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Article

Arbuscular Mycorrhizae Shift Community Composition of N-Cycling Microbes and Suppress Soil N_2O Emission

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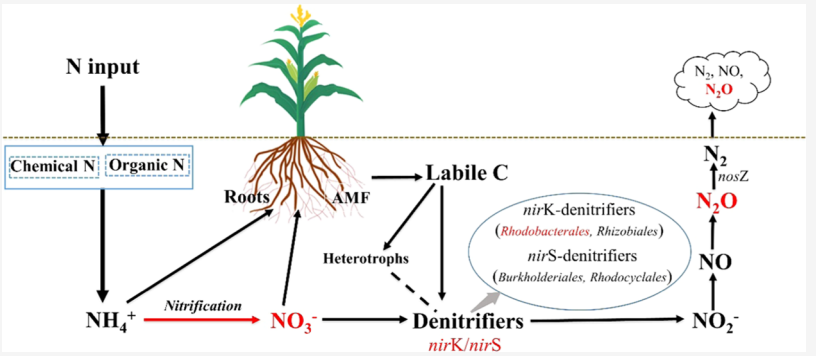

ABSTRACT: Mycorrhizae are ubiquitous symbiotic associations between arbuscular mycorrhizal fungi (AMF) and terrestrial plants, in which AMF receive photosynthates from and acquire soil nutrients for their host plants. Plant uptake of soil nitrogen (N) reduces N substrate for microbial processes that generate nitrous oxide (N_2O), a potent greenhouse gas. However, the underlying microbial mechanisms remain poorly understood, particularly in agroecosystems with high reactive N inputs. We examined how plant roots and AMF affect N_2O emissions, N_2O-producing (*nir*K and *nir*S) and N_2O-consuming (*nos*Z) microbes under normal and high N inputs in conventional (CONV) and organically managed (OM) soils. Here, we show that high N input increased soil N_2O emissions and the ratio of *nir*K to *nir*S microbes. Roots and AMF did not affect the (*nir*K + *nir*S)/*nos*Z ratio but significantly reduced N_2O emissions and the *nir*K/*nir*S ratio. They reduced the *nir*K/*nir*S ratio by reducing *nir*K-*Rhodobacterales* but increasing *nir*S-*Rhodocyclales* in the CONV soil while decreasing *nir*K-*Burkholderiales* but increasing *nir*S-*Rhizobiales* in the OM soil. Our results indicate that plant roots and AMF reduced N_2O emission directly by reducing soil N and indirectly through shifting the community composition of N_2O-producing microbes in N-enriched agroecosystems, suggesting that harnessing the rhizosphere microbiome through agricultural management might offer additional potential for N_2O emission mitigation.

KEYWORDS: *nitrous oxide, nitrogen fertilizer, arbuscular mycorrhizal fungi, plant roots, denitrifier communities, microbial diversity*

INTRODUCTION

Nitrous oxide (N_2O) is a long-lived potent greenhouse gas that has a global warming potential 2 magnitudes higher than carbon dioxide (CO_2).[1,2] It is also the most important depleter of the stratospheric ozone.[3,4] High chemical and organic nitrogen (N) fertilizer applications in agricultural ecosystems are the primary causes contributing to the increasing atmospheric N_2O.[5−7] Two microbial processes, nitrification and denitrification, are primary sources of N_2O emissions,[4,5,8] but denitrification is the predominant source of N_2O from agricultural soils.[9,10] In denitrification, denitrifying microbes produce various reductases that convert nitrogen oxides (NO_3^-, NO_2^-) into gaseous products (i.e., N_2O and N_2).[8,11] Because denitrifier communities are highly diverse and difficult to quantify, the functional genes that encode reductases have been widely used to characterize the denitrifier communities.[12−15] While *nir*K and *nir*S genes encode nitrite reductases, that produce N_2O as a byproduct,[12,16] gene *nos*Z encodes nitrous oxide reductase that converts N_2O to N_2.[11,17] The ratio between N_2O-producing denitrifiers and N_2O-consuming denitrifiers (*nos*Z-type) is indicative to the source/sink strengths of N_2O. Also, the relative composition of N_2O-producing denitrifiers affects N_2O emissions as they have different efficiencies in producing N_2O. As *nos*Z gene is more likely linked with *nir*S-type denitrifiers,[18] *nir*S-type denitrifiers are believed to be less effective in generating N_2O emissions

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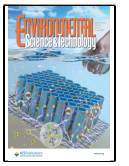

than *nir*K-type ones.[18−20] For example, Jones et al.[18] showed that soil N_2O sink capacity was positively related to the *nir*S:*nir*K gene abundance ratio. Therefore, the ratio of *nir*K/*nir*S has also been used as an indicator for potential N_2O emissions.[21−23]

Plant roots and their associated microbes, especially arbuscular mycorrhizal fungi (AMF), compete against nitrifiers and denitrifiers for N substrates and likely suppress N_2O emission.[21,24,25] AMF form symbiotic associations with roots of over 80% plant species, in which AMF obtain soil nutrients in exchange of photosynthates from host plants.[26,27] Yet, only a few studies have investigated the effects of plant roots and their associated AMF on soil N_2O emission, and these have yielded variable results.[21,25,28,29] Using either AMF-deficient tomato mutants or soil sterilization to manipulate the presence of AMF, for example, Bender et al.[21] showed that AMF significantly reduced soil N_2O emission. Other studies, however, showed or suggested that root exudates and labile C from plant roots and AMF may enhance denitrifiers and thus increase soil N_2O production.[30,31] Because of their extensive mycelial network, changes in C and N inputs induced by AM fungi can also significantly affect the composition and activities of the microbial community in the mycorhizosphere.[32−34] Qiu et al.[35] showed that under elevated atmospheric CO_2, plant roots with AMF reduced *nir*K abundance and the (*nir*K + *nir*S)/*nos*Z ratio, although AMF alone had no significant effects. Changes in soil denitrifiers induced by plant roots and/or AMF may further exert a major control over N_2O emission. Few studies, however, have explicitly examined the effects of AMF and roots on denitrifier communities and their N_2O emissions, particularly in N-enriched agroecosystems.

Modern agriculture is often characterized by high reactive N input either through chemical N fertilizers or organic manures, which may profoundly affect plant−microbe interactions, and the abundance and community composition of denitrifiers, regulating soil N_2O emission.[4,15,36] Previous studies showing AMF reduction of soil N_2O emission were mainly conducted in natural ecosystems where relatively low nutrient availability promotes the formation of mycorrhizae.[21,25,37] Applications of N fertilizers to agricultural soils often negatively affect AMF by reducing mycorrhizal colonization of roots, sporulation, and development of AM-fungal extraradical mycelia.[38,39] High N input can suppress root AM colonization and reduce the abundance and diversity of AMF.[40,41] However, neutral or even positive N effects on AMF have also been observed.[42−44] Yet, it remains largely unexplored how N fertilizer applications interact with plant roots and their associated AMF to affect N_2O emission.

AMF can play a role in plant N uptake and transfer in both conventionally and organically managed systems,[45,46] but are often believed to be less important in conventional systems where high chemical inputs often suppress AMF infection of plant roots and nutrient uptake.[39,45,47] The indirect effects of AMF on N-cycling microbes and N_2O emissions in soils with high N inputs, however, are seldom tested directly. We used both conventional and organic soils for two purposes. First, it is often believed that AMF are suppressed in conventional agricultural soils due to high N and fungicide input,[45,46] and their role in plant nutrient uptake is limited. Indeed, a few studies have examined the potential roles through which AMF affect other microbes such as denitrifiers and modify important functions such as N_2O emissions. Second, organic farming uses manures and other organic inputs which are typically less suppressive to AMF.[47,48] It remains largely unclear how AMF affect denitrifiers and N_2O emissions under high manure input. Our long-term field sites provide a unique opportunity to assess whether AMF differentially affect N_2O emissions under conventional and organic farming systems. Therefore, we conducted a mesocosm experiment to assess the impacts of AMF alone or roots with AMF on soil denitrifiers and N_2O emission in two contrasting soils that had been under distinct farming practice regimes (conventionally or organically managed) for 14 years at Center for Environmental Framing Systems, Goldsboro, North Carolina.[49,50] Two N input levels were also designed for each soil so that the response of soil denitrifiers to different N inputs can be assessed. We hypothesized that (1) the presence of AMF and roots would reduce the abundances of N_2O-producing denitrifiers, corresponding to decreases in N_2O emission, and (2) the composition of denitrifiers would differ between conventional and organic soils, but AMF and roots would influence it in a similar way in both soils.

MATERIALS AND METHODS

Experimental Design. This mesocosm experiment was performed in the greenhouse at North Carolina State University (NCSU), Raleigh, NC (Figure S1). The air temperature in the greenhouse was maintained at 25 ± 1 °C during the day and 23 ± 1 °C during the night.

Two distinct soils [conventionally managed (CONV) soil and organically managed (OM) soil] were used for this experiment. Field CONV and OM treatments were initiated at the Center for Environmental Farming Systems at NCSU (35°22′48″N, 78°02′36″W) in Goldsboro, NC, in 1999.[49,50] While the CONV field had been supplied only with chemical N, the OM field had received organic manures and cover crops since 1999.[49,50] Prior to our soil sampling in November 2013, maize (*Zea mays* L.) was the summer crop in both systems (i.e., fields). Both soils were partially air-dried and sieved to 4 mm to remove stones and plant roots before being used for the microcosm experiment (see Supplementary Materials and Methods for details).

This experiment was a full-factorial design with two N input levels and three mycorrhizal treatments. All treatments were replicated three times. The two levels of N fertilizers for each soil type were normal N input (NN) and high N input (HN: that is 2 × the normal rate). The CONV soil was supplied with urea at either180 (NN) or 360 kg N ha^{-1} (HN). The OM soil was supplied with chicken manure at either 12.5 (NN) or 25.0 (HN) t ha^{-1}, respectively (more details in Supplementary Materials and Methods).

To isolate the effects of AMF alone or roots with AMF on soil N_2O emissions, we used plexi-glass mesocosms (Figure S1) to control the ingrowth of AMF hyphae and/or host roots. Each mesocosm was divided into six compartments.[50,51] Three on one side were used as HOST compartments where host plants grew with AMF inoculum (Figure S1). The other three were used as TEST compartments where static chambers were installed to collect gas samples over the duration of the experiment (Figure S1). The HOST and TEST compartments were separated by three different size mesh to create three mycorrhizae treatments: the no-AMF or no-root control (CK) (0.45 μm), the AMF treatment (20 μm) that allowed AMF growing through (AMF), and the root with AMF treatment (1.6 mm) allowing the ingrowth of plant roots and AMF

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hyphae (RAMF) (see Supplementary Materials and Methods for details).

We adopted this method to isolate the effects of AMF and roots because AMF are obligate biotrophs and need to colonize plant roots to grow and reproduce.[51,52] Consequently, the effects of roots and AMF on N-cycling microbes and N_2O emissions are confounded in natural soils. Our goal was to assess the impact of AMF and roots on denitrifiers and N_2O emissions in real soils with intact native microbial communities, which is the unique aspect of our study. Because the traditional method through sterilization and reinoculation of AMF greatly alters soil microbial communities and soil physical/chemical conditions, including soil N availability, the results obtained would poorly reflect what happens in reality. The method we used here selectively allows or prevents the penetration of roots or AMF but allows soil solutions to move freely.[52] This method has been widely used by other scientists in the last two decades,[33,53] to isolate and/or assess the effects of roots and/or AMF on nutrient transfer[50] and organic carbon decomposition.[54,55]

Plant Growing Conditions. All of the mesocosms were placed in a temperature-controlled greenhouse (Figure S1). Each HOST and TEST compartment was filled with 3.5 kg of soil. The HOST compartment was inoculated with 100 g inoculum of a mixture of different AMF species.[35,54] Two maize seeds (*Zea mays* L.) were sown to each of the HOST compartments on 4 March 2014 (0 day after sowing, DAS 0). Both HOST compartments with plants and TEST compartments were periodically watered with deionized water as needed (more details in Supplementary Materials and Methods).

Measurements of Soil N_2O and CO_2 Emissions. We collected gas samples from each TEST compartment using a modified static chamber method as described in Qiu et al.[35] Gas emission rates were measured separately at 3−6, 14−19, 35−40, 47, and 56−66 DAS (more details in Supplementary Materials and Methods). It needs to note that this mesocosm study was designed to determine soil N_2O emission mainly from denitrification, and N_2O emission from nitrification process was not considered because N_2O emissions had been monitored at our field site for over two years, and N_2O emissions was not detectable when soil moisture was low (see Supplementary Materials and Methods for more details). Therefore, in each TEST compartment, deionized water was applied to reach ∼70 to 80% of soil water-filled pore space (WFPS) to mimic a rain event in the evening prior to collection of gas samples and create an anaerobic environment for the microbial denitrification (see Supplementary Materials and Methods for more details).[35] The soil moisture was also periodically determined by Hydrosense II probe (Campbell Scientific, Logan, UT).

Plant Biomass Harvest, Plant N Analyses, and Quantification of AMF Colonization of Plant Roots. Maize plants were allowed to grow for 66 days until being harvested. Aboveground plant biomass and its N concentration, root biomass, and colonization rate of maize roots by AMF were measured (see Supplementary Materials and Methods for details).

Soil Sampling and Analyses. Soil samples of all treatments were taken from TEST compartments after maize plants were harvested. A subsample (each at ∼30 g) from each treatment was immediately stored at −20°C for analyses of the denitrifying microbes, and the remainder was kept at 4°C for chemical and microbial measurements. Soil pH, soil extractable organic C (SEOC), and concentrations of soil NH_4^+-N and NO_3^--N were quantified (see Supplementary Materials and Methods for details).

Abundances of Functional Genes Responsible for Denitrification in TEST Compartment Soil. Soil DNA was extracted from all samples using a FastDNA SPIN kit (MP Bio, Solon, OH) following the manufacturer's instruction. A subsample of each frozen TEST soil (0.50 g, dry weight equivalent) was used, and the quality and size of extracted soil DNA were checked by electrophoresis on a 1% agarose gel. The quantity of extracted DNA was further determined on a NanoDrop spectrophotometer (Thermo Scientific, Wilmington, DE). The abundance of *nir*S-, *nir*K-, and *nos*Z-type denitrifier was determined using quantitative real-time polymerase chain reaction (qPCR) (CFX96 real-time PCR detection system, Bio-Rad, Hercules, CA) with the primers given in Table S1 using a previously described method[35,50] (more details in Supplementary Materials and Methods).

Illumina MiSeq Sequencing and Analyses of *nir*K- and *nir*S-type Denitrifier Communities. To characterize *nir*K- and *nir*S-type denitrifier communities through high-throughput sequencing analysis, the *nir*K- and *nir*S-type genes were amplified using the same primer sets as for quantitative PCR (Table S1). We used a set of 8 bp barcode reads to identify sequences from different soil samples. All PCR reactions were conducted in triplicate and contained 5 μL of 2 × reaction buffer, 5 μL of 2 × GC buffer, 1 μL of each primer, 2 μL of dNTPs (2.5 mM), 0.25 μL of Q5 DNA Polymerase, 2 μL of template DNA, and 4.75 μL of ddH_2O with a total volume of 25 μL. We performed PCR amplifications on an ABI Gene Amp 2720 PCR thermocycler (ABI, CA). Three replicate PCR products of each sample were pooled and purified with a DNA gel extraction kit (Axygen, China). Purified PCR products were then pooled in equimolar amounts and sequenced on the Illumina MiSeq platform (Illumina, San Diego, CA).

The raw high-throughput sequencing data were analyzed using the Quantitative Insights Into Microbial Ecology (QIIME) toolkit.[56] Low-quality sequences were excluded from consideration if they (1) were shorter than 200 bp in length, (2) did not exactly match to barcode and primers, (3) contained ambiguous bases, or (4) had a low average quality score ($Q \leq 30$). We downloaded all of the draft and completed microbial genome nucleotide sequences to obtain reference sequences for *nir*S- and *nir*K-type genes from NCBI (http://www.ncbi.nlm.nih.gov/) as previously described.[18,57] After quality filtering, our final sequencing of the *nir*K and *nir*S genes resulted in 973,920 and 1,409,479 reads, respectively. All of these sequences were then clustered at 97% nucleotide similarity, resulting in 2896 and 2847 operational taxonomic units (OTUs) for *nir*K and *nir*S genes, respectively. The number of sequences per sample was then normalized to the smallest size. It bears noting that the filtered sequences were also assigned to amplicon sequence variants (ASVs) by DEBLUR.[58] Taxonomy was assigned to ASVs using the BLAST+ consensus taxonomy classifier with default parameters.[59] No significant differences in the diversity and community compositions of both *nir*K- and *nir*S-type denitrifiers were observed between OTUs and ASVs methods (Figures S11−S15).

Statistical Analysis. All of the data sets of this experiment were analyzed using two-way ANOVA to test the significance

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Table 1. Comparison of Plant Biomass, Root Biomass, Plant N Accumulation, Root Colonization Rate, Soil Inorganic N, and Soil pH among CK, AMF, and RAMF with Normal N Input and High N Input in CONV and OM Soils[a]

			plant biomass ($g\ m^{-2}$)	root biomass ($g\ m^{-2}$)	plant biomass N ($g\ N\ m^{-2}$)	AMF colonization (%)	NH_4^+-N ($mg\ N\ kg^{-1}$)	NO_3^--N ($mg\ N\ kg^{-1}$)	pH
CONV soil	NN	CK	2756 ± 56a	528 ± 112a	23.2 ± 2.0a	41.2 ± 7.5b	0.9 ± 0.3a	33.9 ± 5.7c	4.7 ± 0.06bc
		AMF	2975 ± 143ab	655 ± 36a	28.2 ± 3.0b	41.1 ± 10.8b	0.9 ± 0.3a	25.2 ± 2.7b	4.8 ± 0.07c
		RAMF	4165 ± 346c	866 ± 198ab	30.2 ± 1.7b	39.6 ± 4.2b	0.7 ± 0.1a	3.7 ± 0. 7a	5.7 ± 0.08e
	HN	CK	3174 ± 267ab	936 ± 172ab	41.9 ± 2.3c	27.9 ± 5.0ab	1.9 ± 0.3b	40.4 ± 1.1d	4.5 ± 0.04a
		AMF	3684 ± 57bc	1109 ± 291b	50.9 ± 2.5d	28.0 ± 6.6ab	1.7 ± 0.2b	27.9 ± 3.6b	4.6 ± 0.10ab
		RAMF	5189 ± 812d	1145 ± 359b	53.6 ± 0.4d	21.6 ± 7.7a	1.7 ± 0.2b	3.5 ± 0.2a	5.5 ± 0.11d
OM soil	NN	CK	2652 ± 122a	771 ± 40a	21.3 ± 2.3a	46.4 ± 2.8bc	0.8 ± 0.1a	35.8 ± 2.5c	6.3 ± 0.08bc
		AMF	3343 ± 392ab	1159 ± 583ab	24.0 ± 2.8ab	48.5 ± 0.5c	0.9 ± 0.1a	30.3 ± 2.2b	6.3 ± 0.11ab
		RAMF	5149 ± 970cd	1155 ± 177ab	30.1 ± 4.9b	42.2 ± 2.2abc	1.2 ± 0.2b	8.1 ± 0.5a	6.5 ± 0.10cd
	HN	CK	3407 ± 159ab	939 ± 165ab	38.0 ± 2.0c	36.4 ± 2.3a	1.3 ± 0.2b	55.4 ± 2.2e	6.3 ± 0.16ab
		AMF	4079 ± 78cd	987 ± 63ab	50.4 ± 1.7d	40.2 ± 7.0ab	1.9 ± 0.1c	47.0 ± 1.6d	6.1 ± 0.08a
		RAMF	5992 ± 1113d	1403 ± 180b	59.3 ± 6.8e	38.4 ± 4.1a	2.0 ± 0.3c	8.9 ± 1.5a	6.6 ± 0.03d

[a]NN, normal N input, HN, high N input. Different letters among the treatments indicated the significance at 0.05 level, Duncan's LSD test.

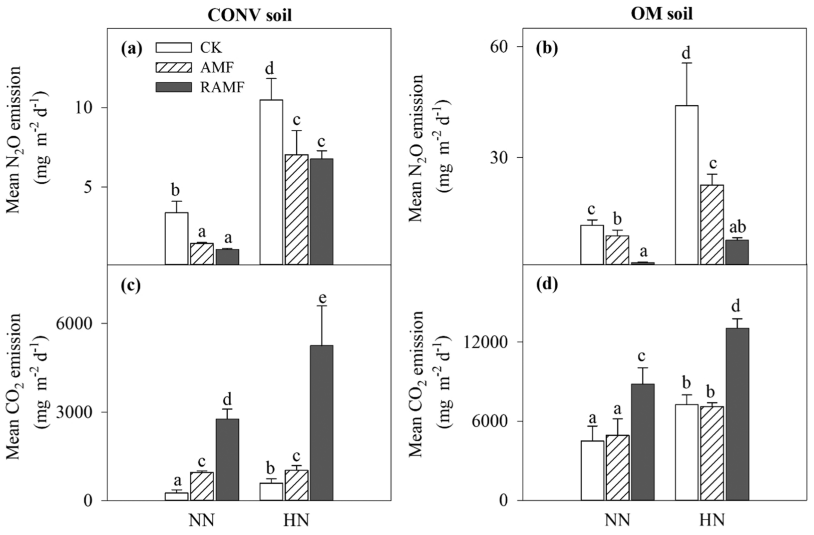

Figure 1. Effects of N fertilizer input and AMF or roots on mean N_2O (a, b) and CO_2 emission (c, d) in CONV and OM soils. Values are mean ± SE (n = 3). NN, normal N input, HN, high N input. Different letters among the treatments indicated the significance at 0.05 level, Duncan's LSD test.

of N input and plant roots and/or AMF effects. Effects of treatment, sampling time, and their interaction on soil N_2O and CO_2 emissions were tested as repeated measures ANOVA, using the software SPSS10.0 (SPSS, Inc., Chicago, IL).

Principal coordinate analyses (PCoA) from pairwise Bray–Curtis distances were employed to characterize the distribution patterns of the beta diversity of denitrifiers between different treatments. Using the function adonis from the R package vegan,[60] two-way permutational multivariate analyses of variance (PERMANOVA) were also conducted to ascertain the effects of N fertilizer input, plant roots, and/or AMF on the beta diversity or community composition of *nir*K- and *nir*S-type denitrifier. The first axis of the PCoA was taken as an indicator of the beta diversity of *nir*K- and *nir*S-type denitrifiers in the linear regression analysis. Linear regression analyses were conducted to assess the relationships between soil N_2O emissions and abundances or β diversities of *nir*K- and *nir*S-type denitrifiers. All analyses were performed using R version 4.1.1 (R Core Team, 2021).[61]

RESULTS

Effects of N Input, and Penetration of Roots and Mycorrhizal Fungi on Maize Plant Biomass, Root Biomass, Plant N Accumulation, and Mycorrhizal Fungal Colonization of Roots. Both N input level and AMF or root presence significantly affected maize plant biomass and biomass N (Tables 1 and S2). Compared with their respective normal N input in both CONV and OM soils, high N input significantly enhanced plant biomass and biomass N (Tables 1 and S2). Similarly, both AMF and RAMF significantly increased plant biomass and biomass N in both soils, compared with the no-AMF control (CK) (Tables 1 and S2).

The AMF colonization rate of host plant roots in the HOST compartments was similar between the two soils under normal N inputs (NN) (41 and 46% in CONV and OM soils, respectively) (Tables 1 and S2). However, high N input significantly reduced root AMF colonization by 36 and 16% in CONV and OM soils, respectively (Tables 1 and S2). There was no significant difference in AMF colonization rate among the AMF and RAMF treatments (Tables 1 and S2).

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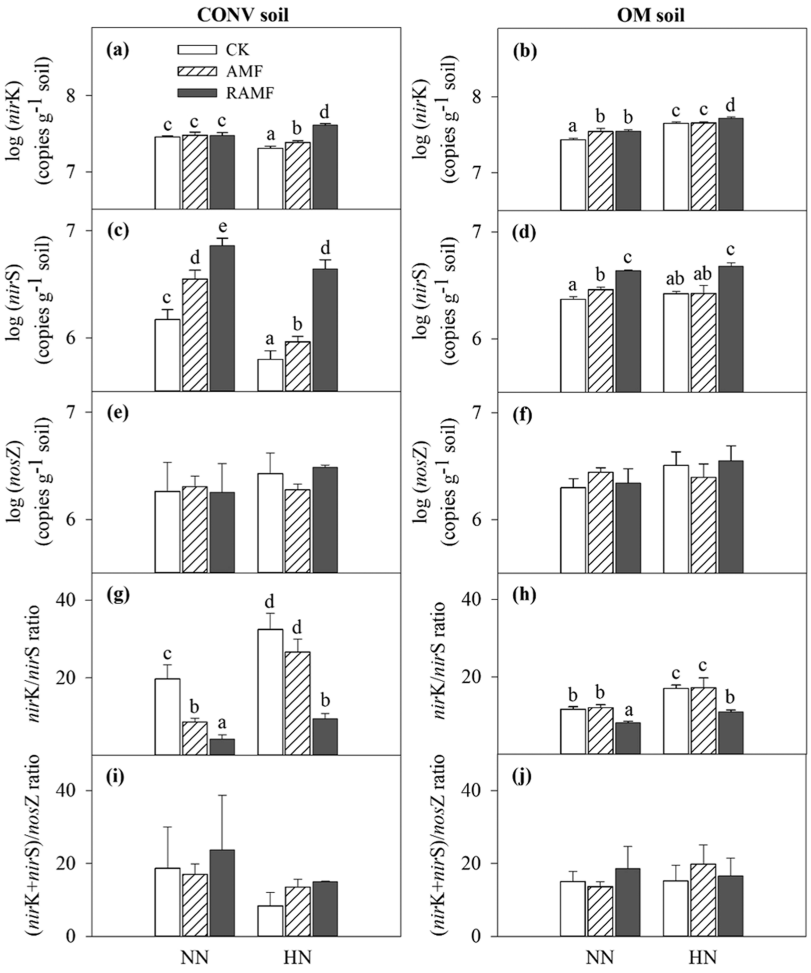

Figure 2. Effects of N fertilizer input and AMF or roots on TEST soil *nir*K (a, b), *nir*S (c, d), and *nos*Z (e, f) gene copy numbers (log-transformed); the ratio of *nir*K/*nir*S (g, h); and the ratio of (*nir*K+*nir*S)/*nos*Z (i, j) in CONV and OM soils. Values are mean ± SE (n = 3). NN, normal N input, HN, high N input. Different letters among the treatments indicated the significance at 0.05 level, Duncan's LSD test.

Soil pH, and Extractable Soil C and N in the TEST Compartments. In both CONV and OM soils, root presence significantly reduced SEOC (Figure S2) but increased soil pH (Table 1). Compared with normal N input, high N input significantly increased soil extractable inorganic N (NH_4^+-N + NO_3^--N) concentrations by 18 and 51% (Table 1), and SEOC by 8 and 39% (Figure S2) in CONV and OM soils, respectively. Compared with the CK, AMF and RAMF reduced soil inorganic N by 28 and 88% in the CONV soil, and by 14 and 78% in the OM soil (Table 1), respectively. Finally, soil inorganic N in CK and AMF treatments were quite high (25−55 mg N kg^{-1}) and the majority existed in the form of NO_3^--N (Table 1), indicating that NO_3^--N was not limiting for denitrification.

Soil N_2O and CO_2 Emissions in the TEST Soil. Both N_2O and CO_2 emission rates showed large temporal variation during the experimental period (Figures S3 and S4) and varied significantly among sampling times (Table S3). High N input significantly increased soil N_2O and CO_2 rates by 310 and 73% in the CONV soil, and 234 and 50% in the OM soil, respectively (Figure 1). Although RAMF consistently increased soil CO_2 emission in both CONV and OM soil, AMF alone only increased soil CO_2 emission in the CONV soil (Figure 1). In addition, the presence of AMF alone did not significantly increase the global warming potential (GWP) either under normal or high N input in both CONV and OM soil, and however, the presence of RAMF increased GWP in the CONV soil but reduced GWP under high organic N input in OM soil (Figure S5).

N_2O emissions were significantly higher in OM than CONV soil (Figure 1). In the CONV soil, AMF and RAMF reduced soil N_2O emission rate by 57 and 68% under normal N input, and by 33 and 35% under high N input, respectively (Figure 1), compared to the CK. In the OM soil, AMF and RAMF reduced N_2O by 24 and 86% under normal N input, and by 49 and 83% under high N input, respectively (Figure 1). These results indicated that AMF reduced N_2O emission more efficiently under normal N input in the CONV soil but more efficiently under high N input in the OM soil.

Abundances of Denitrification Genes. In the CONV soil, high N input decreased the average gene abundance of *nir*S by 51% but had no significant effect on *nir*K, leading to an increase of the *nir*K/*nir*S ratio by 111%, compared with the means of normal N input (Figure 2a,c,g). In the OM soil, however, high N input significantly increased the abundance of *nir*K gene by 46% but had no effect on *nir*S abundance, increasing the *nir*K/*nir*S ratio by 42% (Figure 2b,d,h). Interestingly, both AMF alone and with roots increased the abundances of *nir*K and *nir*S genes in both soil types (Figure 2a−d). While AMF significantly reduced the *nir*K/*nir*S ratio

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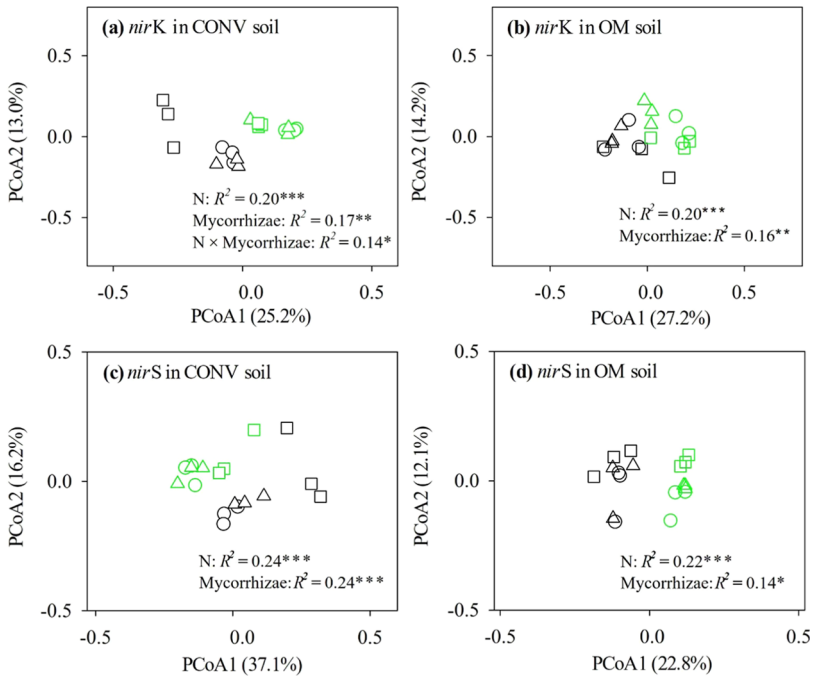

Figure 3. Beta diversity of *nir*K-, *nir*S-type denitrifier communities in CONV (a, c) and OM (b, d) soils, respectively. Beta diversity was assessed by PCoA based on Bray−Curtis similarity distance. The effects of N inputs, mycorrhizae, and their interaction on denitrifier community composition were assessed using PERMANOVA (R^2 and P values: *P < 0.05; **P < 0.01; ***P < 0.001). Different colored symbols represent different N inputs (black symbols, normal N input; green symbols, high N input), and different shapes represent mycorrhizae treatments (circles, no-AMF control; triangles, AMF; squares, roots with AMF).

only in the CONV soil, RAMF significantly reduced it in both CONV and OM soils (Figure 2g,h).

***β* Diversity of *nir*K- and *nir*S-Type Denitrifiers.** Our PERMANOVA showed that N input significantly altered the *β* diversity (community composition) of *nir*K- and *nir*S-type denitrifiers (Figure 3; Table S4). Compared with the CK, AMF alone had no significant effect on either *nir*K- or *nir*S-type denitrifier community composition (Figure 3; Table S4). However, RAMF significantly altered the community composition of *nir*S-denitrifier community composition in the OM soil and both *nir*K- and *nir*S-denitrifiers in the CONV soil (Figure 3; Table S4). Also, there was a significant interaction effect between N input and AMF or RAMF on the community composition of *nir*K-type denitrifiers in the CONV soil (Figure 3; Table S4).

Taxonomic profiling showed that *Rhizobiales* (78−92%) was the predominant order in the *nir*K-type communities in both CONV and OM soils, with *Rhodobacterales*, *Burkholderiales*, and *Nitrosomonadales* accounting for 0.1−15%, respectively (Figure 4). While high N input increased the abundance of *Rhodobacterales* and *Burkholderiales* by 12-fold and 75% in the CONV soil, it reduced the abundance of *Rhizobiales* by 6% and increased *Nitrosomonadales* by 64% in the OM soil, respectively (Figures 4 and S6; Table S5). Compared to the CK, AMF and RAMF reduced *Rhodobacterales* by 56 and 63% in the CONV soil, and *Burkholderiales* by 26 and 24% in the OM soil, respectively (Figures 4 and S6).

The major orders of *nir*S-type denitrifier community were *Burkholderiales* (19−38%), *Rhodocyclales* (16−27%), *Nitrosomonadales* (9−16%), and *Rhizobiales* (4−9%) in the CONV and OM soils (Figure 4). High N input significantly reduced the abundance of *Burkholderiales* (−21%) and *Rhodocyclales* (−24%) in the CONV soil (Figures 4 and S7; Table S5), respectively. However, it increased *Rhizobiales* in both CONV (42%) and OM (39%) soils. Compared to the CK, AMF and RAMF increased *Burkholderiales* (3 and 33%) and *Rhodocyclales* (6 and 30%) in the CONV soil, and *Rhizobiales* by 5 and 29% in the OM soil, respectively (Figures 4 and S7).

Relationships among Plant and Soil Properties, Abundances and Community Composition of Denitrification Genes, and Soil N_2O Emission. In both CONV and OM soils, soil N_2O emission negatively correlated with the copy numbers of *nir*S gene (Figure S8) but positively correlated with the ratio of *nir*K/*nir*S (Figure 5a,b), indicating that soil N_2O emissions were significantly mediated by the community composition of N_2O-producing microbes. In the CONV soil, N_2O emission positively correlated with the *β* diversity of *nir*K-type denitrifiers but negatively with the *β* diversity of *nir*S-type denitrifiers (Figure 5e,g). In comparison, N_2O emission positively correlated with the *β* diversity of both *nir*K and *nir*S-type denitrifiers in the OM soil (Figure 5f,h).

We further examined the relationships between N_2O emission and the abundances of specific denitrifier groups (Figures S9 and S10). In the CONV soil, soil N_2O emission was positively correlated with the abundance of *nir*K-type *Burkholderiales* or *Rhodobacterales*, *nir*S-type *Nitrosomonadales* or *Rhizobiales* but negatively with the abundance of *nir*K-type *Rhizobiales*, *nir*S-type *Burkholderiales* or *Rhodocyclales* (Figures S9 and S10). In the OM soil, however, N_2O emission positively correlated with *nirK*-type *Nitrosomonadales* but negatively correlated with *Rhizobiales* (Figure S9d,e).

DISCUSSION

Our results showed that the presence of AMF alone and/or with plant roots significantly reduced soil N_2O emission (Figures 1 and S16). These AMF and/or root effects on soil

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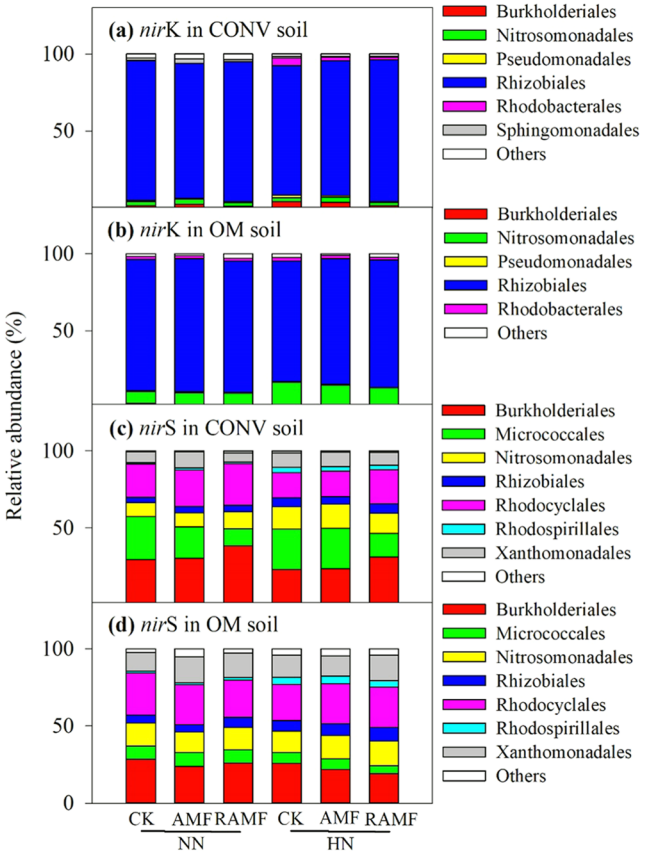

Figure 4. Taxonomic composition of *nir*K- and *nir*S-type denitrifier community composition at the order level in CONV and OM soils. NN, normal N input, HN, high N input. CK, no-AMF control; AMF, presence of AMF; RAMF, presence of roots with AMF.

N_2O emission occurred under both normal and high N inputs in both conventionally and organically managed soils (Figures 1 and S16). In addition, reductions in N_2O emission in both systems corresponded to decreases in the *nir*K/*nir*S ratio of the denitrifying communities (Figure 2). Considering the high level of soil NO_3^- remained in soil (Table 1), these results were surprising, which also suggests that the roles of AMF in N-rich ecosystems may have been underappreciated.

AMF- and Root-Mediation of Soil N_2O Emission: Impacts of N Quantity and Types. Plant roots and their symbiotic mycorrhizal fungi are largely responsible for plant nutrient acquisition from soil. Plant roots and AMF may suppress or stimulate soil N_2O emission because they can differentially affect N-cycling microbes.[18,35,50,62] On the one hand, roots take up soil N that would otherwise be available for nitrification and/or denitrification that generate N_2O.[18,24] Similarly, AMF often form a large extraradical mycelial network,[27,63] enhancing host plant N uptake and reducing N substrate for soil nitrifiers and denitrifiers, and their N_2O production.[18,24] Also, AMF hyphae are N-rich and have a high concentration of N (with a C:N of ca. 10)[64,65] and a low to moderate N input often enhances AMF growth and root colonization,[41,51] which in return facilitate plant N uptake and may outcompete nitrifiers and/or denitrifiers.[50,54] On the other hand, most of denitrifiers are heterotrophs and labile C derived from roots and AMF may stimulate denitrifier activities and their N_2O emission, when soil N substrate is not limiting. Several studies have shown that plant roots stimulated denitrifier activities and N_2O emission,[30,31,66] likely due to root-derived C availability in the rhizosphere.[67]

The role of AMF in plant nutrition in N-rich agroecosystems is less certain. High N input mitigates N limitation to plant growth and often suppresses AMF colonization and/or extraradical biomass likely by reducing plant photosynthate allocation belowground[38,39,68] and/or inducing soil acidity.[41] Consequently, it is assumed that the effect of AMF on soil N_2O emission might be less significant in cropping systems with high N input. However, other studies did show that AMF were not sensitive to external N input in some agricultural soils.[69,70] For example, Williams et al.[69] observed no significant N fertilization effect on AMF biomass in soils from four sites managed under N input for 55 years in Skåne, Sweden. Also, Liu et al.[70] reported that high N input had no effect on soil AMF richness from a winter wheat-summer maize rotation field under continuous fertilization over a 13-year period. Further, emerging evidence showed that N-induced acidity, rather than N-nutrient itself, may be mainly responsible for the suppression of AMF under high N input.[39,41] Together, these results suggest that some AMF species may be resistant or tolerant to external N input, and N-induced decreases in mycorrhization alone may not be indicative of the decline of hyphal biomass and their nutrient uptake.[35,51]

Different types of N input under conventionally and organically managed systems may also mediate the effects of plant roots and AMF on soil N_2O emission. Although N_2O is generated mainly by soil nitrifiers and denitrifiers, microbial communities that perform denitrification in general play a dominant role in soil N_2O emissions, particularly in agricultural soil (also see Supplementary Materials and Methods for more details).[9,10] While chemical N to the conventional soil only provides N substrate, organic manures introduce both C and N substrates for denitrifiers.[71−73] Denitrifiers are predominantly heterotrophic bacteria that require energy from organic matter to convert NO_3^- to N_2O.[36,71,74] Organic manure input provides C source for microbial growth and promotes oxygen depletion,[75] creating temporary anaerobic microsites that favor denitrification and N_2O production.[71] We also observed a significant difference in the composition of the denitrifying communities between CONV and OM soils (Figures 3 and 4), with the relative abundance of *Nitrosomonadales* in the *nir*K-type communities 2- to 3-fold higher in OM than CONV soil (Figure 4). Yet, what was really surprising is that in spite of the differences in soil pH and the community composition (Table S2, Figure 4) and reduced AMF colonization of roots, AMF and RAMF consistently reduced N_2O emission from both CONV and OM soils under high N input (Figure 1) where soil extractable N was plentiful and should not limit N_2O emission (Table 1). These results indicate that the role of AMF and roots in mediating N_2O emission may be underappreciated, which also suggests that in addition to N removal, other mechanisms may contribute to the suppression of N_2O emission induced by AMF and roots with AMF.

Effects of AMF and Roots on Soil N_2O Emission: Linkages to the Composition of Denitrifying Microbes. There is a concern that decreases in AMF abundance and diversity due to intensive agricultural management, fungicide and fertilizer applications in particular, may cascade up to affect belowground plant−microbe interactions and stimulate soil N_2O emission.[25,76] However, emerging evidence suggests that in N-rich systems, plant roots and their associated AMF may

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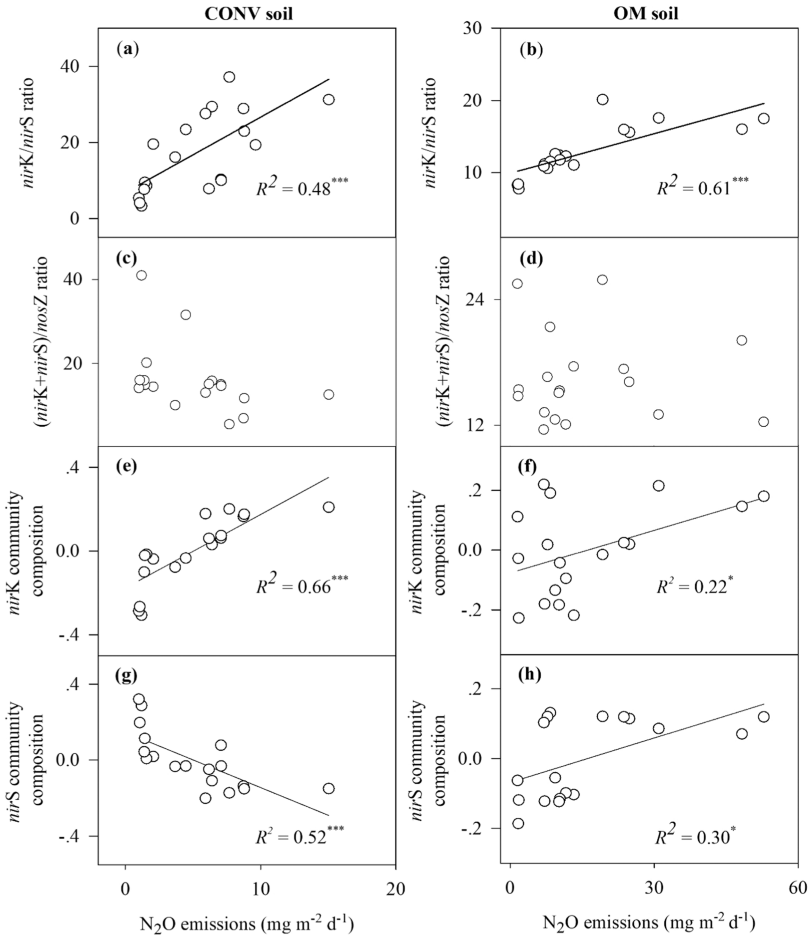

Figure 5. Correlations between soil N_2O emission and the ratio of *nir*K/*nir*S (a, b), the ratio of (*nir*K + *nir*S)/*nos*Z (c, d), and β diversities of *nir*K- (e, f) and *nir*S-type denitrifiers (g, h) in CONV and OM soils. β diversity is indicated by the first axis of PCoA. The significance levels are labeled as: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$, respectively.

play a large role in modulating N_2O emission by altering the composition of the denitrifying community and its N_2O production potential. Bender et al.[21] reported that mycorrhizal inoculation to tomato plants significantly reduced soil N_2O emission, compared with soils under tomato mutants that were nonmycorrhizal, and that the AMF abundance correlated negatively with the abundance of *nir*K but positively with the *nos*Z. Also, Qiu et al.[35] showed that plant roots significantly reduced *nir*K-type denitrifiers but not *nos*Z-type denitrifiers, resulting in a decrease in soil N_2O emission. Unfortunately, these previous studies did not assess whether plant roots and/or AMF had affected the composition of the N-cycling microbial community. In this study, the abundance and composition of denitrifying communities were characterized (Figures 2−4), allowing us to assess whether alteration in soil N_2O emission was linked to AMF- or to root-induced changes in the abundance and community composition of denitrifying microbes (Figure 5). Partially contrary to our first hypothesis, our results showed that the total abundance of denitrifiers was not indicative of N_2O emission. Rather, it was the ratio of *nir*K to *nir*S (but not the (*nir*K + *nir*S)/*nos*Z ratio) that was significantly correlated with soil N_2O emissions (Figure 5), indicating that the composition of the N_2O-producing microbes was more important in explaining the effects of AMF and roots on soil N_2O emission. The positive correlation between the *nir*K/*nir*S ratio and soil N_2O emission (Figure 5) was consistent with findings in other N-rich fertile agricultural soils.[18,31] For example, Jones et al.[18] also showed that the N_2O sink capacity of soil was correlated with the proportion between the *nir* genes, with a higher N_2O consumption with increasing the ratio of *nir*S/*nir*K, and the relative abundance and phylogenetic diversity of the *nos*Z community, and the ratio of *nir*S/*nir*K-type denitrifiers were the strongest drivers of soil N_2O sink capacity.

Different functional groups of the denitrifier community may respond differently to AMF- and root-induced changes in C and N availability.[24,77,78] In this study, we found that AMF and plant roots reduced the relative abundance of *nir*K-type *Rhodobacterales* and increased the *Rhodocyclales* for *nir*S-type denitrifier in the conventional soil, and increased the *Rhizobiales* for *nir*S-type denitrifiers in the OM soil (Figures 4, S6, and S7). Previous studies have revealed that both *Rhodobacterales* and *Rhodocyclales* were important contributors to the denitrifying process.[31,79,80] For example, Ai et al.[31] showed that the *nir*S-type denitrifier communities, such as *Pseudomonadaceae* and *Rhodobacterales*, dominated the reduction of NO_2^- to N_2O in a wheat rhizosphere. Wang et al.[79] reported that *nir*S gene in the *Rhodocyclales* order contained the downstream genes of *nor*B and *nos*Z, which may contribute to reducing N_2O to N_2. Moreover, *Rhizobiales* often carry the nitrous oxide reductase-encoding clade I *nos*Z, which reduces N_2O emission through converting N_2O to N_2.[81] We postulate

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that AMF- and root-induced enhancement of *Rhodocyclales* and *Rhizobiales* in *nir*S-type denitrifier communities may have contributed to the decrease in soil N_2O emission (Figures 4 and S16). Regardless of the community composition and dominant species, a common trend of reduced *nir*K/*nir*S ratio corresponding to a decrease in N_2O in the presence of AMF and roots with AMF (Figure 3) emerged in our study. A few other studies have also shown that a decreased *nir*K/*nir*S ratio correlated with a reduction in N_2O emissions in N-rich agricultural soils,[18,31] though they did not examine the role of roots and AMF *per se*. Together, these results suggest that plant roots and their associated fungi may alter N_2O emission by modifying the structure and composition of soil denitrifying microbial communities. Although the exact mechanisms underlying root and AMF modification of the denitrifying microbes are currently unclear, these findings suggest that the suppressive roles of roots and AMF on N_2O emissions might be amplified through agricultural practices. Root exudates from some plants and cultivars have been shown to selectively inhibit nitrifiers and nitrification,[82] and it remains to explore whether similar inhibition from roots and AMF may occur on denitrifying microbes.

Our results showed that the presence of AMF or roots with AMF consistently reduced soil N_2O emission through increasing plant N uptake and decreasing the ratio of *nir*K/*nir*S in both conventionally and/or organically managed soils, even under higher N fertilizer application (Figure S16). In particular, AMF- and root-induced reductions in N_2O emission concurred with the alterations in the community composition of soil denitrifiers. These results indicate that plant roots and their symbiotic mycorrhizal fungi may interact with a wide range of other soil microorganisms such as nitrifiers and denitrifiers to modify nutrient cycling processes in the rhizosphere or mycorrhizosphere. These findings also suggest that AMF may play a larger role in mediating N cycling and N_2O emission in N-rich agroecosystems than previously appreciated.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.est.2c03816.

Supplementary Materials and Methods; mesocosm units for N_2O and CO_2 sampling (Figure S1); effects of N fertilizer input and penetration of AMF or roots on TEST soil extractable organic C (Figure S2), N_2O, CO_2 emission rate (Figures S3 and S4) and global warming potential (Figure S5); relative abundance of *nir*K- and *nir*S-type denitrifiers at order level based on OTU (Figures S6 and S7); relationships between N_2O emission and *nir*S (log-transformed) gene copy numbers (Figure S8) and the relative abundance of the *nir*K- and *nir*S-type denitrifiers at order level based on OTU (Figures S9 and S10); β diversity of *nir*K- and *nir*S-type denitrifier communities based on ASVs (Figure S11); taxonomic composition (at the order level) of *nir*K- and *nir*S-type denitrifier community composition based on the ASVs (Figure S12); relationships between N_2O emission and the β diversity of *nir*K- and *nir*S-type denitrifiers based on the ASVs (Figure S13) and the relative abundance of the relative abundance of *nir*K- and *nir*S-type denitrifiers at the order level based on ASVs (Figures S14 and S15); conceptual framework of N fertilizer input, and plant roots and AMF on soil properties, denitrifiers, and soil N_2O emissions (Figure S16); primers and qPCR conditions for *nir*K, *nir*S, and *nos*Z genes (Table S1); results of two-way ANOVA for the effects of N fertilizer input and mycorrhizae on plant, soil, and microbial parameters (Table S2); results of analysis of repeated measures linear mixed models of N fertilizer input, mycorrhizae, and time effects and all interactions on N_2O and CO_2 emission rates (Table S3); effects of N fertilizer input and penetration of AMF or roots on soil *nir*K- and *nir*S-type denitrifiers communities at the OTU level assessed by PERMANOVA analysis (Table S4); and effects of N fertilizer input and penetration of AMF or roots on soil *nir*K- and *nir*S-type denitrifiers communities at order level assessed by two-way ANOVA (Table S5) (PDF)

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Author Contributions

[▽]X.Z. and Y.Q. contributed equally. X.Z., Y.Q., and S.H. designed the study. X.Z., Y.Q., and C.T. performed experiment and lab work. X.Z. and Y.Q. conducted the bioinformatic and statistical analysis. X.Z., Y.Q., F.S.G., C.G., C.R., and S.H. wrote the manuscript with inputs from all authors.

Notes

The authors declare no competing financial interest.

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