

Effects of Arbuscular mycorrhizal fungi on leguminous crop growth and soil N₂O emissions

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ABSTRACT

Arbuscular mycorrhizal fungi (AMF), which form symbiotic associations with 80% of land plants, provide host plants with soil phosphorus (P) and nitrogen (N) in exchange for carbon. The AMF often reduce nitrous oxide (N₂O) emissions from soils with non-legume plants, as they acquire N from soils to plants. However, the effect of AMF on soil N₂O emissions is still unknown in the legume system, a major source of this potent greenhouse gas. Therefore, we conducted the field and pot experiments to investigate the effects of AMF on plant growth and soil N₂O emissions in legume systems. The results showed that AMF significantly increased soil N₂O emissions from soils with soybean by 39% in the field experiment and by 63% in the pot experiment. The AMF significantly enhanced the root biomass, aboveground biomass, grain yield, plant N uptake, plant P uptake, and the nodule numbers. The AMF increased soil extractable N concentrations and DOC concentrations, but reduced soil available P concentrations. The AMF stimulated the N₂O-producing microbes (*nirS*- and *nirK*-type) whereas they did not affect the N₂O-consuming (*nosZ*-type) denitrifiers. The AMF reduced the yield-scaled N₂O emissions in the field experiment and increased the net ecosystem economic budget. Our findings indicate that AMF increase soil N₂O emissions from legume system through enhancing P acquisition for biological N₂ fixation and suggest that AMF benefit crop production for higher-yield with less N₂O emissions.

Keywords: N₂O emissions, Greenhouse gas emissions; Arbuscular mycorrhizal fungi; Food security; Phosphorus

1. Introduction

Nitrous oxide (N_2O) is the third most important greenhouse gas with a 265 times greater global warming potential than carbon dioxide (CO_2) (IPCC, 2013). Nitrous oxide is estimated to contribute approximately 6%~8% of global warming (IPCC, 2007) and enhances stratospheric ozone (O_3) depletion (Ravishankara et al., 2009; Millar et al., 2018). The atmospheric N_2O concentration has increased by 20% since industrial times and is predicted to continue to increase in the future (IPCC, 2013). Agricultural soils are responsible for nearly 60% of the anthropogenic N_2O emissions (Syakila and Kroeze, 2011; Jahangir et al., 2021). In agricultural soils, N_2O is often emitted from soil N, N fertilizer, biological N_2 -fixation, and crop residue (Rochette and Janzen, 2005; Itakura et al., 2013). Soil N_2O is mainly produced through microbial processes of nitrification (i.e., oxidation of NH_4^+ into NO_3^-) and denitrification (i.e., reduction of NO_3^- to gaseous forms, NO , N_2O , and N_2) (Bouwman, 1998; Barnard et al., 2005; Zhu et al., 2013; Guillermo et al., 2018), which is affected by soil C and N availability, soil moisture, plants species, and soil microbes (Barnard et al., 2005; Abalos et al., 2014; Chen et al., 2016).

Arbuscular mycorrhizal fungi (AMF) are widespread in most terrestrial ecosystems and form associations with roots of ~80% of terrestrial plant species (Kiers et al., 2011; Voriskova et al., 2016; Ghorchiani et al., 2018). AMF often stimulate the uptake of several soil nutrients (i.e. N, P, and Zn) via extensive hyphal networks and transfer of nutrients from the fungus to the plant root in exchange for C (Smith and Read, 2008; Fellbaum et al., 2012; Xu et al., 2018). As AMF often increase plant N uptake (Cheng et al., 2012; Cavagnaro et al., 2015), they are widely expected to reduce soil N_2O emissions (Bender et al., 2014; Storer et al. 2018; Teutscherova et al., 2019; Bender et al., 2019). However, all these studies were conducted using

non-legume species, such as *Lolium multiflorum*, *Solanum lycopersicum* L., *Z. mays* L.,
Brachiaria decumbens Stapf., *P. australis*, and *Oryza sativa* L. (Lazcano et al., 2014; Bender et
al., 2014; Bender et al., 2015; Zhang et al., 2015; Storer et al. 2018; Liang et al., 2019; Okiobe
et al., 2019; Teutscherova et al., 2019). As such, the effects of AMF on N₂O emissions in legume
systems are still unknown.

Legumes play a significant role in maintaining global food security and ecosystem
resilience, because they can enhance soil N supply and improve crop yields through biological
N₂ fixation (Tilman et al., 2002; Siddique et al., 2012; Foyer et al., 2016). The harvested area
of grain legumes is around 1/3 of total cereal harvested area (Foyer et al., 2016), and legume
systems are estimated to emit around 10% of total agricultural N₂O emissions (Stehfest and
Bouwman, 2006). In legume ecosystems, N₂O is also emitted from the degradation of the root
nodules. Organic N inside the nodules is mineralized to NH₄⁺, followed by nitrification and
denitrification that produces N₂O (Rochette and Janzen, 2005; Inaba et al., 2009; Barton et al.,
2011; Itakura et al., 2013). AMF often stimulate the biological N₂ fixation through improving
P uptake (Wang et al., 2011; Wang et al., 2016; Püschel et al., 2017). Therefore, in legume
ecosystems, AMF may improve P restrict and increase root nodules infection, which can
indirectly change N substrates and the response of microbial processes related to nitrogen
transformation (e.g., N₂O production). Based on these hypotheses, AMF may result in higher
N₂O emissions in legume ecosystems.

Here, we conducted pot and field experiments to assess the effects of AMF on N₂O
emissions in the legume system. First, soybeans were planted to enable comparison of the soil

N₂O emissions between AMF and non-AMF treatments. Second, we measured the N₂O emissions under field condition that AMF hyphae were either allowed or prevented access to a chamber containing P fertilizer. We tested whether AMF increased N₂O emissions in legume system through improving P uptake. To the best of our knowledge, this is the first study to report on the effects of AMF on N₂O emissions from legume systems.

2. Material and methods

2.1 Experimental design

Pot experiment

We conducted a pot experiment to investigate the effects of AMF on N₂O emissions under open field conditions at the Jiangxi Academy of Agricultural Science (28.6°N, 115.9°E), Nanchang, China. This experiment consisted of AMF inoculum and non-AMF inoculum treatments, and three replicates for each treatment.

The test soil was collected from the plow layer of a cropland and was air dried, sieved (6 mm mesh size) and mixed. The main soil properties were as follows: soil organic C 26.2 g kg⁻¹, total N 2.8 g kg⁻¹, and total P 1.0 g kg⁻¹. The soils were sterilized using an autoclave at 120 °C for 30 min. Plastic pots (diameter, 30 cm; depth, 30 cm) were filled with 9 kg of sterilized soil and 2 kg sterilized sand that were mixed together. To recover the requisite soil microflora, 100 ml of soil microbial solution without AMF was added into each pot. The microbial solutions were produced from the same fresh soil of this experiment. In the AMF treatments, we added 150 g AMF inoculum sand into soil. The sand included six common AMF species (i.e. *Glomus mosseae*, *Gigaspora margarita*, *Acaulospora scrobiculata*, *Corymbiglomus tortuosum*,

Diversispora epigaea and *Rhizophagus intraradices*). In the non-AMF treatments, 150 g sand without AMF and a solution without AMF prepared from AMF inoculum was added. We also added 5 ml rhizobium solution into each pot and did not apply any fertilizer. The soybean (*Glycine max* (Linn.) Merr.) seeds were surface-sterilized. In a pot, we sowed five soybean germinated seeds. Ten days later, plants were thinned to only three seedlings in each pot. When almost all pods became yellow and the bottom leaves began to fall, the soybean plants were harvested at early full ripening stage (90 days after seeding). We placed an open bottom and top chamber (diameter, 8 cm; height, 10 cm) into the soil (2 cm depth) to measure the emitted N₂O gas.

Field experiment

In this experiment, we tested whether AMF increased N₂O emissions in legume systems through improving P uptake under field conditions at Jinxian, Jiangxi province (28.3°N, 116.3°E). The main soil properties recorded were: soil organic C 13.3 g kg⁻¹, total N 1.2 g kg⁻¹, total P 0.5 g kg⁻¹ and available P 6.4 mg kg⁻¹.

We designed the “phosphorus chamber” (length, 60 cm; width, 20 cm; height, 30 cm) with four mesh windows (Fig. 1). The pore sizes of the meshes were either 20 μm or 0.45 μm. AMF hyphae were allowed access to the chambers with two mesh window pore size of 20 μm (nonprevented) and were prevented access to chambers with two mesh window pore size of 0.45 μm (prevented). For each chamber, we put 46 kg air dried soils mixed with 1.44 g P₂O₅.

We sowed 12 rows of soybean seeds. For each row, we sowed 20 hills soybean with hill spacing of 0.25 m× 0.15 m. Twenty days after seeding, we selected adjacent two rows with

healthy soybean plant and placed a P chamber into the soil close to soybean plant. The distance of two P chamber was more than 1 m. An open bottom and top chamber (diameter, 8 cm; height, 10 cm) was placed into the soil (2 cm depth) close to the plant with chamber to collect the N₂O gas. The soybean plants were harvested at early full ripening stage (90 days after seeding) after almost all pods became yellow.

2.2 Sampling and measurement methods

In both experiments, the static closed chamber technique was used to measure the N₂O fluxes from 10-20 days after thinning to harvest at 5-10-day intervals. Two gas samples (40 ml) were collected from each static sampling chamber at 0 and 30 min after the chamber was closed (Wu et al., 2017; Qiu et al., 2019). The N₂O concentrations were determined with a gas chromatograph (GC, Agilent 7890A, USA) and the fluxes calculated as follows: $F = \Delta C / \Delta T \times V / A$, where $\Delta C / \Delta T$ is the change rate of N₂O concentration ($\mu\text{g L}^{-1} \text{h}^{-1}$) in the chamber. We assumed a linear rate of N₂O accumulation in the chambers over a short period (Wu et al., 2017; Qiu et al., 2019). V is the volume of the chamber (L) and A is surface area of chamber (m²). Cumulative N₂O emissions were estimated using the trapezoidal method (Jiang et al., 2018).

At the harvest, soils were collected to determine dissolved organic carbon (DOC), extractable nitrogen (NH₄⁺ and NO₃⁻), available P concentrations and the abundances of *nirS*, *nirK* and *nosZ* genes related to N₂O emission. We measured the DOC concentrations by using a TOC analyzer (multi N/C UV, Analytik Jena AG, Germany). The flow autoanalyzer (Auto Analyzer 3, BRAN LUEBBE, Germany) was used to measure the soil NH₄⁺ and NO₃⁻

concentrations. Soil available P concentrations were measured by the Olsen method for alkaline soils (Olsen et al., 1954). We used a Power Soil DNA Isolation Kit (MoBio, USA) to extract soil DNA from 0.25 g of fresh soil. The quantification of *nirS*, *nirK* and *nosZ* genes was decided by the primer pairs *cd3aF/R3cd*, *nirK1F/nirK5R* and *nosZF/nosZR*, respectively (Throbäck et al., 2004). The *nirS* and *nirK* genes represent the process of N₂O production, and the *nosZ* gene represents the process of N₂O consumption. The quantitative real-time PCR was performed using a QuantStudio 3 (Bio-Rad, USA).

When At harvest, we counted the nodule numbers and AMF vesicle numbers of plant roots (Phillips and Hayman, 1970) and measured the root biomass, above-ground biomass and grain yield. Plants were oven-dried at 70°C no less than 48 hours to achieve a constant weight. The plant N concentrations were measured using an elementary analyzer (vario PYRO, Elementar, Germany) and plant P concentrations were measured from H₂SO₄-H₂O₂ digestion and molybdenum-antimony colorimetric method (Thomas et al., 1967).

Yield-scaled N₂O emissions and relative net ecosystem economic budget (NEEB) between AMF and non-AMF treatments were calculated as follows:

$$\text{Yield-scaled N}_2\text{O emission} = \text{N}_2\text{O emission} / \text{grain yield}$$

Relative NEEB = (yield income of AMF- agricultural activity of AMF- N₂O cost of AMF)- (yield income of non-AMF- agricultural activity of non-AMF- N₂O cost of non-AMF), where yield incomes were calculated using the grain yield and the price of soybean (0.84 US\$ kg⁻¹) and N₂O emission costs were calculated using total N₂O emissions and carbon trade price (17 US\$ t⁻¹ CO₂-eq). Agricultural activity costs were the same between the AMF and non-AMF treatments in this study.

2.5 Statistical analysis

In pot experiment, the data was analyzed by independent sample t-test. In the field experiment, we used the paired sample t-test. All analyses were performed using the SPSS 22.0 statistical software package. Differences between treatments were considered significant at $P < 0.05$.

3. Results

3.1 Plant traits

In the pot experiment, the vesicle numbers on the roots were two orders of magnitude higher in the AMF treatment than in the non-AMF treatment. The AMF increased root biomass by 38%, and also significantly stimulated above-ground biomass, plant N and P accumulation, and nodule numbers by 37%, 33%, 116% and 192% respectively. The AMF enhanced the grain yield by 41% (Table 1).

In the field experiment, the AMF vesicle numbers in the roots were similar between the allowed and prevented AMF hyphal access treatments. The AMF hyphal access significantly stimulated root biomass, above-ground biomass, plant N and P accumulation and nodule numbers by 100%, 146%, 200%, 113% and 213% respectively. The AMF hyphal access increased the grain yield of soybean by 97% (Table 1).

3.2 N_2O emissions and NEEB

In the pot and field experiments, AMF significantly increased the soil N_2O emissions (Fig. 2 and 3a). In the pot experiment, AMF increased soil N_2O emissions by 33%. In the field experiment, the AMF hyphae accesses significantly increased soil N_2O emissions by 24%. The

AMF reduced the yield-scaled N₂O emissions by 34% under the field condition, whereas they did not affect the yield-scaled N₂O emissions in the pot experiment (Fig. 3b). The AMF increased the NEEB by 1475 US\$ ha⁻¹ in the field experiment and by 1005 US\$ ha⁻¹ in the pot experiment (Fig. 3c).

3.3 Soil properties

In the pot experiment, AMF significantly increased the soil DOC and NH₄⁺ concentrations, but did not affect the soil NO₃⁻ concentrations for soybean (Table 2). AMF reduced the soil available P concentrations by 17%. AMF did not affect the *nirS* and *nosZ* abundances, but increased the *nirK* abundance by 80% and (*nirS*+ *nirK*)/ *nosZ* by 62% (Fig. 4).

In the field experiment, AMF hyphal access increased the soil DOC concentrations and soil NO₃⁻ concentrations, but did not affect the soil NH₄⁺ concentrations. The AMF enhanced the *nirS* abundance by 64% and *nirK* abundance by 34%. The *nosZ* abundance was similar between the allowed and prevented AMF hyphal access treatments. Finally, AMF hyphal access increased the (*nirS*+ *nirK*)/ *nosZ* by 27%.

4. Discussion

The results showed that AMF significantly increased N₂O emissions from soils with soybean. However, several previous studies indicated that AMF significantly reduced soil N₂O emissions from non-legume system (e.g. Bender et al., 2014; Storer et al. 2018; Teutscherova et al., 2019). AMF played an important role in plant N uptake (Smith and Read, 2008; Cavagnaro et al., 2015). The extraradical mycelium of AMF takes up NH₄⁺ and NO₃⁻ from the soils and transfers them to the host plants (Hodge and Fitter, 2010; Cheng et al., 2012; Fellbaum

et al., 2012). The AMF hyphae can extend more than 10 cm beyond the root surface, which allows AMF to transfer N extensively and quickly (Li et al., 1991; Cavagnaro et al., 2015). Thus, the presence of AMF increased plant N accumulation and reduced soil N availability in non-legume systems (Bender et al., 2014; Liang et al., 2019). The denitrification is primarily limited by the soil N availability (Zhu et al., 2013), resulting in lower N₂O emissions of AMF treatments. Besides reducing soil N availability, AMF can decrease N₂O emissions from nonlegume systems through other underlying mechanisms. For instance, Bender et al. (2014) indicated that increased N immobilization by the soil microbial biomass can contribute to lower N₂O emissions because of AMF. Lazcano et al. (2014) found that control over N₂O emissions by AMF seemed to be driven by a higher use of soil water. Storer et al. (2018) found that AMF hyphae directly reduced N₂O production because it may be outcompeting slower-growing nitrifiers for ammonium.

In the legume system, N₂O is mainly emitted as a consequence of biological N₂ fixation (Itakura et al., 2013). The rhizobia symbiosis imposes great P demand on the host plants, probably due to nitrogenase demand for ATP and high P concentration in microbial tissue (Jakobsen, 1985; Divito and Sadras et al., 2014; Kleinert et al., 2014). AMF can stimulate P uptake via their extensive hyphal networks (Smith and Read, 2008, Püschel et al., 2017), as indicated by lower availability of P concentrations from soil and higher plant P accumulation in the AMF treatments. Furthermore, Zhang et al. (2016) showed that AMF increased soil P mobilization. Thus, AMF increased the weight of nodules in both pot and field experiments, indicating that AMF stimulated biological N₂-fixation (Püschel et al., 2017). Higher biological N₂-fixation can release more N into soils through exudates and decomposition of root nodules (Itakura et al., 2013), as indicated by higher NH₄⁺ or NO₃⁻ concentrations in AMF treatments.

Moreover, AMF increased root growth and soil DOC concentrations, which may provide more C for denitrification. Higher soil C and N availability can stimulate the microbial role in nitrification and denitrification (Barnard et al., 2005; Zhu et al., 2013). The results also showed that AMF stimulated the *nirS* and/or *nirK* abundances in the soils for soybean. In other words, AMF stimulated soil C and N availability in the legume systems, through improving biological N₂-fixation and plant growth, and resulted in higher N₂O emissions.

Because of symbiotic atmospheric nitrogen fixation, grain legumes benefit the agricultural sustainability in developed and developing countries. In this study, AMF stimulated the grain yields of soybean, in accordance with several previous studies (e.g. Lazcano et al., 2014; Liu et al., 2018; Liang et al., 2019). The global meta-analysis showed that AMF inoculation increased crop grain yield by about 20% (Lekberg and Koide, 2005; Pellegrino et al., 2015). These results suggest that AMF may benefit to break the yield ceiling for ensuring food security. Furthermore, the results showed that AMF reduced the yield-scaled N₂O emissions under field condition and increased the NEEB, indicating that benefits of crop yield improvement of AMF can cover the enhancement of N₂O emissions.

The results indicated that AMF increased N₂O emissions for legume systems due to higher biological N₂-fixation. Yet, higher biological N₂-fixation may reduce N fertilizer input of the next season, which can directly and indirectly reduce N₂O emissions (Shcherbak et al., 2014). AMF also affect the soil organic matter decomposition (Cheng et al., 2012), which may affect soil organic C stock. Thus, we suggest that the effect of AMF on net greenhouse gas emissions should be investigated in future research.

5. Conclusion

In conclusion, we found that AMF increased the soil N₂O emissions from soybean. The AMF increased plant biomass, grain yield, plant N and P uptake, and biological N₂ fixation. AMF stimulated soil NH₄⁺ or NO₃⁻ concentrations, DOC concentrations, and the abundances of *nirS* and/or *nirK* involved in N₂O production. AMF reduced the yield-scaled N₂O emissions under field condition and increased the NEEB. Our findings indicate AMF stimulate N₂O emissions from legume plants mostly through improving P acquisition for biological N₂ fixation, and suggest AMF benefit soybean production for higher-yield with less N₂O emissions.

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Tables and figures:

Table 1 Root biomass, above-ground biomass, yield, plant N and P accumulation, nodule numbers and AMF vesicle numbers.

Table 2 Soil dissolved organic C (DOC), NH_4^+ , NO_3^- and available P concentrations. **Fig. 1**

"Phosphorus chamber" - a device for controlling the absorption of phosphorus by arbuscular mycorrhizal fungi (AMF) in the field experiment.

Fig. 2 N_2O emissions flux as affected by AMF in the pot (a) and field (b) experiments. AMF, with AMF inoculum; non-AMF, without AMF inoculum; non-prevented, allowed AMF hyphal access; prevented, prevented AMF hyphal access. Error bars indicate standard errors (n=3).

Fig. 3 Cumulative N_2O emissions (a), yield-scaled N_2O emissions (b) and relative net ecosystem economic budget (c) as affected by AMF. AMF, with AMF inoculum; non-AMF, without AMF inoculum; non-prevented, allowed AMF hyphal access; prevented, prevented AMF hyphal access. * indicates significant differences at $P < 0.05$. Error bars indicate standard errors (n=3).

Fig. 4 The abundance of *nirS* (a), *nirK* (b), *nosZ* (c) and (*nirS* + *nirK*)/*nosZ* (d) as affected by AMF. AMF, with AMF inoculum; non-AMF, without AMF inoculum; non-prevented, allowed AMF hyphal access; prevented, prevented AMF hyphal access. * indicates significant differences at $P < 0.05$. Error bars indicate standard errors (n=3).

Table 1 Root biomass, above-ground biomass, grain yield, plant N and P accumulation, nodule numbers and AMF vesicle numbers.

	Pot		Field	
	non-AMF	AMF	Prevented	non-prevented
Root biomass (g plant ⁻¹)	1.3±0.1	1.8±0.3	0.6±0.0*	1.2±0.1
Aboveground biomass (g plant ⁻¹)	18.0±0.2*	24.6±0.2	3.9±0.2*	9.6±0.9
Yield (g plant ⁻¹)	7.8±0.4*	11.0±0.1	1.7±0.1*	3.3±0.4
N accumulation (g plant ⁻¹)	0.6±0.0*	0.8±0.1	0.1±0.0*	0.3±0.1
P accumulation (mg plant ⁻¹)	30.6±2.9*	66.1±3.9	10.0±0.8*	21.3±1.4
Nodule number (No. plant ⁻¹)	6.4±2.9*	18.7±2.0	8.0±1.0*	25.0±5.1
AMF vesicle (No. cm ⁻¹)	0.3±0.1*	31.2±4.7	18.1±0.8	20.7±6.7

Notes. Mean \pm standard error (n=3). AMF, soil with AMF inoculum; non-AMF, soil without AMF inoculum; non-prevented, allowed AMF hyphal access; prevented, prevented AMF hyphal access. * indicates significant differences within AMF at $P < 0.05$.

Table 2 Soil dissolved organic C (DOC), NH_4^+ , NO_3^- and available P concentrations.

	Pot		Field	
	non-AMF	AMF	Prevented	non-prevented
DOC (mg kg^{-1})	53.4 \pm 1.8*	66.8 \pm 2.2	28.7 \pm 1.7*	35.8 \pm 3.3
Soil NH_4^+ (mg kg^{-1})	7.1 \pm 0.4*	10.4 \pm 0.9	4.3 \pm 0.1	4.5 \pm 0.1
Soil NO_3^- (mg kg^{-1})	7.0 \pm 0.4	7.3 \pm 1.4	16.5 \pm 0.7*	19.5 \pm 0.4
Available P (mg kg^{-1})	31.9 \pm 1.2	26.5 \pm 0.4*	NA	NA

Notes. Mean \pm standard error (n=3). AMF, soil with AMF inoculum; non-AMF, soil without AMF inoculum; non-prevented, allowed AMF hyphal access; prevented, prevented AMF hyphal access. NA, not acquirement. * indicates significant differences within AMF at $P < 0.05$.

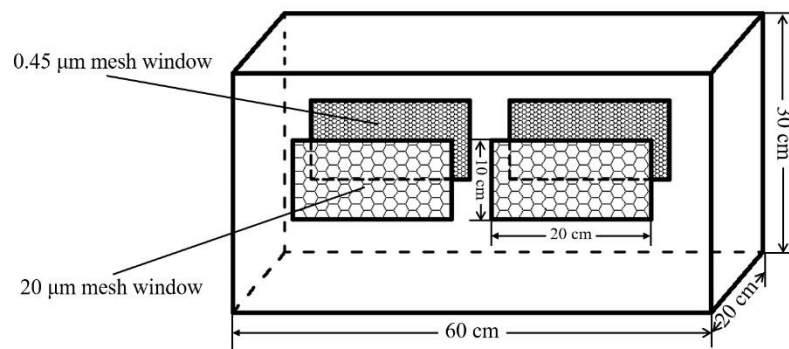


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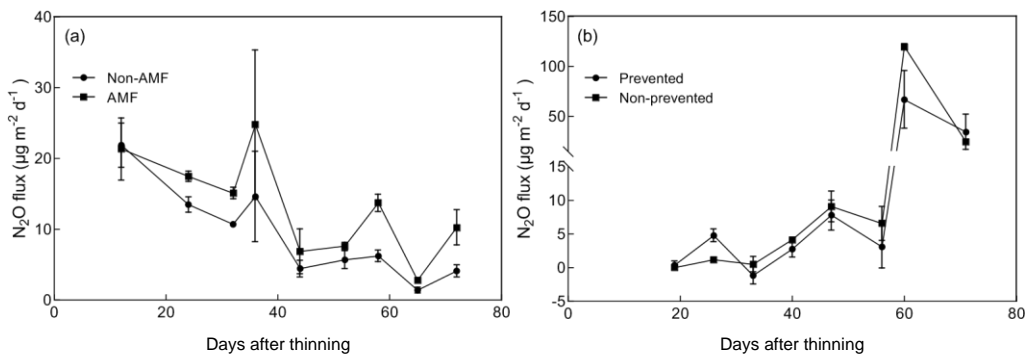


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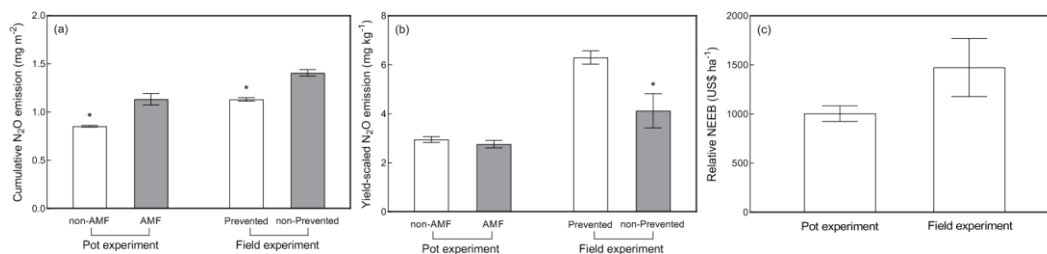


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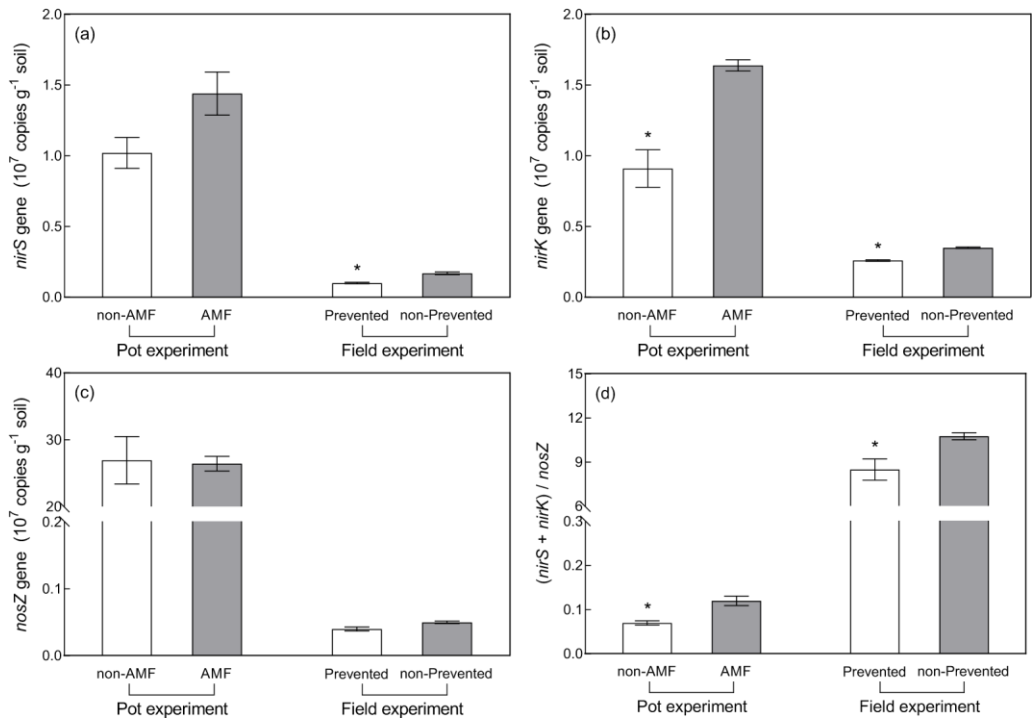


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