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ORIGINAL ARTICLE

Symbiotic relationships between soil fungi and plants reduce N_2O emissions from soil

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N_2O is a potent greenhouse gas involved in the destruction of the protective ozone layer in the stratosphere and contributing to global warming. The ecological processes regulating its emissions from soil are still poorly understood. Here, we show that the presence of arbuscular mycorrhizal fungi (AMF), a dominant group of soil fungi, which form symbiotic associations with the majority of land plants and which influence a range of important ecosystem functions, can induce a reduction in N_2O emissions from soil. To test for a functional relationship between AMF and N_2O emissions, we manipulated the abundance of AMF in two independent greenhouse experiments using two different approaches (sterilized and re-inoculated soil and non-mycorrhizal tomato mutants) and two different soils. N_2O emissions were increased by 42 and 33% in microcosms with reduced AMF abundance compared to microcosms with a well-established AMF community, suggesting that AMF regulate N_2O emissions. This could partly be explained by increased N immobilization into microbial or plant biomass, reduced concentrations of mineral soil N as a substrate for N_2O emission and altered water relations. Moreover, the abundance of key genes responsible for N_2O production (*nirK*) was negatively and for N_2O consumption (*nosZ*) positively correlated to AMF abundance, indicating that the regulation of N_2O emissions is transmitted by AMF-induced changes in the soil microbial community. Our results suggest that the disruption of the AMF symbiosis through intensification of agricultural practices may further contribute to increased N_2O emissions.

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Introduction

N_2O is a potent greenhouse gas contributing to global warming with a 300 times higher global warming potential than CO_2 and is involved in the destruction of the protective ozone layer in the stratosphere (Forster *et al.,* 2007; Ravishankara *et al.,* 2009). N_2O has, after CO_2 and CH_4, the highest impact on the greenhouse effect and its importance is expected to increase due to its longevity and a predicted increase in future emissions (Montzka *et al.,* 2011). Approximately 57% of global N_2O emissions are thought to derive from terrestrial soils (Mosier *et al.,* 1998). A major process producing N_2O in soils is denitrification, a microbial respiratory process that reduces nitrogen oxides (NO_3^-, NO_2) to the gaseous products N_2O and N_2 when oxygen is limiting (Seitzinger *et al.,* 2006; Philippot *et al.,* 2007). It is well established that denitrification depends on soil nitrogen and carbon substrate availability and quality, soil water content, pH and temperature (Knowles, 1982). However, the knowledge of ecological interactions among the vast variety of soil biota on denitrification and N_2O emissions is mostly limited to effects of earthworms and nematodes (Djigal *et al.,* 2010; Lubbers *et al.,* 2013), while the effects of other soil invertebrates on N_2O emissions are just recently being discovered (Kuiper *et al.,* 2013).

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A potential effect of arbuscular mycorrhizal fungi (AMF) on N_2O emissions has been hypothesized (Cavagnaro *et al.,* 2012; Veresoglou *et al.,* 2012a), but has, to our knowledge, never been thoroughly tested. This is surprising because AMF associate with two thirds of all land plants and are among the most abundant functional groups of soil microorganisms being present in almost any ecosystem investigated. They are obligate plant symbionts and are known to improve plant nutrition and influence plant diversity and ecosystem functioning (van der Heijden *et al.,* 1998; Smith and Read, 2008; van der Heijden, 2010; Cheng *et al.,* 2012).

AMF induce changes in soil structure and soil aggregation (Rillig and Mummey, 2006), soil water relations (Auge, 2001), pH (Bago *et al.,* 1996), and the availability and quality of labile carbon (Graham *et al.,* 1981; Hooker *et al.*, 2007), all being factors affecting denitrification. Several studies also show that AMF influence bacterial communities inhabiting the rhizosphere and mycorrhizosphere (Ames *et al.,* 1984; Scheublin *et al.,* 2010), including shifts in denitrifying communities (Amora-Lazcano *et al.,* 1998; Veresoglou *et al.,* 2012b). AMF influence the N cycle and can take up significant amounts of nitrogen (Hodge and Fitter, 2010; Veresoglou *et al.,* 2012a). By reducing the availability of soluble N in the soil, AMF could also reduce denitrification and N_2O emission rates. Thus, there is compelling evidence to suggest that AMF influence denitrification.

It has been shown that fungi possess the ability to denitrify and that fungal N_2O emissions through denitrification can be of high ecological relevance (Shoun *et al.,* 1992; Laughlin and Stevens, 2002; Herold *et al.,* 2012), but we know of no study reporting denitrifying ability for arbuscular mycorrhizal fungi.

To test for a functional relationship between AMF abundance and N_2O emissions, we conducted two independent greenhouse experiments with differing approaches and soils. It was hypothesized that (i) a reduced abundance of AMF increases denitrification-related emissions of N_2O, and that (ii) an increase in emissions is driven by a reduction in plant and/or microbial biomass N pools and (iii) is related to alteration in abundance of key genes for denitrification.

Materials and methods

Two experiments (the 'grass experiment' and the 'tomato experiment', see below for details) were conducted in microcosms constructed from PVC tubes with a diameter of 15 cm, a height of 40 cm and a volume of approx. 7 l. Each microcosm had a removable, airtight cap, allowing the headspace to be closed for gas measurements (see Supplementary Figure S1 for details).

Grass experiment

The soil was collected from a long-term grassland site at the Research Station Agroscope ART in Zürich, Switzerland (47°42′78.13″ N, 8°51′78.38″ E). It was a slightly acidic brown earth with a sandy-loam texture. The collected soil was 5 mm sieved, air dried and mixed with quartz sand to a soil to sand ratio of 7:3 (v/v). The mixture was gamma irradiated with a maximum dose of 32 kGy to eliminate indigenous AMF. After irradiation, soil was incubated at room temperature for 4 weeks to allow stabilization of soil chemical properties before the experiment was initiated. The experiment consisted of two treatments, the mycorrhizal (M) treatment and the non-mycorrhizal (NM) treatment, each being replicated 10 times and set up in three randomized blocks. Each microcosm was filled with 5000 ml of the sterilized soil and 270 ml of an inoculum mixture of three common AMF species; the NM microcosms received a non-mycorrhizal control inoculum. Inoculum details are given in Supplementary Information. Soil irradiation not only eliminated indigenous AMF but will also have removed a significant proportion of other soil biota. Therefore, to include microbes from natural grassland and to allow a similar microbial background among the AMF and control inoculums, a microbial wash was mixed into the substrate for each microcosm (Koide and Li, 1989; van der Heijden *et al.,* 2006). The microbial wash was produced from the same fresh field soil used to fill the microcosms and from all inocula used in the experiment. In addition, 400 ml sterilized soil-sand mixture was added on top of the microcosms to reduce the risk of contamination between pots. Seeds of *Lolium multiflorum* var. oryx were surface-sterilized by stirring in 1.25% bleach for 10 min and rinsing them with deionized water. They were allowed to germinate on 1.5% water agar for 1 week before planting 30 evenly spaced seedlings into each microcosm. After planting, pots were transferred to a climate chamber, under the conditions of 16 h, 22 °C day, 200 μmol m^{-2} s^{-1} light intensity and 8 h, 16 °C night. Relative humidity was 65% at day and 85% at night. Microcosms were watered regularly by weight with deionized water to 40% water filled pore space (WFPS). Plant shoots were cut 6 weeks after planting, ~3 cm above soil surface, and were allowed to re-grow. The experiment was started on 5 November 2010.

Tomato experiment

The soil was collected from a regularly manured long-term pasture on a calcareous brown earth with a sandy-loam texture of an organic farm near the Research Station Agroscope ART in Zürich, Switzerland (47°43′11.83″N, 8°53′65.25″E). The soil was sieved through a 5-mm sieve to homogenize and to remove large stones, plant material, earthworms and other macrofauna that could cause undesired variation. Microcosms were filled with 6000 ml of the sieved field soil. In addition to this, 550 ml of an additional AMF inoculum was mixed with this soil

to assure a high AMF root infection potential. Inoculum details are given in Supplementary Information. Hyphal bags made from 30 μm nylon mesh and filled with 40 g autoclaved quartz sand were buried approximately 5 cm below soil surface. The fine mesh prevented roots from entering the bag, but allowed AMF hyphae to pass. Two genotypes of tomato (*Solanum lycopersicum L.* cv. Micro-Tom), the BC1 mutant and its progenitor wild type, were planted into the microcosms. The BC1 mutant exhibits a strongly reduced AMF root colonization compared to its wild-type progenitor (Meissner *et al.,* 1997). This mutant/wild-type pair was created by fast-neutron mutagenization (David-Schwartz *et al.,* 2001) and hybridization and has been demonstrated to be very suitable for studies in AMF ecology (Rillig *et al.,* 2008). The tomato seeds were germinated in a sterilized 1:1 (v/v) sand-soil mixture and then transplanted into the microcosms. A test for equal performance of both tomato genotypes in absence of AMF was conducted and is described in the Supplementary Information (Supplementary Table S1).

The plants were grown in a greenhouse with an average daily temperature of 24 °C, nightly temperature of 18 °C and 16 h of light per day. Supplemental light was provided by 400 W high-pressure sodium lights when natural irradiation was lower than 300 W m^{-2}. Plants were regularly watered to 40% WFPS with deionized water. The tomato experiment consisted of two treatments, the M treatment planted with the wild type and the NM treatment planted with the BC1 mutant, each replicated 10 times and was established in three randomized blocks. One replicate of the NM treatment failed and was irretrievably lost. The blocks were set up during two-week intervals, starting 26 July 2011.

In the field, both soils used in this study were regularly subjected to waterlogging under wet weather conditions. The characteristics of the substrates being filled into the microcosms of both experiments are summarized in Supplementary Table S2. When filling the microcosms, substrate dry weights were determined gravimetrically. The exact weight of the pots was noted to be able to calculate the WFPS as described in the Supplementary Information.

Fertilization and water pulse. In the grass experiment, after 13 and 14 weeks of plant growth, each pot received 10 ml of a nutrient solution with a low NO_3^-–N concentration (9.98 mM KNO_3, 1 mM $MgSO_4$, 1.5 mM KH_2PO_4, 2 mM $CaCl_2$, 50 μM KCl, 25 μM H_3BO_3, 2 μM $MnSO_4$, 2 μM $ZnSO_4$, 0.5 μM $CuSO_4$ and 0.5 μM Na_2MoO_4). After 15 weeks, microcosms were watered to 100% WFPS with deionized water mixed with 10 ml of a nutrient solution (778 mM KNO_3, 59 mM KH_2PO_4, 1 mM $MgSO_4$, 2 mM $CaCl_2$, 50 μM KCl, 25 μM H_3BO_3, 2 μM $MnSO_4$, 2 μM $ZnSO_4$, 0.5 μM $CuSO_4$ and 0.5 μM Na_2MoO_4). This corresponded to a fertilizer pulse of 60 kgN ha^{-1} and 10 kgP ha^{-1}. The higher water and nutrient loadings were introduced to provide conditions conducive for denitrification.

In the tomato experiment, after 10 weeks of plant growth, the microcosms were watered to 94% WFPS with deionized water mixed with 10 ml of nutrient solution as applied in the grass experiment after 15 weeks. After fertilization, gas fluxes were measured.

Gas sampling. To measure the fluxes of N_2O and CO_2 from the microcosms, the headspace was adjusted to a height of 20 cm above soil surface (4 l volume) and closed for a period of 10 min with the headspace gas pumped through a sample loop, first into a LI-820 CO_2 Gas Analyzer (LI-COR Biosciences, Lincoln, NE, USA) and, subsequently, to a TEI46c-automated N_2O analyzer (Thermo Fisher Scientific, Waltham, MA, USA). The cap used to close the headspace was non-transparent. At every gas sampling, the respective pot was weighed to determine the actual WFPS.

In the grass experiment, after fertilization, lights remained on to avoid diurnal variation in gas fluxes. Headspace gas was analyzed for CO_2 and N_2O emissions at approximately every 6 h for 72 h and once at 89 h after the fertilization pulse, resulting in 13 flux measurements per microcosm.

In the tomato experiment, gas fluxes were measured three times per day (morning, noon and evening) starting 24 h after fertilization for 6 days, and once on the seventh day (noon), resulting in 19 flux measurements per microcosm.

Harvest. Before the final harvest and after the gas measurements, microcosms in both greenhouse experiments were watered, received artificial rainfall and leachates were collected as described in van der Heijden (2010). Shoots were cut at the soil surface. The microcosms were emptied and the roots were collected thoroughly from the soil, rinsed with water, cut into pieces <2 cm and a subsample was weighed and stored in 50% ethanol. Shoots and remaining roots were dried at 60 °C and weighed. In the tomato experiment, the hyphal bags were extracted and frozen for real-time PCR analyses. The remaining substrate was mixed thoroughly and soil samples were taken for soil analyses and assessment of AMF extraradical hyphal length.

Analyses. Soil, leachate and plant samples were chemically analyzed and AMF root colonization and extraradical hyphal length were determined as described in Supplementary Information.

Gene copy numbers. To test if AMF affect the bacterial communities involved in denitrification, we quantified copy numbers of key genes involved in denitrification and N_2O production, encoding cd1 and copper nitrite reductases (*nir*S and *nir*K) and nitrous oxide reductase (*nos*Z) (Zumft, 1997) from hyphal bag samples in the tomato experiment.

Bacterial *16S rRNA* gene abundance was determined to assess the size of the total bacterial community in the samples.

Gene copy number estimations were performed using relative real-time estimation against a reference target to increase accuracy and sensitivity of detection (Daniell *et al.,* 2012). Briefly, DNA was extracted from the hyphal bag samples by a modified phenol chloroform extraction method with bead beating (Deng *et al.,* 2010) with the addition of the reference target. Bacterial *16S*, reference target and denitrification gene amplification was performed essentially as described in Daniell *et al*. (2012) with the primer pairs and reaction conditions shown in Supplementary Table S3. All amplifications were performed using the SYBR green I master mix (Roche, Burgess Hill, UK) with the recommended conditions and 10 pmol μl^{-1} of each primer on a lightcycler 480 (Roche) with associated relative quantification software with three technical replicates performed per sample.

Statistical analyses. Repeated gas-flux measurements were analyzed using the mixed procedure in SPSS version 20 (IBM corp., Armonk, NY, USA). This approach uses the Satterthwaite approximation to obtain the degrees of freedom (Satterthwaite, 1946). The linear mixed effect models for N_2O and CO_2 fluxes included measurement time, AMF treatment and the interaction as fixed effects, and the measurement time nested within each microcosm as the repeated compound. The repeated measurements taken on the same pot were assumed to be correlated. We fit several models using different correlation structures. The adequate correlation structure was chosen by minimizing the Akaike information criterion and performing log-likelihood tests. To reduce calculation effort in the tomato experiment, the repeated measurements taken on the same day were averaged. This reduced the number of repeated measures from 19 to 7. Cumulative gas emissions were calculated by linear interpolation between measurements. Plant biomass and N content, soil data, WFPS, microbial biomass C and N contents, their molar ratio and AMF parameters were statistically analyzed using linear mixed effects models with the AMF treatment as factor and the Block as random effect. Non-parametric Kruskal–Wallis tests were performed to test the differences in AMF parameters between treatments in the grass experiment. Gene copy numbers of denitrification genes and their ratio in the tomato experiment were analyzed similarly, but the three technical replicates were nested within each individual pot. Pearson correlations of AMF parameters with N_2O emissions, microbial biomass and gene copy numbers and their ratio were performed. Data were checked for normality and homoscedasticity and log-transformed where necessary.

For the tomato experiment, a multiple regression was performed to identify the most influential pathways by which the presence of AMF affected N_2O emissions, as described in Supplementary Table S4. As no gene copy number data was available, no multiple regression was performed for the grass experiment. All statistical analyses, except for gas-fluxes, were done using the software R version 2.14.1 and the R-package 'nlme' (Pinheiro *et al.,* 2011).

Results

Grass experiment

Gas emissions. Immediately after fertilization and watering, the N_2O emission curves in both treatments increased in the grassland microcosms (Figure 1a). After this initial phase, N_2O fluxes varied significantly between the treatments (time:AMF interaction $F_{12,18.03} = 8.65$, $P < 0.001$, see Table 1a). The peak of N_2O flux was both attained earlier and was lower in the M treatment compared to the NM treatment (Figure 1a). Cumulatively, N_2O emissions were 42.4% higher in microcosms without AMF compared to mycorrhizal microcosms. Emissions of CO_2 also differed significantly between treatments (time:AMF interaction $F_{12,15.35} = 3.88$, $P = 0.007$, Table 1a, Figure 2a). Cumulative CO_2 emissions were reduced by 5% in the NM treatments.

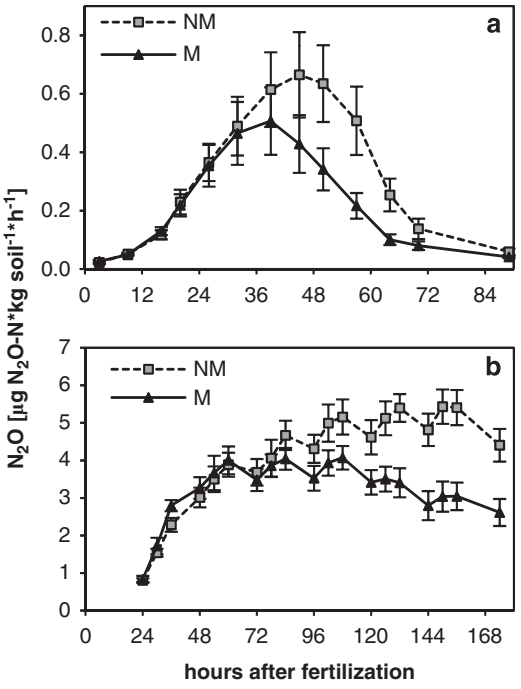

Figure 1 N_2O fluxes from mycorrhizal (M) and non-mycorrhizal (NM) microcosms after a water and fertilization pulse corresponding to 60 kg N ha^{-1} in the grass experiment (**a**), and the tomato experiment (**b**). Grey squares and dashed line: non-mycorrhizal treatment (NM); black triangles and solid line: mycorrhizal treatment (M). Error bars = ± 1 s.e.m. ($n = 10$ for the grass experiment; for the tomato experiment, $n = 9$ for the NM and $n = 10$ for the M treatment).

Table 1 ANOVA output of the repeated-measures analysis for the N_2O and CO_2 fluxes in the grass experiment (a) and the tomato experiment (b)

Variable	*num df*	*den df*	F-*value*	P-*value*
(a) Grass-experiment				
log(N_2O)				
(Intercept)	1	18.00	87.06	<0.0001
Time	**12**	**18.03**	**23.06**	**<0.0001**
AMF	1	18.00	1.70	0.209
Time:AMF	**12**	**18.03**	**8.65**	**<0.0001**
CO_2				
(Intercept)	1	17.98	6094.03	<0.0001
Time	**12**	**15.35**	**262.90**	**<0.0001**
AMF	1	17.98	2.52	0.130
Time:AMF	**12**	**15.35**	**3.88**	**0.007**
(b) Tomato-experiment				
N_2O				
(Intercept)	1	17	406.99	<0.0001
Time	**6**	**17**	**59.98**	**<0.0001**
AMF	**1**	**17**	**6.71**	**0.019**
Time:AMF	**6**	**17**	**5.35**	**0.003**
CO_2				
(Intercept)	1	17.49	512.69	<0.0001
Time	**6**	**14.32**	**3.70**	**0.020**
AMF	**1**	**17.49**	**7.07**	**0.016**
Time:AMF	6	14.32	0.61	0.716

Abbreviation: AMF, arbuscular mycorrhizal fungal treatment.
In the grass experiment, the factor time consisted of 13 levels. In the tomato experiment, time comprised 7 levels (19 timepoints averaged to 1 mean per day. See Materials and methods for detailed description). The factor AMF consisted of two levels, mycorrhizal (M) and non-mycorrhizal (NM) ($n=10$ for the grass experiment; for the tomato experiment, $n=9$ for the NM, and $n=10$ for the M treatment). Significant effects ($P<0.05$) are shown in bold.

Plant and soil measures. There were no significant differences between the treatments in plant biomass and N nutrition and soil N content and pH at the end of the experiment (Table 2). The water content, expressed as the reduction in WFPS during the gas measurements, did also not reveal any differences (Table 2, Supplementary Figure S2). Roots from the NM treatments did not show any colonization with AMF structures. However, some extraradical hyphae were detected in the NM treatment. Those were considered as non-mycorrhizal or dead fungal hyphae.

Soil microbial biomass C and N contents were significantly increased in the M treatment (Table 2). There was a positive correlation ($R^2=0.67$, $P=0.004$) of AMF extraradical hyphal length with soil microbial biomass N (Figure 3).

Tomato experiment

Gas emissions. N_2O emissions differed significantly between treatments (AMF $F_{1,17}=6.71$, $P=0.019$; time:AMF interaction $F_{6,17}=5.35$, $P=0.003$, Table 1b, Figure 1b). Total N_2O emissions were 33.8% higher in the microcosms planted with the non-mycorrhizal tomato mutant compared to the mycorrhizal wild type. Similar to the grass experiment, the peak of N_2O fluxes was reached earlier and was lower in the M treatment (Figure 1b). There was a significant, negative correlation of AMF root colonization to N_2O emissions ($R^2=0.47$, $P=0.001$, Figure 4).

CO_2 emissions differed significantly between treatments (AMF $F_{1,17.49}=7.07$, $P=0.016$, Table 1b, Figure 2b). Cumulative CO_2 emissions were 23.4% lower in the NM treatment compared to the M treatment.

Plant and soil measures. NM plants had a 25.3% lower biomass and 31.1% lower N content than M plants. However, root N contents did not differ significantly between the treatments (Table 2).

Available NO_3^- was 28.1% higher in the NM treatment at the end of the experiment, while soil pH was slightly but significantly reduced (Table 2). The water content during the gas measurements declined faster in the M treatment (Table 2, Supplementary Figure S2).

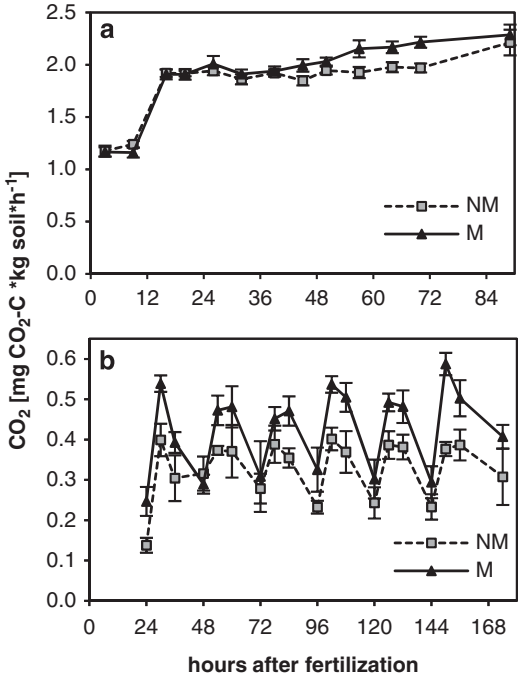

Figure 2 CO_2 fluxes from mycorrhizal (M) or non-mycorrhizal (NM) microcosms after a water and fertilization pulse in the grass experiment (**a**), and the tomato experiment (**b**). The measurements in the grass experiment were made in a climate chamber with lights constantly switched on during the whole measuring period. In contrast, in the tomato experiment, measurements were made in a greenhouse with a 16 h day/8 h night pattern. This resulted in pronounced diurnal CO_2 flux variations in the tomato experiment, while no such pattern was detected in the grass experiment. Grey squares and dashed line: non-mycorrhizal treatment (NM); black triangles and solid line: mycorrhizal treatment (M). Error bars represent ± 1 s.e.m. ($n=10$ for the grass experiment; for the tomato experiment, $n=9$ for the NM and $n=10$ for the M treatment).

Table 2 Plant, soil and AM fungal parameters of the microcosms being inoculated with (M) or without (NM) AMF (grass experiment) or being planted with a mycorrhizal tomato wild type (M) or the non-mycorrhizal BC1 tomato mutant (NM) (tomato experiment)

	Grass experiment			*Tomato experiment*		
	M	*NM*		*M (WT)*	*NM (MT)*	
Plant biomass						
Shoot (g kg soil^{-1})	3.025 (±0.035)	3.115 (±0.040)	$P=0.271$	**0.746 (±0.044)**	**0.529 (±0.040)**	$\mathbf{P=0.002}$
Root (g kg soil^{-1})	3.757 (±0.235)	4.079 (±0.337)	$P=0.691$	0.101 (±0.008)	0.104 (±0.013)	$P=0.859$
Total (g kg soil^{-1})	6.782 (±0.246)	7.194 (±0.334)	$P=0.613$	**0.847 (±0.042)**	**0.632 (±0.047)**	$\mathbf{P=0.002}$
Plant N content						
Shoot (mgN kg soil^{-1})	68.10 (±1.49)	68.96 (±0.91)	$P=0.722$	**29.69 (±1.40)**	**19.70 (±1.53)**	$\mathbf{P<0.001}$
Root (mgN kg soil^{-1})	48.05 (±1.64)	52.82 (±4.25)	$P=0.273$	2.613 (±0.306)	2.553 (±0.355)	$P=0.871$
Total (mgN kg soil^{-1})	116.1 (±2.3)	122.8 (±4.5)	$P=0.213$	**32.30 (±1.38)**	**22.25 (±1.58)**	$\mathbf{P<0.001}$
Soil						
Available NO_3^- (mgNO_3^--N kg soil^{-1})	ND	ND		**31.05 (±1.44)**	**39.77 (±1.17)**	$\mathbf{P<0.001}$
Total soil N (gN kg soil^{-1})	0.863 (±0.022)	0.821 (±0.030)	$P=0.558$	2.390 (±0.041)	2.444 (±0.035)	$P=0.331$
Soil pH	7.373 (±0.033)	7.335 (±0.025)	$P=0.531$	**7.745 (±0.027)**	**7.623 (±0.026)**	$\mathbf{P=0.004}$
rWFPS (% reduction h^{-1})	−0.310 (±0.007)	−0.309 (±0.009)	$P=0.901$	**−0.083 (±0.003)**	**−0.070 (±0.004)**	$\mathbf{P=0.004}$
Soil microbial biomass						
C content (mg kg soil^{-1})	**200.8 (±5.96)**	**180.2 (±6.19)**	$\mathbf{P=0.028}$	743.8 (±10.39)	761.8 (±19.68)	$P=0.417$
N content (mg kg soil^{-1})	**29.98 (±1.15)**	**26.26 (±1.23)**	$\mathbf{P=0.026}$	107.75 (±1.959)	102.92 (±2.529)	$P=0.099$
C/N ratio	6.75 (±0.227)	6.91 (±0.164)	$P=0.434$	**8.06 (±0.129)**	**8.651 (±0.208)**	$\mathbf{P=0.015}$
AM fungal parameters						
HC (%)	**64.53 (±2.37)**	**0 (±0.00)**	$\mathbf{P<0.001}$[a]	**42.05 (±3.335)**	**17.33 (±2.194)**	$\mathbf{P<0.001}$
VC (%)	**4.80 (±0.71)**	**0 (±0.00)**	$\mathbf{P<0.001}$[a]	**1.6 (±0.245)**	**0.3 (±0.147)**	$\mathbf{P<0.001}$
AC (%)	**23.67 (±2.16)**	**0 (±0.00)**	$\mathbf{P<0.001}$[a]	**34 (±2.580)**	**7.9 (±1.213)**	$\mathbf{P<0.001}$
HL (mg soil^{-1})	**16.54 (±1.19)**	**4.387 (±0.28)**	$\mathbf{P<0.001}$[a]	6.78 (±0.519)	5.70 (±0.274)	$P=0.096$
Gene copy numbers						
16srRNA (mg soil^{-1})	NA	NA		34 242 (±12146)	19 288 (±6998)	$P=0.282$[b]
nirK (mg soil^{-1})	NA	NA		109.13 (±24.62)	210.03 (±58.87)	$P=0.094$[b]
nirS (mg soil^{-1})	NA	NA		6.45 (±2.446)	3.37 (±0.805)	$P=0.225$[b]
nosZ (mg soil^{-1})	NA	NA		64.14 (±10.99)	39.20 (±7.881)	$P=0.191$
Ratio *nosZ*/(*nirS* + *nirK*)	NA	NA		1.37 (±0.739)	0.26 (±0.060)	$P=0.194$

Abbreviations: AC, arbuscular root colonization; AMF, arbuscular mycorrhizal fungi; HC, hyphal colonization; HL, extraradical hyphal length; NA, not available; ND, not detectable; VC, vesicular root colonization; WFPS, water-filled pore space.
Values are presented as means (±1 s.e.m.), *P*-values are obtained from linear mixed effects models with the AMF treatment as factor and the block as random effect. Available NO_3^- - was calculated as the sum of soil and leachate NO_3^- contents at the end of the experiment. In the grass experiment no available NO_3^- was detected; in the tomato experiment on average 62.7% (±1.92% s.e.m.) of available NO_3^- was found in the leachate. The WFPS declined nearly linearly during the gas measurements (see Supplementary Figure S2) and the slope of the decline in WFPS over time (% reduction in WFPS*h^{-1}, 'rWFPS') was used to analyze differences between the treatments. Significant differences between the NM and the M treatments are shown in bold ($n=10$ for the grass experiment; for the tomato experiment, $n=9$ for the NM and $n=10$ for the M treatment).
[a]A non-parametric Kruskal–Wallis test was performed to test for differences between treatments.
[b]log-transformed.

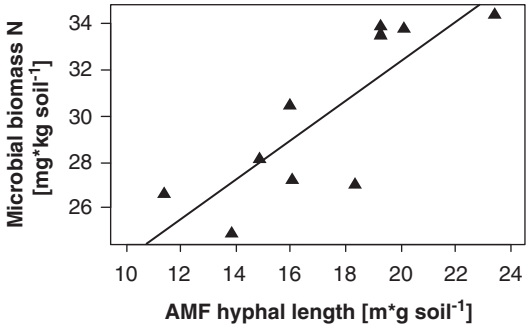

Figure 3 Pearson correlation of AMF extraradical hyphal length with microbial biomass N content ($R^2=0.67$, $P=0.004$) in the M treatment of the grass experiment. The NM treatment was omitted from the correlation analysis, as it did not contain AMF.

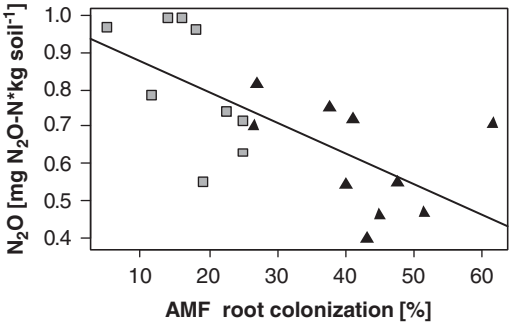

Figure 4 Pearson correlation of AMF root colonization with N_2O emissions in the tomato experiment ($R^2=0.47$, $P=0.001$). Grey squares: tomato mutant (NM), black triangles: tomato wild type (M).

Microbial biomass C and N contents did not differ between the treatments. However, the C/N ratio of the soil microbial biomass was significantly higher in the NM treatment (Table 2).

The BC1 mutant did not completely suppress root colonization by AMF but reduced it significantly. The average root length colonized by AMF were 42.1% and 17.3% for the M and the NM treatment,

respectively. Extraradical hyphal length did not differ significantly between the treatments (Table 2).

To exclude the possibility of any non-target effects resulting from differences between the genotypes independent of AMF, a test for equal performance of the genotypes in the absence of AMF was conducted; this demonstrated no significant differences between genotypes in all measured variables (Supplementary Table S1).

Denitrification gene copy numbers. Copy numbers of *nirK*, *nirS* and *nosZ*, key genes involved in denitrification and N_2O production or consumption, and the ratio of nosZ/(nirK + nirS) did not differ significantly between treatments, but AMF parameters were significantly negatively correlated to the copy numbers of the functional gene *nirK* (Figures 5a and b, Supplementary Table S5). Simultaneously, gene copy numbers of *nosZ* were positively correlated to AMF root colonization measures (Figure 5c, Supplementary Table S5). The ratio of *nosZ* copy numbers to the sum of *nirK* and *nirS* copy numbers (*nosZ*/(*nirK* + *nirS*)) was positively correlated to AMF root colonization measures (Supplementary Table S5). All correlations were strongest with AMF vesicular root colonization (Figures 5b–d, Supplementary Table S5). Correlations of *nirS* and the *16s rRNA* to AMF abundance were mostly absent (Supplementary Table S5).

Most influential parameters affecting N_2O emissions. The multiple regression performed to identify the most influential parameters affecting N_2O emissions included microbial biomass C and N content and the abundance of *nirK* gene copy numbers. Overall, the model significantly ($P = 0.001$) explained 58% of the variance in N_2O emissions (Supplementary Table S4).

Discussion

Soils are the major source of atmospheric N_2O. Still, the role of soil ecological interactions on denitrification and N_2O emissions are poorly understood and are only beginning to be revealed. While it is well established that AMF play a key role in ecosystems and provide a number of ecosystem services, it was unknown, until now, that AMF also influence N_2O emissions. Here, we demonstrate in two complementary experiments that AMF can contribute to reduced emissions of N_2O. As N_2O is a strong greenhouse gas and AMF are a very widespread group of organisms being distributed worldwide, the results suggest that AMF could play a role in the mitigation of climate change.

Our results point to several possible mechanisms by which the AMF symbiosis may reduce N_2O emissions. First, it is known that AMF can acquire significant amounts of nitrogen from soil (Johansen *et al.,* 1993; Bago *et al.,* 1996; Govindarajulu *et al.,* 2005), suggesting that they can reduce substrate availability for denitrifying organisms. In the tomato experiment, plant biomass and N contents were higher in the M treatment, while available soil NO_3^-

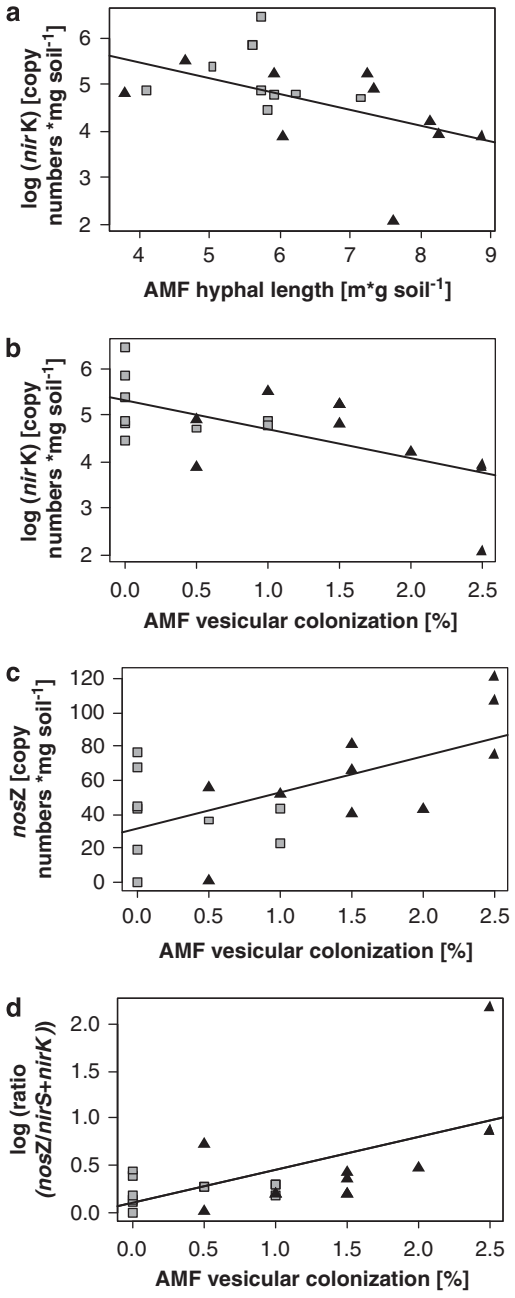

Figure 5 Pearson correlations of AMF structures with denitrification gene copy numbers. Correlation of AMF extraradical hyphal length with gene copy numbers of *nirK* (log-transformed) ($R^2 = 0.26$, $P = 0.025$) (**a**), and of AMF vesicular colonization with gene copy numbers of *nirK* (log-transformed) ($R^2 = 0.39$, $P = 0.004$) (**b**), with gene copy numbers of *nosZ* ($R^2 = 0.38$, $P = 0.005$) (**c**) and with the ratio *nosZ*(*nirK* + *nirS*) (log-transformed) ($R^2 = 0.42$, $P = 0.003$). Correlations of other AMF parameters and denitrification genes are shown in Supplementary Table S5. For all correlations the mean of three technical replicates per pot was used. Grey squares: tomato mutants (NM), black triangles: tomato wild type (M).

was reduced. Consequently, one obvious mechanism by which AMF reduce N_2O emissions could be improved plant N nutrition resulting in the reduction of soil NO_3^- concentration, thus limiting denitrification. However, in the grass experiment, planted with a C3- grass known to show less pronounced responses to AMF (Hoeksema *et al.,* 2010), plant biomass and N content as well as available soil NO_3^- did not differ between treatments. This implies an additional involvement of mechanisms other than improved plant N nutrition to prevent N_2O emissions. The positive correlation of AMF extraradical hyphal length to soil microbial biomass N in this experiment suggests that increased N immobilization by the soil microbial biomass, also including AMF hyphae, may have contributed to reduced N_2O emissions in the grass experiment.

Second, the availability of O_2 in soil is an important control of denitrification (Morley and Baggs, 2010) and is strongly correlated to soil water content (Smith, 1990). In the tomato experiment, the WFPS declined faster in the M treatment during the gas measurements, probably due to enhanced plant transpiration induced by the higher plant biomass, or by enhanced water removal directly induced by AMF (RuizLozano and Azcon, 1995; Auge, 2001; Khalvati *et al.,* 2005). The faster water removal in the M treatment likely increased the oxygen availability in the soil and therefore reduced N_2O emissions, as denitrifying enzymes are expressed under low oxygen conditions to maintain respiration (Berks *et al.,* 1995).

Third, in the tomato experiment, we observed a significant negative correlation of AMF root colonization and extraradical hyphal length with *nirK*, a gene directly being involved in the production of N_2O, and a positive correlation of AMF root colonization with *nosZ*, a main gene consuming N_2O and reducing it to N_2. It has been shown that a relative reduction in denitrifying organisms containing the *nosZ* gene can lead to enhanced N_2O emissions (Philippot *et al.,* 2011). There was a positive correlation of most AMF structures with the *nosZ*/(*nirS* + *nirK*) gene ratio (Supplementary Table S5), indicating a relative increase in organisms containing *nos*Z with increased AMF abundance. Hence, these observations suggest that the presence of AMF is linked to changes in the denitrifier community composition. The absence of a relationship of AMF structures to *16S rRNA* implies that the total bacterial community size was not affected by the presence or absence of AMF, providing further support to our notion that AMF change the denitrifier community composition.

The increased CO_2 emissions in the M treatments confirm other studies (Grimoldi *et al.,* 2006; Nottingham *et al.,* 2010; Cheng *et al.,* 2012) showing that AMF enhance CO_2 emissions from soil and suggest that C cycling was modified by AMF (Drigo *et al.,* 2010). AMF-induced shifts in C allocation into the soil can modify soil bacterial community composition (Toljander *et al.,* 2007) and could also provide an explanation for the observed changes in the denitrifying communities, as suggested by Veresoglou *et al.* (2012b). Moreover, AMF were reported to reduce C exudation from roots (Graham *et al.,* 1981) and to exudate C from their hyphae (Hooker *et al.*, 2007), suggesting that AMF enhance C transport into the bulk soil, where denitrifiers are less abundant and N_2 is the dominant denitrification endproduct (Cheneby *et al.,* 2004). Our observation that the abundance of the *nosZ* gene increased with AMF abundance supports this.

There is increasing evidence that many fungi are capable of denitrification and act as potentially significant sources of N_2O as they appear to lack a nitrous oxide reductase (Shoun *et al.,* 1992; Prendergast-Miller *et al.,* 2011). These studies have focused on ascomycete and basidiomycete species. Glomeromycota form a distinct linage (Schüssler *et al.,* 2001) and direct assessment of any role in denitrification has not been performed. However, our results suggest that this group does not denitrify perhaps explaining why AMF root colonization is often reduced under waterlogged conditions (e.g. Mendoza *et al.,* 2005; Ipsilantis and Sylvia, 2007).

In order to further understand which factors contributed to N_2O production, we performed a multiple regression. Our analysis revealed that microbial biomass C and N contents together with *nir*K abundance were the strongest predictors of N_2O emissions for the tomato experiment, suggesting that the reduced N_2O emissions were caused by AMF-induced changes in soil microbial biomass and community composition.

In conclusion, the results presented here demonstrate that the AMF symbiosis can reduce N_2O emissions from soil. Denitrification and related N_2O emissions are governed by complex interactions of various entangled factors. Also, the effects exerted by the AMF symbiosis on ecosystem processes are the result of complex interactions between fungus and plant. Disentangling these interactions and showing a direct cause–effect relationship is a challenging task that warrants further investigations. We show a hitherto unknown involvement of the AMF symbiosis in the reduction of N_2O emissions. Our results give a starting point for further investigations that should focus on the detailed mechanistic pathways by which the presence of AMF influences denitrifying communities and N_2O emissions.

The abundance of AMF in soil depends on soil nutrient availability and declines with fertilization and intensive land use (Helgason *et al.,* 1998; Egerton-Warburton and Allen, 2000; Oehl *et al.,* 2004). The results obtained here suggest that a reduction of AMF abundance by intensive agricultural management and high fertilizer additions may initiate a cascade of below-ground interactions that further enhance N_2O emission from soil with

potential negative consequences for the ozone layer and the earth's climate.

Conflict of Interest

The authors declare no conflict of interest.

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