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Arbuscular Mycorrhizae Affect Soil Nitrogen Fertilizer Utilization, Denitrification Functional Genes, and N_2O Emissions During Biochar Amendment

Yanfang Wang [1], Jing Liu [1], Xuxian Deng [2], Yuyang Li [1], Jiakai Gao [1] and Ling Liu [1,*]

[1] College of Agriculture, Henan University of Science and Technology, Luoyang 471023, China
[2] Luoyang Seed Industry Development Center, Luoyang 471023, China; hnlydxx@126.com
* Correspondence: liuling1978@haust.edu.cn

Abstract: Arbuscular mycorrhizal fungi (AMF) can form symbionts with plant roots, acquire soil nitrogen, and affect nitrous oxide (N_2O) production. Biochar, as a soil additive for the management of agricultural soil, affects soil nitrogen (N) utilization and plant growth. However, how AMF regulates soil N unitization, the denitrification process, and N_2O emissions in plant–soil systems remains largely unknown, particularly under the biochar amendment. In this study, a microcosm experiment was conducted to investigate the impacts of different mycorrhizal treatments (CK: neither AMF hyphae nor plant roots; AMF: only AMF hyphae; AMF + R: AMF hyphae and plant roots) on plant growth, soil N fertilizer utilization, N_2O production and consumption, functional gene abundance, and N_2O emission at two biochar addition levels (B0: no biochar; B1: biochar addition rate of 10 $g \cdot kg^{-1}$ soil) in a maize planting soil system. The results revealed that AMF alone and AMF with plant root treatments enhanced the fresh weight of maize plants by 10.15% and 19.23% and decreased soil inorganic N contents by 33.28% and 75.56%, respectively. The combination of biochar, AMF, and plant roots showed the largest increase in maize plant biomass. The AMF and AMF with root treatments all significantly decreased the *nir*S + *nir*K/*nos*Z ratio and N_2O emissions at two biochar levels. The presence of AMF and plant roots during biochar amendment showed the smallest *nir*S + *nir*K/*nos*Z ratio and N_2O emissions. The AMF combined with biochar and AMF and plant roots combined with biochar treatments increased *nir*S + *nir*K/*nos*Z by 24.32% and 26.90% and decreased N_2O accumulation emission by 21.12% and 38.13%, respectively. The results imply that biochar, AMF, and plant roots reduced N_2O emissions directly by reducing soil N and increasing soil N unitization efficiency and indirectly by shifting the N_2O production and consumption gene abundance in agroecosystems. These findings suggest that the addition of biochar and AMF and/or the presence of plant roots can interact to alleviate soil N_2O emissions by manipulating plant inorganic N acquisition and the soil denitrification process.

Keywords: arbuscular mycorrhizal fungi; denitrification; plant biomass; nitrogen uptake; nitrogen retention

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1. Introduction

Nitrous oxide (N_2O) is a potent greenhouse gas that can destroy the ozone layer in the stratosphere and cause climate warming [1,2]. Due to the large amount of nitrogen (N) fertilizer applied, agricultural soils have become the main emission source of N_2O, accounting for about 60% of the global anthropogenic N_2O emission [3]. The production of soil N_2O mainly comes from soil nitrification and denitrification processes [4,5]. In the process of soil nitrification, microorganisms in the soil oxidize the ammonium salt (NH_4^+) to nitrite (NO_2^-) or nitrate (NO_3^-), and N_2O is produced mainly by the oxidation of hydroxylamine (NH_2OH) to NO_2. The microorganisms involved in this process are the ammonia-oxidized archaea (AOA) and ammonia-oxidizing bacteria (AOB), which

are encoded by the ammonia mono oxygen-enzyme gene (*amo*A). Denitrification is the main N_2O emission source in agricultural soils [6]. In the denitrification process, nitrogen oxides (NO_3^- and NO_2^-) are reduced to N_2O and N_2 by various reductases, among which nitrite reductase and nitrous oxide reductase are key, and rate-limiting enzymes in soil denitrification [6,7]. The *nir*S and *nir*K genes encode nitrite reductases, which produce N_2O, and *nos*Z genes encode N_2O reductases that reduce N_2O to N_2 [8]. Therefore, the ratio of *nir*S + *nir*K/*nos*Z has been used as an indicator for N_2O emission potential [9].

Arbuscular mycorrhizal fungi (AMF) are a kind of beneficial soil microorganisms, which are widespread and can form symbionts with the plant roots of over 80% of the land plants [10]. AMF can help plant roots obtain soil nutrients and absorb photosynthates from host plants [11]. Plant roots combined with AMF play a vital role in the soil N cycles and significantly affect the absorption and mineralization of N [12,13]. Yet, only a few researchers have studied the impacts of plant roots and AMF on soil N_2O emission, and they have produced different results. For instance, Zhang et al. [14] revealed that plant roots combined with AMF suppressed soil N_2O emission by changing the N cycling microbial community. Bender et al. [15] also revealed that the AMF significantly reduced soil N_2O emission by shifting the key gene abundance of N_2O emission. Qiu et al. [9] reported that AMF combined with plant roots reduced the *nir*K gene abundance and the (*nir*K + *nir*S)/*nos*Z ratio, decreasing soil N_2O emission. Changes in soil denitrifiers play a major controlling role in the emission of N_2O. However, some studies indicated that plant roots and AMF may increase soil N_2O emission by producing more root exudates and labile carbon from plant roots, which supply high carbon for denitrifiers to stimulate denitrification [16,17]. Therefore, the impacts of plant roots and AMF on the denitrification process and shifting soil N_2O emission need further investigation.

Biochar is a carbon-rich solid material produced through the high-temperature cracking of crop straw, firewood, weeds, and feces in the absence of oxygen conditions [18]. In recent years, biochar has been used as a soil additive for the management of agricultural soil, which produced important impacts on soil quality, crop yield, carbon sequestration, and greenhouse gas emissions [2,14,19,20]. Previous studies have indicated that biochar addition can regulate the nitrification and denitrification process by facilitating biofilm formation and retaining soil moisture [21,22]. Biochar application can reduce N leaching from soil and activate N fixation by increasing soil carbon sequestration [23,24]. Moreover, biochar amendment can increase the soil cation exchange and enhance the adsorption of soil NH_4^+-N, thus reducing soil inorganic N content and inhibiting the nitrification process [25,26]. In addition, biochar could promote soil O_2 content to restrain the denitrification process and reduce N_2O emission [27,28]. However, few studies have explicitly examined the effects of AMF and plant roots on denitrifying genes and N_2O emissions, particularly in biochar amendment. Some recent studies have shown that biochar has a special effect on soil microbes, such as AMF [29–32]. For example, a crop straw-derived biochar application can improve soil physicochemical characteristics and facilitate the germination and growth of AMF spores [33,34], and biochar can regulate plant fungal signal substances that affect the germination and hyphal branching of AMF [35,36]. Zhang et al. [37] also showed that biochar may indirectly influence AMF by affecting the activity of phosphorus bacteria in soil. Yet, it remains largely unexplored how plant roots associated with AMF affect N utilization and N_2O emission under biochar application. Therefore, the microcosm experiment was conducted to investigate the impacts of AMF, plant roots, and biochar on plant growth, soil N utilization, soil denitrification, and N_2O emission in maize planting soil systems. We hypothesized that (1) the biochar addition would enhance the growth of AMF mycelial, increase the activity of AMF mycelial microorganisms, and promote the absorption and transfer of effective nitrogen to plants to reduce the substrate concentration of denitrification microorganisms and then affect the soil denitrification process and N_2O emissions, and (2) the combined of biochar, AMF, and plant roots would reduce N_2O-producing gene abundance and increase soil N utilization, corresponding to decreases in N_2O emission.

2. Materials and Methods

2.1. Biochar, AMF Inocula, Soil, and Plant Preparation

The biochar was produced from wheat straw pyrolyzed under 500 °C in anaerobic conditions for 2 h, and the heating rate was 20 °C min^{-1} (Sanli New Energy Co. Ltd., Shangqiu, China). The organic C and N contents were measured using a Perkin-Elmer 2400 CHN elemental analyzer (Perk-Elmer Co. Ltd., Norwalk, CT, USA). The total K content was determined using acid digestion and elemental analysis through atomic adsorption spectroscopy (Thermo Fisher Scientific Co. Ltd., Waltham, MA, USA). The available P was extracted using 0.5 mol L^{-1} $NaHCO_3$ and measured colorimetrically (Beijing Puxi General Instrument Co. LTD., Beijing, China). The pH was measured for a 1:5 (*w*/*v*) suspension of biochar and deionized water with a compound glass electrode. The surface area and pore size were measured using a TriStar II 3020 automated surface area and pore size analyzer (Micromeritics Co. Ltd., Atlanta, GA, USA). The basic biochar properties were surface area at 8.9 m^2 g^{-1}, average pore size at 3.14 nm, total pore volume at 0.015 cm^3 g^{-1}, organic C content at 46.76%, total N content at 0.59%, available P content at 0.89 g kg^{-1}, K content at 2.72%, and pH at 10.4.

The AMF inoculum, *Rhizophagus intraradices* BJ09, and *Funneliformis mosseae* BJ04A were purchased from the Beijing Academy of Agriculture and Forestry Sciences. The inocula were pot-cultured with Sudangrass [Sorghum sudanese (Piper) Stapf.] in the autoclaved (121 °C for 2 h) mixture of zeolite and river sand at 4:1 (*w*/*w*) in the greenhouse for four months. The ratio of the substrate and AMF inoculum was 100:1 (*w*/*w*). The temperature was maintained at 16–25 °C, with regular watering to maintain plant growth. The AMF inocula contains spores (approximately 30 spores per g^{-1} of substrate), mycelium, and fine root segments.

Surface horizon soil (0–20 cm) was collected from agricultural soil at Henan University of Science and Technology (34°38″ N, 112°28″ E), Luoyang, China, on 5 May 2020. The soil was air-dried and passed through a 5 mm sieve for the microcosm experiment. The soil had a pH of 7.2, 14.09 g kg^{-1} of organic matter, 53.76 mg kg^{-1} of available N, 10.18 mg kg^{-1} of available P, and 112.74 mg kg^{-1} of available K. The planted crop was maize (*Zea mays* L. var. Zhengdan-958).

2.2. Experimental Design

2.2.1. Microcosm Experiment

The microcosm experiment was established as a completely randomized trial with two biochar levels and three mycorrhizal levels, making a total of six treatments. The two levels of biochar were no biochar application (B0) and biochar addition rate of 10 g·kg^{-1} soil (B1). To insulate the impact of AMF hyphae alone or AMF hyphae with roots on N_2O emission, the plexiglass mesocosms (45 cm length, 15 cm width, 18 cm depth) were constructed and separated into three identical compartments by different mesh membrane sizes to create different mycorrhizal treatments as follows: 0.45 μm pore size nylon mesh (neither AMF nor plant roots, CK) (Figure 1A), 20 μm pore size nylon mesh (only AMF hyphae can grow through, AMF) (Figure 1B), and 1.6 mm pore size nylon mesh (AMF hyphae and plant roots can penetrate through, AMF + R) (Figure 1C). The three meshes used in this experiment all allow water, soluble C, and N produced by plant roots to pass through. Each compartment of half of the plexiglass mesocosms was filled with 3.0 kg of soil evenly mixed with 100 g of AMF inoculum. Meanwhile, each compartment of the other half of the plexiglass mesocosms was filled with 3.0 kg of an even mixture of soil and the prepared biochar at a rate of 10 g·kg^{-1}, as well as 100 g AMF of inoculum. Each treatment had four replicates, and there was a total of 24 plexiglass mesocosms. The two compartments at the left and right of the plexiglass mesocosm were named as host compartments, in which maize was planted, and the compartment in the middle of the plexiglass mesocosm was designated as the test compartment, in which N_2O emission was measured (Figure 1).

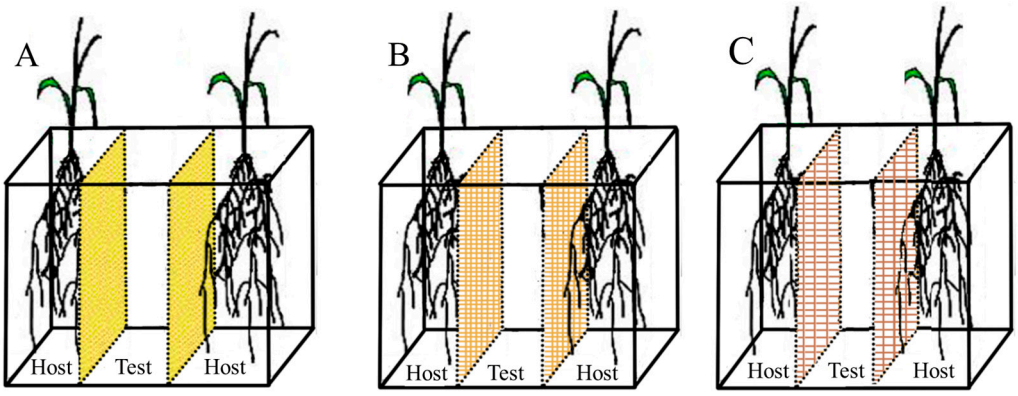

Figure 1. Schematic diagram of root compartments. The plexiglass mesocosms (45 cm length, 15 cm width, 18 cm depth) were constructed and separated into three identical compartments by different mesh membrane sizes to create different mycorrhizal treatments: (**A**) 0.45 μm pore size nylon mesh (neither AMF nor plant roots); (**B**) 20 μm pore size nylon mesh (only AMF hyphae can grow through); (**C**) 1.6 mm pore size nylon mesh (AMF hyphae and plant roots can penetrate through). Host compartments with growing maize and test compartments with N_2O collection.

2.2.2. Plant Growth Conditions

The experiment was conducted in the greenhouse at Henan University of Science and Technology from 10 May to 3 August 2020. The air temperature in the greenhouse ranged between 24.2 °C and 36.4 °C. Two maize seeds were sown in the host compartment at the left and right of the plexiglass mesocosms on 10 May 2020 and thinned to one plant on the seventh day after germination. At the jointing stage of maize, the 10.21% abundance of ^{15}N labeled urea fertilizer was hole-applied into each test compartment of plexiglass mesocosms to achieve a rate of 180 kg N ha^{-1} soil. The plexiglass mesocosms were placed randomly; deionized water was periodically watered for host compartments with plants, as needed. The test compartments without plants were also watered with a small amount of deionized water and usually were observed to ensure that plant roots and AMF could pass through the mesh.

2.3. Measurements

2.3.1. Measurement of Plant Biomass, N Contents, and Root AMF Colonization

The maize plants were harvested after 85 days of growth. The aboveground parts and roots were sampled as described by Zhang et al. [14]. The fresh weight of the maize aboveground parts and roots was determined with an electronic balance. Then, the aboveground parts were dried to obtain a stable weight and ground to measure the N contents. The total N contents were determined using the micro-Kjeldahl method after digestion with H_2SO_4-H_2O_2. The ^{15}N abundance of the maize plant was determined using the isotope mass spectrometer (Elementar Analysensystems GmbH Inc., Langenselbold, Germany). Fresh fibrous roots ($\leq$2 mm in diameter) were cut into 1 cm root segments, which were put into the test tube, added 10% KOH(w/v) for digestion, and then stained with acid fuchsin. The root AMF colonization was observed under a microscope (100×) using the gridline intersection method [38].

2.3.2. Measurement of Soil N_2O Emissions

The N_2O gas samples were collected from the test compartment using the static chamber method [39]. Gas samples were collected from 1 day after fertilization (DAF) and discontinuously collected at 1–5, 7, 9, 11, 13, 18, and 21–28 DAF. The concentrations of N_2O were measured using gas chromatography (Agilent 7890A, Agilent Technologies Co. Ltd., Santa Clara, CA, USA). The calculations of N_2O fluxes and cumulative emissions by the research of Gao et al. [40].

2.3.3. Soil Sampling and Analyses

After the maize plants were harvested, soil samples of the test compartment were collected, and the visible residues and plant roots were carefully removed. Some of the soil samples were stored in a −80 °C refrigerator for soil DNA extraction, and the remainder of the soil was stored in a 4 °C refrigerator for soil total nitrogen, soil ^{15}N abundance, NH_4^+-N, and NO_3^--N measurements. The soil total nitrogen, NH_4^+-N, and NO_3^--N contents were measured using a continuous flow analyzer (SEAL Analytic, Norderstedt, Germany). The soil ^{15}N abundance was determined in a similar manner as that described for the plants' ^{15}N mensurate.

2.3.4. Soil DNA Extracted and Denitrification Functional Gene Abundances

A total of 0.5 g of the soil samples stored in the −80 °C refrigerator to extract soil DNA were taken according to the method and procedure of the Fast DNA SPIN Kit for Soil (MP Biomedicals Co. Ltd., Santa Ana, CA, USA). After extraction, the integrity of the proposed DNA fragments was measured using 1% agarose gel electrophoresis. The extracted DNA purity and concentration were measured using an ultraviolet spectrophotometer (Thermo Fisher Scientific Co. Ltd., Waltham, MA, USA). The abundances of the *nir*S, *nir*K, and *nos*Z genes were measured through quantitative real-time polymerase chain reaction (qPCR) (Applied Biosystems, Waltham, MA, USA). The qPCR primers are given in Table 1.

Table 1. Primers and qPCR cycle conditions.

Target Gene	Primer	Sequence	Reaction Conditions of qPCR	Reference
nirS	*nirSC*d3AF *nirSR*3cd	GTSAACGTSAAGGARACSGG GASTTCGGRTGSGTCTTGA	Procedure 1	[41]
nirK	*nirK*1F *nirK*5R	GGMATGGTKCCSTGGCA GCCTCGATCAGRTTRTGG	Procedure 2	[42]
nosZ	*nosZ*-1F *nosZ*-1622R	CGYTGTTCMTCGACAGCCAG CGSACCTTSTTGCCSTYGCG	Procedure 1	[43]

Procedure 1: 5 min—95 °C, 30 s—95 °C, 30 s—58 °C annealing, 35 cycles, 1 min—72 °C, 5 min—72 °C, Procedure 2: 5 min—95 °C, 30 s—95 °C, 30 s—55 °C annealing, 35 cycles, 1 min—72 °C.

2.4. Calculation of Nitrogen Utilization

The ^{15}N utilization efficiency by plants (NUE), ^{15}N retention rate by soil (NRR), and ^{15}N loss rate (NLR) were calculated according to the following formulas used by Robinson [44].

$$\text{NUE or NRR\%} = \times 100 \quad (1)$$

$$\text{NLR\%} = 100 - \text{NUE} - \text{NRR} \quad (2)$$

$$\text{N1} = \text{N0} \times \text{A} \quad (3)$$

$$\text{A\%} = \frac{\text{APC}(^{15}\text{N})}{\text{C}} \times 100 \quad (4)$$

where NUE is the ^{15}N utilization efficiency for maize plants, NRR is the ^{15}N retention rate for soil, and NLR is the ^{15}N loss rate, which is calculated according to Equations (3) and (4). N1 is the ^{15}N contents from ^{15}N urea in the plant or soil ($g \cdot kg^{-1}$), N2 is the total ^{15}N contents from ^{15}N urea ($g \cdot kg^{-1}$), N0 refers to total N contents in the plant or soil ($g\ kg^{-1}$), A is the percentages of ^{15}N amount from ^{15}N urea of total N in the plant or soil (%), APC (^{15}N) is the ^{15}N-atom percent excess of the plant or soil (%), and C is the ^{15}N-atom percent excess of labeled ^{15}N urea (%).

2.5. Statistical Analysis

All the data analyses were performed using SPSS 25.0 software. The significance was tested using analysis of variance (ANOVA) and LSD for multiple comparisons. Two-way ANOVA was used to analyze the significance of biochar and AMF effects. Correlations

among plant biomass, AMF, soil NH_4^+-N and NO_3^--N, abundance of denitrification functional genes, and N_2O emission were analyzed by using Pearson's correlation.

3. Results

3.1. Maize Plant Biomass, Soil Inorganic N Contents, and AMF Colonization of Roots

Both biochar application and AMF significantly affected maize biomass (Tables 2 and 3). Compared with no biochar input, the biochar application significantly increased maize biomass under the same treatment. Compared with the CK, the AMF and AMF + R treatments all significantly enhanced the growth traits of fresh weight ($p < 0.05$) under two biochar application levels (Table 1).

Table 2. Plant biomass, root colonization rate, and soil inorganic N contents with CK, AMF, and AMF + R treatments under different biochar application levels.

Biochar Levels	Treatment	Fresh Weight of the Root (g plant^{-1})	Fresh Weight of the Shoot (g plant^{-1})	Soil NH_4^+-N (mg N kg^{-1})	Soil NO_3^--N (mg N kg^{-1})	AMF Colonization (%)
B0	CK	110.78 ± 10.23 e	243.12 ± 20.34 d	1.02 ± 0.24 a	45.38 ± 5.12 a	41.17 ± 4.78 c
	AMF	132.07 ± 15.36 cd	246.08 ± 22.67 d	0.91 ± 0.25 b	27.70 ± 3.24 c	40.27 ± 5.28 d
	AMF + R	136.63 ± 11.25 c	266.47 ± 30.89 c	0.87 ± 0.26 b	9.65 ± 1.23 d	38.23 ± 4.17 fde
B1	CK	132.96 ± 16.39 cd	260.46± 28.67 c	0.95 ± 0.14 b	32.20 ± 3.78 b	45.14 ± 4.96 a
	AMF	141.66 ± 15.56 b	304.61 ± 32.78 b	0.73 ± 0.15 c	23.06 ± 2.79 c	44.67 ± 5.02 b
	AMF + R	163.63 ± 21.15 a	338.62 ± 37.59 a	0.68 ± 0.12 c	7.33 ± 1.11 e	43.46 ± 4.79 bc

Values are mean ± SE (n = 4). B0, no biochar application. B1, biochar application, Different letters among the treatments indicated the significance according to Duncan's LSD test at 0.05 level.

Table 3. Results (F-values) of two-way ANOVA for the effects of biochar, AMF, and their interaction on plant and soil-related indices.

Factors	Biochar(B)	AMF	B × AMF
Shoot biomass (g plant^{-1})	16.7 **	42.5 **	12.1 *
Root biomass (g plant^{-1})	14.2 **	5.7	2.7
AMF colonization (%)	29.6 **	2.4	1.5
Soil NH_4^+-N (mg N kg^{-1})	47.9 ***	113.6 ***	16.8 **
Soil NO_3^--N (mg N kg^{-1})	20.78 **	109.7 ***	8.9
N_2O emission flux (μg·m^{-2}·h^{-1})	116.2 ***	79.4 ***	25.7 **
nirK (copies g^{-1} soil)	35.4 ***	78.5 ***	13.7 **
nirS(copies g^{-1} soil)	56.2 ***	82.3 ***	17.5 **
nosZ(copies g^{-1} soil)	38.9 ***	78.5 ***	24.7 ***
*nir*S + *nir*K/*nosZ*	87.2 ***	70.3 ***	19.4 **

Significant effects: * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$.

Compared with the CK, the AMF and AMF + R treatments all decreased the AMF colonization rate of maize roots under both biochar application levels (Tables 2 and 3). The AMF and AMF + R treatments reduced the average AMF colonization rate by 1.87% and 5.68%, respectively. There was no significant difference in the AMF colonization rate between the AMF and AMF + R treatments (Table 2). Compared with no biochar input, the biochar application showed a trend of increasing root AMF colonization. Compared with no biochar input, the CK, AMF, and AMF + R treatments enhanced the AMF colonization rate by 9.64%, 10.93%, and 13.68%, respectively, at the B1 input level.

Compared to B0 addition, B1 application significantly reduced the average soil NH_4^+-N and NO_3^--N contents by 16.43% and 22.62% (Table 2). Compared with the CK, the AMF and AMF + R treatments significantly decreased soil NH_4^+-N and NO_3^--N contents at two biochar input levels. The AMF and AMF + R treatments at the B0 level reduced the soil NH_4^+-N contents by 10.78% and 14.71%, respectively, and the NO_3^--N contents by 38.96% and 78.73%, respectively, and at the B1 level, they reduced the soil NH_4^+-N contents by

23.16% and 30.53%, respectively, and NO_3^--N contents by 28.38% and 77.23%, respectively, compared with the CK treatment (Table 2).

3.2. Soil N_2O Emissions

The N_2O emission rates showed a variation trend of increasing first and then decreasing during the experimental period at both biochar levels (Figure 2), and the N_2O emission peak occurred on the third to fourth days after fertilization. Compared with the CK, the AMF and AMF + R treatments all significantly decreased the N_2O emission peak by 40.62% and 53.23%, respectively, during the B1 application and 27.63% and 34.67%, respectively, during the B0 application. The result showed that biochar application significantly decreased soil N_2O emission rates (Figure 2). Both biochar input and AMF presence significantly affected soil N_2O emission flux (Table 3).

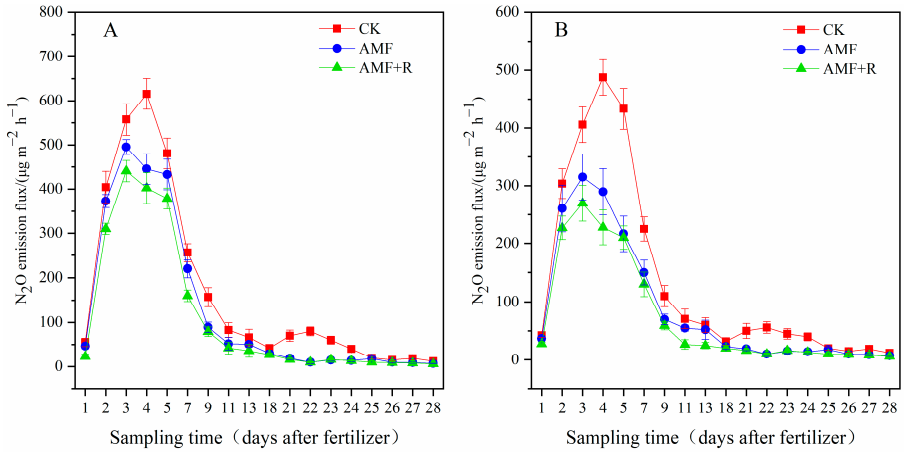

Figure 2. Effects of biochar input, AMF, and roots on soil N_2O emission flux. Values are means ± SE (n = 4) at any given time point. (**A**) No biochar application; (**B**) biochar application.

The soil N_2O accumulation emission during different treatments showed the trend of CK > AMF > AMF + R at both biochar levels (Figure 3). Compared with the CK, the AMF and AMF + R treatments significantly decreased N_2O accumulation emission by 14.41% and 25.02%, respectively, during the B0 application and 21.12% and 38.13%, respectively, during the B1 application. Overall, the soil N_2O cumulative emissions were significantly decreased during AMF and AMF + R treatments compared with CK ($p < 0.05$), and the AMF + R treatment had the best effect in reducing the soil N_2O cumulative emissions.

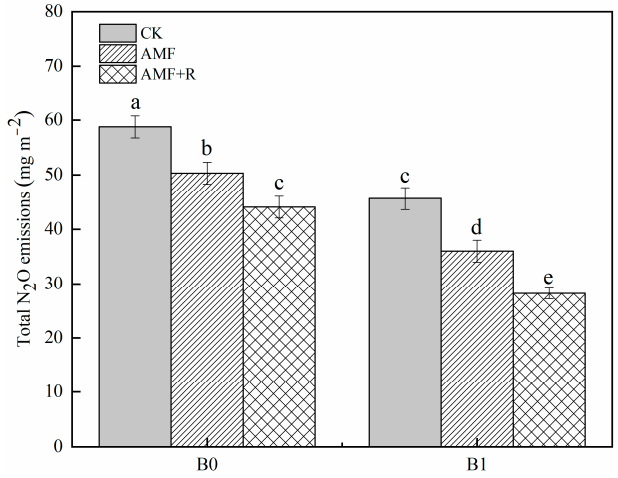

Figure 3. Effects of biochar input, AMF, and roots on soil total N_2O emission. Values are mean ± SE (n = 4). B0, no biochar application; B1, biochar application. Different letters among the treatments indicated the significance according to Duncan's LSD test at 0.05 level.

3.3. Abundances of Denitrification Genes

The AMF and AMF + R treatments enhanced the *nir*S, *nir*K, and *nos*Z gene copy numbers at both biochar application levels (Figure 4). During B1 application, the AMF and AMF + R treatments increased the *nir*S gene copy numbers by 23.83% and 49.10%, respectively, the *nir*K gene copy numbers by 68.01% and 113.75%, respectively, and the *nos*Z gene copy numbers by 110.86% and 175.51%, respectively, compared with CK. During B0 application, the AMF and AMF + R treatments increased the *nir*S gene copy numbers by 27.02% and 45.86%, respectively, the *nir*K gene copy numbers by 62.55% and 127.18%, respectively, and the *nos*Z gene copy numbers by 66.27% and 118.71%, respectively.

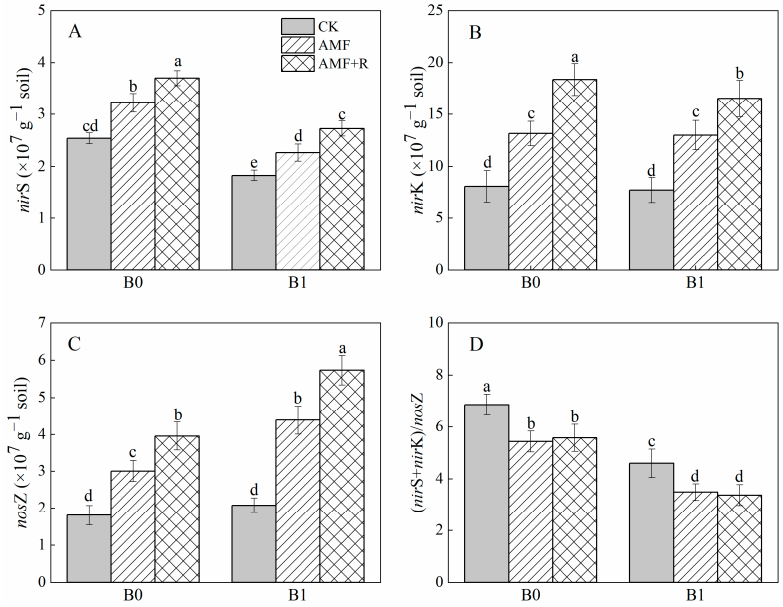

Figure 4. Effects of biochar input, AMF, and roots on soil *nir*K (**A**), *nir*S (**B**), and *nos*Z (**C**) gene copy numbers and *nir*S + *nir*K/*nos*Z ratio (**D**). Values are mean ± SE (n = 4). B0, no biochar application; B1, biochar application. Different letters among the treatments indicated the significance according to Duncan's LSD test at 0.05 level.

The AMF and AMF + R treatments significantly decreased *nir*S + *nir*K/*nos*Z at both biochar levels. The AMF and AMF + R treatments increased *nir*S + *nir*K/*nos*Z by 24.32% and 26.90%, respectively, at the B1 level and 20.83% and 18.84%, respectively, at the B0 level. The results showed that biochar input significantly decreased *nir*S + *nir*K/*nos*Z compared with no biochar addition (Figure 4). Both biochar input and AMF or root presence significantly affected the abundances of the denitrification genes (Table 3).

3.4. Nitrogen Content and Nitrogen Use Efficiency of Maize Plants

The biochar, AMF, and AMF with roots all had significant ($p < 0.05$) effects on the uptake and utilization of N nutrients for maize plants (Table 4). Compared with the CK, the AMF and AMF + R treatments all significantly increased the ^{15}N absorption and ^{15}N utilization efficiency of maize under both biochar addition levels. The AMF and AMF + R treatments increased N utilization efficiency by 18.03% and 25.17%, respectively, at the B0 level and 4.46% and 20.15%, respectively, at the B1 level. Compared with no biochar input, the biochar application significantly increased the N uptake and absorption, ^{15}N content, and nitrogen use efficiency of maize during the same treatment.

The soil ^{15}N retention rate during different treatments showed the trend of AMF + R > AMF > CK at the same biochar levels. The largest value of the soil ^{15}N retention rate was 31.09% during the AMF +R treatment with biochar application (Table 4). Contrarily, the soil ^{15}N loss rate during different treatments showed the trend of CK > AMF > AMF + R at the same biochar levels. Compared with CK, the AMF and AMF + R treatments decreased

the soil ^{15}N loss rate by 10.33% and 14.42%, respectively, at the B0 level and 9.06% and 19.16%, respectively, at the B1 level.

Table 4. Effects of biochar input, AMF, and roots on nitrogen uptake of maize and the fate of ^{15}N-labeled urea N.

Biochar Levels	Treatment	Nitrogen Content of Maize (g kg^{-1})	^{15}N Absorption of Maize (g kg^{-1})	^{15}N Utilization Efficiency (%)	Soil ^{15}N Retention Rate (%)	Soil ^{15}N Loss Rate (%)
B0	CK	16.79 ± 1.04 e	3.35 ±8.93 d	20.02 ± 2.25 d	26.79 ± 3.04 e	53.19 ± 4.28 a
	AMF	22.04 ± 1.23 c	3.79 ±9.23 c	23.63 ±1.40 c	27.78 ± 3.23 d	48.59 ± 4.28 c
	AMF + R	27.56 ± 2.18 a	4.13 ± 8.59 b	25.06 ± 2.09 b	28.56 ± 14.18 c	46.38 ± 4.28 d
B1	CK	18.29 ± 2.27 d	3.75 ±3.10 c	23.72 ± 6.24 c	27.29 ± 2.27 d	48.99 ± 4.28 b
	AMF	24.80 ± 2.47 b	4.36 ±3.43 b	24.74 ± 1.63 b	29.80 ± 1.47 b	45.46 ± 4.28 d
	AMF + R	28.09 ± 1.77 a	4.98 ±7.07 a	28.50 ± 3.37 a	31.09 ± 3.77 a	40.41 ± 4.28 e

Values are mean ± SE (n = 4). B0, no biochar application; B1, biochar application. Different letters among the treatments indicated the significance according to Duncan's LSD test at 0.05 level.

3.5. Factors Affecting Soil N_2O Emissions

N_2O emission was correlated positively with the soil NH_4^+-N, NO_3^--N, and (*nir*K + *nir*S)/*nos*Z ratio. The *nir*K, *nir*S, and *nos*Z gene abundance, AMF colonization, and shoot biomass were correlated negatively with N_2O emission (Figure 5).

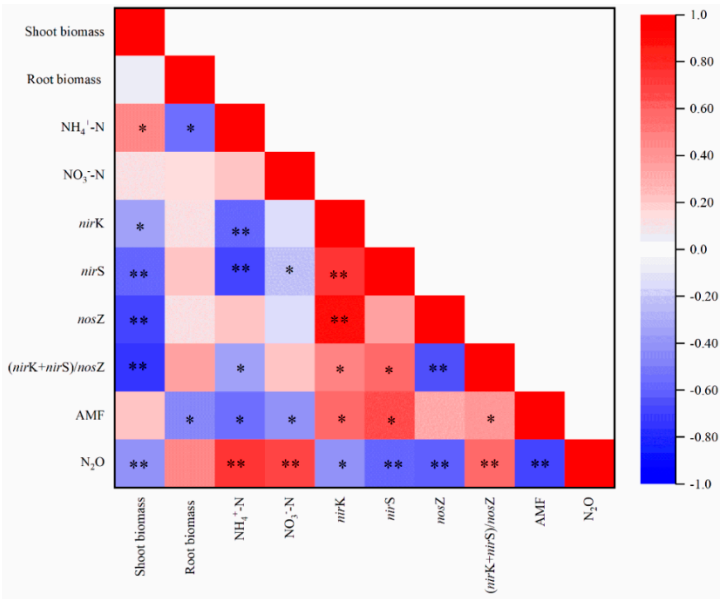

Figure 5. Relationships among plant biomass, soil inorganic N, denitrification gene abundance, AMF, and soil N_2O emission. ** $p < 0.01$ and * $p < 0.05$.

4. Discussion

4.1. Effects of Biochar, AMF, and Roots on Maize Growth

The studies revealed that the AMF alone or AMF with plant roots increased maize biomass (Table 2). The positive effects on maize growth are likely because AMF and plant roots promote nutrient acquisition and water uptake [30,34], which are beneficial to plant growth. Consistent with some previous studies [32,37], in this study, biochar application also promoted maize biomass. This may be because biochar could supply valuable macronutrients (e.g., N and P) and metal ions (e.g., Ca^{2+} and Mg^{2+}), which directly promote plant growth [45]. The second reason may be that biochar has the characteristics of a porous structure and large surface area, which can increase nutrient retention and reduce nutrient loss, thus indirectly promoting plant growth [46,47]. The results showed that biochar

combined with AMF and/or plant roots had the highest shoot and root biomass. There was likely an interaction between biochar and AMF, which is consistent with previous studies [48,49]. In this study, biochar application enhanced AMF colonization, which increased the acquisition of soil nutrients and water and improved maize growth.

4.2. Effects of Biochar, AMF, and Roots on Soil N Utilization

Using the ^{15}N labeling technique, this study revealed that biochar input decreased soil NH_4^+-N and NO_3^--N concentrations and increased the ^{15}N utilization efficiency of maize and ^{15}N retention rate in soil. The first reason may be that biochar improved the soil's physical and chemical properties, which enhanced the inorganic N uptake of plants, ^{15}N utilization efficiency, and ^{15}N retention rate and resulted in a reduction of soil inorganic N content [50–53]. Additionally, previous studies have shown that biochar addition could increase the ion exchange capacity of soil and improve the adsorption and fixation of soil NH_4^+-N and NO_3^--N. These inorganic N were absorbed by biochar and could be released again for the absorption and utilization of plants, thus improving N utilization efficiency [32,54,55]. The third possible explanation for the increase in ^{15}N utilization efficiency is that biochar can increase nutrient availability, stimulate beneficial soil microbial activity, and reduce nutrient leaching [56–58]. Some studies have indicated that biochar addition increased soil pH and supplied a more suitable environment for the growth of nitrifying microbes, which could improve the nitrification process and decrease soil NH_4^+-N and NO_3^--N contents [59,60]. The results also showed that AMF and AMF + R treatments significantly decreased soil NH_4^+-N and NO_3^--N contents (Table 2). The AMF and/or plant roots play a vital role in soil N utilization and plant N uptake [15,61,62]. The extraradical mycelia of AMF and plant roots can absorb inorganic N from soil and transport them to host plants [63]. Moreover, AMF can promote the degradation of nitrogen by soil microorganisms, thus improving N uptake in plants and reducing soil N content [64,65]. Therefore, the AMF significantly reduced soil inorganic N contents and enhanced N utilization efficiency in this study.

The biochar combined with AMF alone or AMF with plant roots all significantly decreased soil NH_4^+-N and NO_3^--N contents and increased the ^{15}N utilization efficiency of maize and the ^{15}N retention rate of the soil, compared with no biochar input or biochar input with no AMF treatments. There are some reasons for the abovementioned results. Firstly, biochar application can improve the physiochemical properties of soil and promote the germination and growth of AMF spores. In addition, biochar has the properties of a porous structure, providing a favorable habitat for AMF [35,66]. Secondly, biochar may affect the germination and branching of AMF by regulating plant fungal signal substances [58,67]. Thirdly, the biochar indirectly affects AMF by affecting the activity of phosphorus bacteria in the soil [67]. Therefore, AMF can improve plants to absorb more soil inorganic N with biochar application.

4.3. Effects of Biochar, AMF, and Roots on N_2O Emissions

In the present study, the application of AMF alone or AMF with plant roots significantly decreased soil N_2O emission, compared with CK treatment (Figures 2 and 3). The application of biochar and AMF with plant roots showed the largest decrease in soil N_2O emission, which was consistent with the study by Hao et al. [36]. There are several reasons that biochar, AMF, and plant roots suppress soil N_2O emissions. On the one hand, biochar, AMF, and plant roots have inherent characteristics that help plants acquire nutrients from the soil [20,68]. In this study, biochar, AMF, and plant roots all reduced soil NH_4^+-N and NO_3^--N concentrations and increased the utilization of soil N. On the other hand, biochar application facilitates the germination of AMF spores and the extension of extraradical mycelial, which enhance N uptake in the host plant, reduce the N substrate of soil nitrifiers and denitrifiers, and decrease N_2O emissions [8,35].

In agricultural soil, N_2O is mainly produced through the participation of soil nitrifiers and denitrifiers. The denitrification process, in general, plays a dominant role in soil N_2O

emissions [8,69]. In the process of denitrification, the denitrifiers acquire energy from organic matter and convert NO_3^- to N_2O. In this study, the abundance of denitrifying functional genes was measured, which was helpful in estimating whether the change in soil N_2O emission was related to biochar-, AMF-, and plant root-induced changes in the abundance of denitrifying functional genes (Figure 4). This research showed that AMF and AMF + R treatments all enhanced *nir*S, *nir*K, and *nos*Z abundance and reduced the ratio of *nir*K + *nir*S/*nos*Z at both biochar application levels (Figure 4). Zhang et al. [14] showed that AMF alone or AMF and plant root treatments all enhanced *nir*S abundance and reduced the *nir*k/*nir*S ratio at high N and normal N input levels with conventionally managed soil. The present research showed that AMF with plant roots decreased N_2O emission directly by reducing soil N and indirectly by changing the abundance of N_2O-producing functional genes in the agroecosystem. Qiu et al. [9] reported that AMF with plant roots reduced *nir*K abundance and the (*nir*K + *nir*S)/*nos*Z ratio, leading to a reduction in soil N_2O emissions. In summary, the effect of AMF and plant roots on N_2O emission was mainly exhibited through regulating denitrifying functional genes and the denitrification process. This study showed that biochar addition decreased *nir*S and *nir*K abundance and enhanced *nos*Z abundance during AMF and AMF + R treatments (Figure 4). The positive correlation between the soil's N_2O emission and the (*nir*K + *nir*S)/*nos*Z ratio (Figure 5) was consistent with the results from other agricultural soils [9,36]. For instance, Hao et al. [36] reported that biochar addition and/or AMF inoculation decreased N_2O emissions in wheat-planted soil, compared with soil in the absence of biochar and AMF addition. This decrease in soil emission was mainly associated with the diminished abundance of N_2O-producing functional genes (*nir*K and *nir*S) and the increased abundance of N_2O-consuming functional genes (*nos*Z), and the (*nir*K + *nir*S)/*nos*Z ratio was the strongest driver for soil N_2O. In brief, the results suggest that the application of biochar, AMF, and plant roots may alter N_2O emission by modifying the denitrifying functional gene abundance. Furthermore, the detailed mechanisms of the interaction of biochar, AMF, and plant roots on soil denitrifying microbes and functional genes are still unclear and need further research.

5. Conclusions

The biochar application and presence of AMF or AMF with plant roots reduced soil N_2O emissions by increasing soil N unitization efficiency and decreasing the (*nir*K + *nir*S)/*nos*Z ratio in maize-planted soils. The presence of AMF and plant roots during biochar amendment showed the highest decrease in N_2O emission and an increase in soil N unitization efficiency. The biochar, AMF, and plant roots may interact to shift plant growth, N utilization, denitrifying functional genes, and N_2O emissions. These results suggest that biochar, in combination with AMF, may be considered an effective method for mediating N cycling and reducing N_2O emissions in agroecosystems. This study was performed under microcosm conditions, which were different from the field environment (fertilization, precipitation, and tillage practices). Therefore, it is necessary to conduct field experiments to study the effects and mechanisms of biochar and AMF on soil N cycle and N_2O emission.

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