emphasize the variations in the microbial community composition and their functional potentials. In a study by Kuang et al. (2022), it was discovered that *Nocardioides* harbors multiple genes that encode key enzymes involved in the processes of denitrification and assimilatory and dissimilatory nitrate reduction [54]. Consistent with these prior findings, our study also uncovered the involvement of *Nocardioides* in the N-cycling processes through the presence of genes related to the AN and DE processes. Although our study confirms previous studies that found that *Bradyrhizobium* has the ability to fix atmospheric N into forms that are readily available for other organisms to utilize, our investigation uncovered a distinct pattern. At the same time, we observed a low distribution of NF genes and a high distribution of OD genes in *Bradyrhizobium*. The high distribution of OD genes in *Bradyrhizobium* indicates that it may be involved in the decomposition of organic materials in soil, such as biochar. The low number of N-fixing genes in *Bradyrhizobium* may be due to the fact that the tested plant in this experiment was maize. Although symbiotic N fixation primarily occurs in legumes, numerous studies have demonstrated that maize and other grass crops facilitate combined N fixation through the secretion of organic substances [55]. Epiphytic diazotrophs are a combination of N-fixing bacteria that colonize the root surfaces of plants.

While a more recent study suggests that *Pseudomonas* is a common epiphytic N-fixing bacterium found in wheat, previous studies show that *Pseudomonas* participates in organic decomposition and denitrification [56,57]. Our findings align with the previous studies, indicating that *Pseudomonas* harbors multiple genes associated with N cycling, potentially contributing to the denitrification, N fixation, and organic matter decomposition of soil. In addition, numerous previous studies have consistently emphasized the significant role of *Arthrobacter* in the N cycle, particularly in denitrification, N fixation, and organic matter decomposition [58,59]. Nevertheless, a recent study revealed the adaptability of *Arthrobacter* to N-depleted conditions through both the assimilatory and dissimilatory nitrate reduction pathways [60]. Our study further supports this finding by identifying the expression of genes related to the assimilatory N processes in *Arthrobacter*. Multiple studies have consistently established a substantial correlation between *Anaeromyxobacter* and *Geobacter* with NF genes [61,62]. This association underscores their potential contributions to the N fixation processes within diverse ecosystems. Our study detected a substantial expression of NF genes in *Geobacter* and *Anaeromyxobacter*. Among the genera analyzed, *Geobacter* displayed the greatest abundance of NF genes. *Geobacter* is a prominent epibiotic diazotroph in other grass crops like rice and wheat. These results imply that *Geobacter* might exert a dominant influence on the N fixation in maize soil [63,64]. Our study identified the differential expressions of genes related to the DE process in major genera such as *Nitrospira*, *Streptomyces*, *Sphingomonas*, and *Dechloromonas*. This observation strongly suggests their active participation in soil denitrification processes, signifying their pivotal roles within the soil ecosystem. While many genes were not found in *Burkholderia* in the taxonomy analysis, module 1 highlighted the presence of numerous genes in *Geobacter* and *Pseudomonas*. By analyzing the abundance of the N-cycling microorganism, we found similar results to those of the total N-cycling genes. The AMBC-P treatment significantly promoted the microbial abundance of N cycling compared with the CN-P treatment, and the promoted abundance was close to

the proportion of gene upregulation. Furthermore, the Mantel test results demonstrated a significant positive correlation between all the N-cycle genes and N-cycle microorganisms, as well as among the AN, TN, root uptake, and shoot uptake. These findings indicate that AM fungus inoculation and biochar application can effectively upregulate soil N-cycle gene expressions by enhancing the abundances of N-cycling microorganisms such as *Geobacter* and *Pseudomonas*, leading to increased soil N content and enhanced maize N uptake.

5. Conclusions

The coupling action of AMF and biochar facilitated the N uptake of the shoots and roots and increased the soil N content by enhancing the abundances of N-cycle genera in the rhizosphere of maize. Specifically, the abundances of the N-cycle genera involved in the N conversion processes, such as *Pseudomonas* and *Geobacter*, were significantly increased under the coupling action of AMF and biochar, particularly when no P was applied. This increase in the abundances of the N-cycle microorganisms led to the upregulation of the N-cycle gene expressions. These findings suggest that the coupling action of AMF and biochar can effectively promote the abundances of N-cycle microorganisms, resulting in upregulated N-cycle genes and N uptake in maize. This study offers a promising approach for increasing the soil nutrient contents in sustainable agricultural production and contributing to climate change mitigation.

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References

1. Zhao, B.; Jia, X.; Yu, N.; Murray, J.D.; Yi, K.; Wang, E. Microbe-dependent and independent nitrogen and phosphate acquisition and regulation in plants. *New Phytol.* **2023**. [CrossRef] [PubMed]
2. Zhou, J.; Zhang, L.; Feng, G.; George, T.S. Arbuscular mycorrhizal fungi have a greater role than root hairs of maize for priming the rhizosphere microbial community and enhancing rhizosphere organic P mineralization. *Soil Biol. Biochem.* **2022**, *171*, 108713. [CrossRef]
3. Meng, L.; Zhang, A.; Wang, F.; Han, X.; Wang, D.; Li, S. Arbuscular mycorrhizal fungi and rhizobium facilitate nitrogen uptake and transfer in soybean/maize intercropping system. *Front. Plant Sci.* **2015**, *6*, 339. [CrossRef] [PubMed]
4. Zhang, L.; Zhou, J.; George, T.S.; Limpens, E.; Feng, G. Arbuscular mycorrhizal fungi conducting the hyphosphere bacterial orchestra. *Trends Plant Sci.* **2022**, *27*, 402–411. [CrossRef] [PubMed]
5. Hestrin, R.; Hammer, E.C.; Mueller, C.W.; Lehmann, J. Synergies between mycorrhizal fungi and soil microbial communities increase plant nitrogen acquisition. *Commun. Biol.* **2019**, *2*, 233. [CrossRef] [PubMed]
6. Zhang, R.; Mu, Y.; Li, X.; Li, S.; Sang, P.; Wang, X.; Wu, H.; Xu, N. Response of the arbuscular mycorrhizal fungi diversity and community in maize and soybean rhizosphere soil and roots to intercropping systems with different nitrogen application rates. *Sci. Total Environ.* **2020**, *740*, 139810. [CrossRef] [PubMed]
7. Zhu, C.; Tian, G.; Luo, G.; Kong, Y.; Guo, J.; Wang, M.; Guo, S.; Ling, N.; Shen, Q. N-fertilizer-driven association between the arbuscular mycorrhizal fungal community and diazotrophic community impacts wheat yield. *Agric. Ecosyst. Environ.* **2018**, *254*, 191–201. [CrossRef]

8. Cheng, Z.; Meng, L.; Yin, T.; Li, Y.; Zhang, Y.; Li, S. Changes in Soil Rhizobia Diversity and Their Effects on the Symbiotic Efficiency of Soybean Intercropped with Maize. *Agronomy* **2023**, *13*, 997. [CrossRef]
9. Sun, R.; Wang, F.; Hu, C.; Liu, B. Metagenomics reveals taxon-specific responses of the nitrogen-cycling microbial community to long-term nitrogen fertilization. *Soil Biol. Biochem.* **2021**, *156*, 108214. [CrossRef]
10. Sun, Y.; Li, Y.; Chang, J.; Tian, L.; Ji, L.; Guo, L.; Gao, Q.; van Veen, J.A.; Tian, C. Chemotaxis mediates nitrogen acquisition of maize under long-term nitrogen input. *Soil Biol. Biochem.* **2023**, *184*, 109118. [CrossRef]
11. Gui, H.; Gao, Y.; Wang, Z.; Shi, L.; Yan, K.; Xu, J. Arbuscular mycorrhizal fungi potentially regulate N_2O emissions from agricultural soils via altered expression of denitrification genes. *Sci. Total Environ.* **2021**, *774*, 145133. [CrossRef]
12. Li, J.; Meng, B.; Yang, X.; Cui, N.; Zhao, T.; Chai, H.; Zhang, T.; Sun, W. Suppression of AMF accelerates N(2)O emission by altering soil bacterial community and genes abundance under varied precipitation conditions in a semiarid grassland. *Front Microbiol.* **2022**, *13*, 961969. [CrossRef] [PubMed]
13. Liu, W.; Zhang, Y.; Jiang, S.; Deng, Y.; Christie, P.; Murray, P.J.; Li, X.; Zhang, J. Arbuscular mycorrhizal fungi in soil and roots respond differently to phosphorus inputs in an intensively managed calcareous agricultural soil. *Sci. Rep.* **2016**, *6*, 24902. [CrossRef]
14. Deepika, S.; Kothamasi, D. Soil moisture—A regulator of arbuscular mycorrhizal fungal community assembly and symbiotic phosphorus uptake. *Mycorrhiza* **2015**, *25*, 67–75. [CrossRef]
15. Azcón, R.; Ambrosano, E.; Charest, C. Nutrient acquisition in mycorrhizal lettuce plants under different phosphorus and nitrogen concentration. *Plant Sci.* **2003**, *165*, 1137–1145. [CrossRef]
16. Liao, D.; Sun, C.; Liang, H.; Wang, Y.; Bian, X.; Dong, C.; Niu, X.; Yang, M.; Xu, G.; Chen, A.; et al. SlSPX1-SlPHR complexes mediate the suppression of arbuscular mycorrhizal symbiosis by phosphate repletion in tomato. *Plant Cell* **2022**, *34*, 4045–4065. [CrossRef]
17. Gul, S.; Whalen, J.K.; Thomas, B.W.; Sachdeva, V.; Deng, H.Y. Physico-chemical properties and microbial responses in biochar-amended soils: Mechanisms and future directions. *Agric. Ecosyst. Environ.* **2015**, *206*, 46–59. [CrossRef]
18. Liu, M.; Zhao, Z.; Chen, L.; Wang, L.; Ji, L.; Xiao, Y. Influences of arbuscular mycorrhizae, phosphorus fertiliser and biochar on alfalfa growth, nutrient status and cadmium uptake. *Ecotoxicol. Environ. Saf.* **2020**, *196*, 110537. [CrossRef] [PubMed]
19. Hammer, E.C.; Balogh-Brunstad, Z.; Jakobsen, I.; Olsson, P.A.; Stipp, S.L.S.; Rillig, M.C. A mycorrhizal fungus grows on biochar and captures phosphorus from its surfaces. *Soil Biol. Biochem.* **2014**, *77*, 252–260. [CrossRef]
20. Gao, S.; DeLuca, T.H. Wood biochar impacts soil phosphorus dynamics and microbial communities in organically-managed croplands. *Soil Biol. Biochem.* **2018**, *126*, 144–150. [CrossRef]
21. Van Zwieten, L.; Kimber, S.; Morris, S.; Chan, K.Y.; Downie, A.; Rust, J.; Joseph, S.; Cowie, A. Effects of biochar from slow pyrolysis of papermill waste on agronomic performance and soil fertility. *Plant Soil* **2009**, *327*, 235–246. [CrossRef]
22. Gao, S.; DeLuca, T.H.; Cleveland, C.C. Biochar additions alter phosphorus and nitrogen availability in agricultural ecosystems: A meta-analysis. *Sci. Total Environ.* **2019**, *654*, 463–472. [CrossRef] [PubMed]
23. Xiao, Z.; Rasmann, S.; Yue, L.; Lian, F.; Zou, H.; Wang, Z. The effect of biochar amendment on N-cycling genes in soils: A meta-analysis. *Sci. Total Environ.* **2019**, *696*, 133984. [CrossRef] [PubMed]
24. Zhang, L.; Jing, Y.; Chen, C.; Xiang, Y.; Rezaei Rashti, M.; Li, Y.; Deng, Q.; Zhang, R. Effects of biochar application on soil nitrogen transformation, microbial functional genes, enzyme activity, and plant nitrogen uptake: A meta-analysis of field studies. *GCB Bioenergy* **2021**, *13*, 1859–1873. [CrossRef]
25. Tian, J.; Kuang, X.; Tang, M.; Chen, X.; Huang, F.; Cai, Y.; Cai, K. Biochar application under low phosphorus input promotes soil organic phosphorus mineralization by shifting bacterial phoD gene community composition. *Sci. Total Environ.* **2021**, *779*, 146556. [CrossRef] [PubMed]
26. Li, S.; Chi, S.; Lin, C.; Cai, C.; Yang, L.; Peng, K.; Huang, X.; Liu, J. Combination of biochar and AMF promotes phosphorus utilization by stimulating rhizosphere microbial co-occurrence networks and lipid metabolites of Phragmites. *Sci. Total Environ.* **2022**, *845*, 157339. [CrossRef] [PubMed]
27. Kjeldahl, J. Neue Methode zur Bestimmung des Stickstoffs in organischen Körpern. *Z. Anal. Chem.* **1883**, *22*, 366–382. [CrossRef]
28. Nell, M.; Votsch, M.; Vierheilig, H.; Steinkellner, S.; Zitterl-Eglseer, K.; Franz, C.; Novak, J. Effect of phosphorus uptake on growth and secondary metabolites of garden sage (*Salvia officinalis* L.). *J. Sci. Food Agric.* **2009**, *89*, 1090–1096. [CrossRef]
29. Dudhagara, P.; Bhavsar, S.; Bhagat, C.; Ghelani, A.; Bhatt, S.; Patel, R. Web Resources for Metagenomics Studies. *Genom. Proteom. Bioinform.* **2015**, *13*, 296–303. [CrossRef]
30. Barna, G.; Makó, A.; Takács, T.; Skic, K.; Füzy, A.; Horel, Á. Biochar Alters Soil Physical Characteristics, Arbuscular Mycorrhizal Fungi Colonization, and Glomalin Production. *Agronomy* **2020**, *10*, 1933. [CrossRef]
31. Solaiman, Z.M.; Abbott, L.K.; Murphy, D.V. Biochar phosphorus concentration dictates mycorrhizal colonisation, plant growth and soil phosphorus cycling. *Sci. Rep.* **2019**, *9*, 5062. [CrossRef] [PubMed]
32. Chang, Y.; Rossi, L.; Zotarelli, L.; Gao, B.; Shahid, M.A.; Sarkhosh, A. Biochar improves soil physical characteristics and strengthens root architecture in Muscadine grape (*Vitis rotundifolia* L.). *Chem. Biol. Technol. Agric.* **2021**, *8*, 1–11. [CrossRef]
33. Jabborova, D.; Annapurna, K.; Azimov, A.; Tyagi, S.; Pengani, K.R.; Sharma, P.; Vikram, K.V.; Poczai, P.; Nasif, O.; Ansari, M.J.; et al. Co-inoculation of biochar and arbuscular mycorrhizae for growth promotion and nutrient fortification in soybean under drought conditions. *Front. Plant Sci.* **2022**, *13*, 947547. [CrossRef] [PubMed]

34. Jabborova, D.; Annapurna, K.; Paul, S.; Kumar, S.; Saad, H.A.; Desouky, S.; Ibrahim, M.F.M.; Elkelish, A. Beneficial Features of Biochar and Arbuscular Mycorrhiza for Improving Spinach Plant Growth, Root Morphological Traits, Physiological Properties, and Soil Enzymatic Activities. *J. Fungi* **2021**, *7*, 571. [CrossRef] [PubMed]
35. Xiang, Y.; Deng, Q.; Duan, H.; Guo, Y. Effects of biochar application on root traits: A meta-analysis. *GCB Bioenergy* **2017**, *9*, 1563–1572. [CrossRef]
36. Kuypers, M.M.M.; Marchant, H.K.; Kartal, B. The microbial nitrogen-cycling network. *Nat. Rev. Microbiol.* **2018**, *16*, 263–276. [CrossRef]
37. Hartman, K.; van der Heijden, M.G.A.; Wittwer, R.A.; Banerjee, S.; Walser, J.C.; Schlaeppi, K. Cropping practices manipulate abundance patterns of root and soil microbiome members paving the way to smart farming. *Microbiome* **2018**, *6*, 14. [CrossRef]
38. Toussaint, J.-P.; St-Arnaud, M.; Charest, C. Nitrogen transfer and assimilation between the arbuscular mycorrhizal fungus Glomus intraradices Schenck & Smith and Ri T-DNA roots of Daucus carota L. in an in vitro compartmented system. *Can. J. Microbiol.* **2004**, *50*, 251–260. [CrossRef]
39. Lehmann, J.; Rillig, M.C.; Thies, J.; Masiello, C.A.; Hockaday, W.C.; Crowley, D. Biochar effects on soil biota—A review. *Soil Biol. Biochem.* **2011**, *43*, 1812–1836. [CrossRef]
40. Caranto, J.D.; Lancaster, K.M. Nitric oxide is an obligate bacterial nitrification intermediate produced by hydroxylamine oxidoreductase. *Proc. Natl. Acad. Sci. USA* **2017**, *114*, 8217–8222. [CrossRef]
41. Yu, H.; Liu, X.; Yang, C.; Peng, Y.; Yu, X.; Gu, H.; Zheng, X.; Wang, C.; Xiao, F.; Shu, L.; et al. Co-symbiosis of arbuscular mycorrhizal fungi (AMF) and diazotrophs promote biological nitrogen fixation in mangrove ecosystems. *Soil Biol. Biochem.* **2021**, *161*, 108382. [CrossRef]
42. Wunsch, P.; Herb, M.; Wieland, H.; Schiek, U.M.; Zumft, W.G. Requirements for Cu(A) and Cu-S center assembly of nitrous oxide reductase deduced from complete periplasmic enzyme maturation in the nondenitrifier *Pseudomonas putida*. *J. Bacteriol.* **2003**, *185*, 887–896. [CrossRef] [PubMed]
43. Wunsch, P.; Zumft, W.G. Functional domains of NosR, a novel transmembrane iron-sulfur flavoprotein necessary for nitrous oxide respiration. *J. Bacteriol.* **2005**, *187*, 1992–2001. [CrossRef] [PubMed]
44. Lanciano, P.; Vergnes, A.; Grimaldi, S.; Guigliarelli, B.; Magalon, A. Biogenesis of a respiratory complex is orchestrated by a single accessory protein. *J. Biol. Chem.* **2007**, *282*, 17468–17474. [CrossRef] [PubMed]
45. Bartnikas, T.B.; Wang, Y.; Bobo, T.; Veselov, A.; Scholes, C.P.; Shapleigh, J.P. Characterization of a member of the NnrR regulon in *Rhodobacter sphaeroides* 2.4.3 encoding a haem–copper protein. The GenBank accession number for nnrS is U62403. *Microbiology* **2002**, *148*, 825–833. [CrossRef]
46. Jones, C.M.; Stres, B.; Rosenquist, M.; Hallin, S. Phylogenetic analysis of nitrite, nitric oxide, and nitrous oxide respiratory enzymes reveal a complex evolutionary history for denitrification. *Mol. Biol. Evol.* **2008**, *25*, 1955–1966. [CrossRef]
47. Kits, K.D.; Klotz, M.G.; Stein, L.Y. Methane oxidation coupled to nitrate reduction under hypoxia by the Gammaproteobacterium *Methylomonas denitrificans*, sp. nov. type strain FJG1. *Environ. Microbiol.* **2015**, *17*, 3219–3232. [CrossRef]
48. Hu, L.T.; Foxall, P.A.; Russell, R.; Mobley, H.L. Purification of recombinant Helicobacter pylori urease apoenzyme encoded by ureA and ureB. *Infect. Immun.* **1992**, *60*, 2657–2666. [CrossRef] [PubMed]
49. Saez, L.P.; Cabello, P.; Ibanez, M.I.; Luque-Almagro, V.M.; Roldan, M.D.; Moreno-Vivian, C. Cyanate Assimilation by the Alkaliphilic Cyanide-Degrading Bacterium Pseudomonas pseudoalcaligenes CECT5344: Mutational Analysis of the cyn Gene Cluster. *Int. J. Mol. Sci.* **2019**, *20*, 3008. [CrossRef]
50. Ensign, S.A.; Hyman, M.R.; Arp, D.J. In vitro activation of ammonia monooxygenase from Nitrosomonas europaea by copper. *J. Bacteriol.* **1993**, *175*, 1971–1980. [CrossRef]
51. Hooper, A.B.; Terry, K.R. Hydroxylamine oxidoreductase of Nitrosomonas. Production of nitric oxide from hydroxylamine. *Biochim. Biophys. Acta* **1979**, *571*, 12–20. [CrossRef] [PubMed]
52. Gardner, P.R.; Gardner, A.M.; Martin, L.A.; Dou, Y.; Li, T.; Olson, J.S.; Zhu, H.; Riggs, A.F. Nitric-oxide dioxygenase activity and function of flavohemoglobins. Sensitivity to nitric oxide and carbon monoxide inhibition. *J. Biol. Chem.* **2000**, *275*, 31581–31587. [CrossRef] [PubMed]
53. Daims, H.; Lucker, S.; Wagner, M. A New Perspective on Microbes Formerly Known as Nitrite-Oxidizing Bacteria. *Trends Microbiol.* **2016**, *24*, 699–712. [CrossRef] [PubMed]
54. Kuang, B.; Xiao, R.; Hu, Y.; Wang, Y.; Zhang, L.; Wei, Z.; Bai, J.; Zhang, K.; Acuna, J.J.; Jorquera, M.A.; et al. Metagenomics reveals biogeochemical processes carried out by sediment microbial communities in a shallow eutrophic freshwater lake. *Front Microbiol.* **2022**, *13*, 1112669. [CrossRef]
55. Bennett, A.B.; Pankievicz, V.C.S.; Ané, J.-M. A Model for Nitrogen Fixation in Cereal Crops. *Trends Plant Sci.* **2020**, *25*, 226–235. [CrossRef]
56. de Lima, D.R.M.; Dos Santos, I.B.; Oliveira, J.T.C.; da Costa, D.P.; de Queiroz, J.V.J.; Romagnoli, E.M.; Andreote, F.D.; Freire, F.J.; Kuklinsky-Sobral, J. Genetic diversity of N-fixing and plant growth-promoting bacterial community in different sugarcane genotypes, association habitat and phenological phase of the crop. *Arch. Microbiol.* **2021**, *203*, 1089–1105. [CrossRef]
57. Yan, Y.; Yang, J.; Dou, Y.; Chen, M.; Ping, S.; Peng, J.; Lu, W.; Zhang, W.; Yao, Z.; Li, H.; et al. Nitrogen fixation island and rhizosphere competence traits in the genome of root-associated *Pseudomonas stutzeri* A1501. *Proc. Natl. Acad. Sci. USA* **2008**, *105*, 7564–7569. [CrossRef]

58. Sellstedt, A.; Richau, K.H. Aspects of nitrogen-fixing Actinobacteria, in particular free-living and symbiotic Frankia. *FEMS Microbiol. Lett.* **2013**, *342*, 179–186. [CrossRef] [PubMed]
59. Zhang, M.; Pan, L.; Liu, L.; Su, C.; Dou, L.; Su, Z.; He, Z. Phosphorus and nitrogen removal by a novel phosphate-accumulating organism, Arthrobacter sp. HHEP5 capable of heterotrophic nitrification-aerobic denitrification: Safety assessment, removal characterization, mechanism exploration and wastewater treatment. *Bioresour. Technol.* **2020**, *312*, 123633. [CrossRef]
60. Liu, Y.; Zhang, Y.; Huang, Y.; Niu, J.; Huang, J.; Peng, X.; Peng, F. Spatial and temporal conversion of nitrogen using Arthrobacter sp. 24S4-2, a strain obtained from Antarctica. *Front. Microbiol.* **2023**, *14*, 1040201. [CrossRef]
61. Calderoli, P.A.; Collavino, M.M.; Behrends Kraemer, F.; Morras, H.J.M.; Aguilar, O.M. Analysis of nifH-RNA reveals phylotypes related to Geobacter and Cyanobacteria as important funsctional components of the $N(_2)$ -fixing community depending on depth and agricultural use of soil. *Microbiol. Open* **2017**, *6*, e00502. [CrossRef] [PubMed]
62. Masuda, Y.; Shiratori, Y.; Ohba, H.; Ishida, T.; Takano, R.; Satoh, S.; Shen, W.; Gao, N.; Itoh, H.; Senoo, K. Enhancement of the nitrogen-fixing activity of paddy soils owing to iron application. *Soil Sci. Plant Nutr.* **2021**, *67*, 243–247. [CrossRef]
63. Liu, Y.; Guo, Z.; Xue, C.; Gao, W.; Wang, G.; Liu, X. Changes in N_2-fixation activity, abundance and composition of diazotrophic communities in a wheat field under elevated CO_2 and canopy warming. *Appl. Soil Ecol.* **2021**, *165*, 104017. [CrossRef]
64. Ma, J.; Bei, Q.; Wang, X.; Liu, G.; Cadisch, G.; Lin, X.; Zhu, J.; Sun, X.; Xie, Z. Paddy System with a Hybrid Rice Enhances Cyanobacteria Nostoc and Increases N_2 Fixation. *Pedosphere* **2019**, *29*, 374–387. [CrossRef]