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Native arbuscular mycorrhizal fungi increase the abundance of ammonia-oxidizing bacteria, but suppress nitrous oxide emissions shortly after urea application

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Abstract

The potential of the symbiosis between plants and arbuscular mycorrhizal fungi (AMF) to reduce emissions of the greenhouse gas N_2O has gained scientific attention in the last years. Given the high nitrogen (N) requirements of AMF and their role in plant N uptake, they may reduce the availability of mineral N that could be subject to N_2O emissions and leaching losses. We investigated the impact of AMF on the growth of tropical grass *Brachiaria decumbens* Stapf. and on N_2O released after fertilization with urea in a mesocosm study. To evaluate the role of nitrification in N_2O emissions, we used nitrification inhibitor dicyandiamide (DCD). The study included a full-factorial design (n=6) with two AMF treatments (with and without AMF inoculation) and three fertilization treatments (control, urea and urea+DCD), applied after 92 days of growth. Plant growth, soil properties and N_2O emissions were measured during the

following two weeks and the abundance of nitrifiers was quantified one and two weeks after fertilization. The production of N_2O increased after urea application but only without DCD, indicating the importance of nitrification in N_2O emissions. The emissions of N_2O after urea application were reduced by 46% due to the presence of AMF. Nevertheless, the abundance of ammonia-oxidizing bacteria (AOB) was increased by urea and AMF, while plant growth was reduced by the AMF. The increased root:shoot ratio of the biomass in AMF pots suggests competition between AMF and plants. This study demonstrated that immobilization of N by AMF can reduce N_2O emissions after fertilization, even when plant growth is reduced. The inverse relationship between (higher) AOB abundance and (lower) nitrification rates suggests that changes in the activity of AOB, rather than abundance, may be indicative of the impact of the AMF-*Brachiaria* symbiosis on N cycling in tropical grasslands. Alternatively, the difference between N_2O emissions from AMF and non-AMF pots may be explained by increased reduction of N_2O in the presence of AMF. Longer-term studies are needed to verify whether the effects of AMF on N_2O emissions and/or plant growth persist over time or are limited to initial immobilization of N by AMF in N-limited systems.

Key words

arbuscular mycorrhizal fungi; nitrification; nitrous oxide; tropical grasses; urea

1. Introduction

The productivity of tropical grasslands, which are prone to degradation upon overgrazing, is generally limited by nitrogen (N) availability. Therefore, synthetic fertilizers, most commonly in the form of urea, are applied to meet the plants nutritional requirements. However, in many soils, applied urea is rapidly decomposed and release ammonium (NH_4^+) which prone to nitrification and linked increased risk of leaching losses. Furthermore, during nitrification and heterotrophic denitrification (Hu et al., 2015), nitrous oxide (N_2O) is produced which

50 contributes to global warming. In tropical grasslands, the nitrification-related pathway of N_2O
51 production is a significant source of N_2O emissions from soil (Byrnes et al., 2017). The first
52 and rate-limiting step of nitrification is the oxidation of ammonium (NH_4^+) to nitrite (NO_2^-) by
53 ammonia oxidizers (AOs), followed by oxidation of NO_2^- to nitrate (NO_3^-) by nitrite oxidizing
54 bacteria, while releasing part of nitrogen as N_2O (Davidson, 1991; Firestone and Davidson,
55 1989). If not taken up by plants or soil microorganisms, NO_3^- is prone to leaching causing
56 eutrophication of ground and surface waters. Furthermore, NO_3^- is the substrate used by soil
57 denitrifiers further contributing to N_2O emissions. Uptake of NH_4^+ is energetically beneficial
58 when compared to NO_3^- (Salsac et al., 1987) and is therefore often the preferred form of N for
59 soil microorganisms and several plant species, as observed for example in case of *Brachiaria*
60 *humidicola* (Rendle) Schweick (Rao et al., 1996). Immobilization of NH_4^+ by plants and other
61 soil biota decreases the substrate availability for AOs and thus suppresses N_2O emissions from
62 nitrification, and, subsequently, decreases the availability of substrate for denitrifiers mediating
63 N_2O emissions from denitrification. The interactions between plants and soil microbes can thus
64 exert a strong control on N_2O emissions from soils.
65 Arbuscular mycorrhizal fungi (AMF), which form symbiotic associations with two-thirds
66 of all land plants and are widespread in most terrestrial ecosystems (Smith and Read, 2008),
67 can improve host plant nutrition by enhanced uptake of several soil nutrients, such as
68 phosphorus (P), N and zinc (Zn), via extensive hyphal networks in soil and transfer of nutrients
69 from fungus to plant root in exchange for assimilated carbon (C) (Fellbaum et al., 2012; Smith
70 et al., 2008). By increasing the N uptake of plant, either by N transfer or by reduced limitation
71 of other nutrients and linked increased N uptake by plant itself, AMF symbiosis can potentially
72 play an important role in controlling N availability for N_2O production. As the growth of both
73 partners and the efficiency of the symbiosis can be improved only if no other nutrient is limiting,

74 the uptake of N by either plant or AMF fungi depends on many interrelated factors with soil
75 nutrient stoichiometry playing a key role (Johnson et al., 2015).

76 Although AMF have been observed to absorb N in various forms including NH_4^+ (Tanaka
77 and Yano, 2005), NO_3^- (Cavagnaro et al., 2012) and organic N (Whiteside et al., 2012), their
78 preferred N source seems to be NH_4^+ (Govindarajulu et al., 2005; Read and Perez-Moreno,
79 2003). It has been suggested that AMF can have an adverse effect on AOs as the latter are
80 considered to be weak competitors for NH_4^+ (Bollmann et al., 2002). Nevertheless, inconsistent
81 results have been found with respect to the potential competition between AMF and
82 microorganism involved in N-cycling (Amora-Lazcano *et al.*, 1998; Cavagnaro *et al.*, 2007;
83 Veresoglou *et al.*, 2011; Chen *et al.*, 2013; Storer *et al.*, 2017) and the conditions explaining
84 such seemingly conflicting observations remain unclear.

85 While Storer et al. (2017) observed a reduction in N_2O emissions after the application of
86 organic material due to the presence of AMF hyphae and hypothesized that AMF outcompeted
87 slow-growing AOs, the vast majority of studies addressing the potential of mycorrhizal
88 symbiosis to reduce N_2O emissions from soil focused on the emissions under high water-filled
89 pore space (WFPS) conditions applying NO_3^- as a substrate for denitrifiers (Bender et al., 2014;
90 Lazcano et al., 2014). Under such conditions of high soil moisture content, mycorrhizal
91 symbiosis was observed to lower the N_2O emissions, which the authors speculated to be related
92 to (i) increased N immobilization in microbial and/or plant biomass resulting in reduced pool
93 of available N for denitrifiers, (ii) reduction of C exudation from the roots and increased C
94 release in the hyphosphere, or (iii) changes in soil-water relations due to the improved soil
95 structure and increased water uptake by AMF plants (Bender et al., 2014; Lazcano et al., 2014).

96 Urea is the most frequently used N fertilizer in agriculture (Glibert et al., 2006) and the key
97 N input in pastures where urea is deposited as urine. To our best knowledge, all studies
98 investigating AMF effects on N_2O release from grassland soil have focused on temperate

climates and the majority of studies has considered the denitrification pathway of N_2O production using NO_3^--based fertilizers at high soil water contents, which is not a realistic scenario in tropical grasslands under well drained conditions, as plant water uptake and evapotranspiration rates are very high. No studies have related AMF-induced changes in AOs abundance with N_2O released via the nitrification pathway.

The aim of the present study was to quantify the effect of AMF in a *Brachiaria* grassland soil on N_2O emissions under aerobic conditions after urea application. To obtain insight into the mechanisms that can explain AMF-induced changes, N uptake by plant and microbial biomass were quantified as well as functional *amoA* gene involved in NH_4^+ oxidation. We hypothesized that the presence of AMF reduces N_2O emissions after urea application, and that this effect can be attributed to the (i) a negative effect of AMF on the abundance of AOs as a result of competition for NH_4^+, and (ii) an overall reduction of mineral N in soil due to increased plant N uptake and microbial N immobilization in the presence of AMF.

2. Materials and Methods

2.1. Experimental design

A pot experiment (106 days since the plant sowing until the final harvest) with *Brachiaria decumbens* Stapf., which is one of the most common forage grasses grown the tropics. Only in Brazilian savanna, the estimated surface covered by *Brachiaria* pastures was 50 million of ha (Sano et al., 2002). Many *Brachiaria* genotypes have the potential to be used to restore degraded grasslands while improving cattle nutrition. The experiment was established in the greenhouse to test the effects of the following factors and their interactions: (i) AMF (with and without AMF) and (ii) fertilization (control without fertilization, urea and urea+dicyandiamide (DCD)). All pots were distributed in a completely randomized block design with eight repetitions per treatment. Fertilizer treatments were applied on day 92 of the

plant growth and the emissions of N_2O and CO_2 were determined right before fertilizer treatments application and during 14 days after fertilization (between day 92 and 106 of plant growth) when the N_2O emissions dropped to the values close to pre-fertilization values. As synthetic fertilizers are commonly applied after grazing, plant aboveground biomass was cut before the application of the fertilizer treatments. Seven and 14 days after fertilization, four plants were destructively sampled for biomass quantification and N content (Supplementary material Fig. S1).

2.2. Soil sterilization and microbial inoculation

The study was performed in a controlled greenhouse at the International Centre for Tropical Agriculture (CIAT) in Palmira, Colombia. Plastic pots (17 cm height, 18 cm diameter) were filled with two kilograms of soil (Vertisol) collected from the experimental fields at CIAT. Soil samples were analyzed by the Analytical Services Laborayory at CIAT and contained 152 mg kg^{-1} of available P (P-BrayII), 1467 mg kg^{-1} of calcium, 469 mg kg^{-1} of magnesium, and 628 mg kg^{-1} of available potassium. Soil was obtained from the margins of pastoral field trial at CIAT HQ in Palmira, Colombia, where Brachiaria hybrid (cv. Cayman) is grown. The bulk density of the field is 1.40 g cm^{-3}, which was also the packing density of the soil in the pot experiment. Field moist soil was homogenized and sieved (<5 mm) prior sterilization with autoclave (121° C, 90 min). All pots were re-inoculated with a microbial extract of the fresh soil collected within the same area, which was filtered to exclude AMF spores and prepared as follows: shaking (30 min) of one kilogram of soil with five liters of deionized water, followed by sieving through 125 µm, 40 µm and 20 µm sieves. The extract was then filtered twice through Whatman 2 filter paper. Fifty ml of final filtrate, corresponding to an extract from 5 grams for each kilogram of sterile soil, was added to all pots. The moisture content was adjusted to 60% of water-filled pore space (WFPS) and pots were placed in the greenhouse (22-28°C) for two weeks for microorganisms to utilize the substrate released by soil sterilization and to

colonize the whole pot area. Water filled pore space (WFPS) was calculated by dividing the volumetric water content (calculated as the gravimetric water content * soil bulk density/water density) by total soil porosity, while total soil porosity was calculated according to: soil porosity = 1 – (soil bulk density/2.65) assuming a soil particle density of 2.65 g cm^{-3}. The soil bulk density in the pots was 1.40 g cm^{-3}. Soil moisture content was controlled gravimetrically and adjusted every 1-2 days.

2.3. Seeding and inoculation with AMF

Two weeks after microbial wash application, seeds of *Brachiaria decumbens* Stapf. were surface-sterilized with ethanol (50%, 30s) and bleach (2.5%, 5 min) and washed three times with deionized water. Seeds were pre-germinated in sterile Petri dishes for three days and then transplanted to sterilized sand. At the two-leaves stage, the plantlets were transplanted to the pots and on the same day (day one of the experiment) half of the pots was inoculated with commercial AMF inoculum obtained from Abonamos Micorrizas (registration number ICA 3556) containing inert substrate, clay, mycorrhizal roots, mycelium and spores. Same amount of sterilized (autoclave, 121°C twice 1h in two consecutive days) inoculum was applied to control non-mycorrhizal pots. The success of the inoculation was checked two weeks later in the additional pots. However, no root colonization or AMF growth was detected. The pots with seedling were re-inoculated with native AMF collected from the area of soil collection on day 14 of the experiment. In brief, AMF spores were extracted from one kg of fresh soil using wet-sieving and decanting method followed by sucrose centrifugation (Sieverding, 1991). Obtained spores were washed, isolated and applied to the pots at a density of approximately 500 spores pot^{-1}. As the AMF spores originated from the same soil as the microbial inoculum, no surface sterilization of spores was performed.

A total number of 72 pots was prepared: 24 AMF-inoculated pots (+M), 24 non-AMF (-M) pots and 24 additional pots. The additional pots were used to confirm the soil moisture content and the presence of AMF by destructive sampling throughout the experiment. Plants were watered every one or two days to 60% WFPS. Pots were fertilized twice with a composite fertilizer containing 6 mg N (all applied N in the form of urea), 0.25 mg Mg, 0.25 mg Ca, 5 mg P, 5 mg K, 0.3 mg S and 0.1 mg Zn pot^{-1} (corresponding to 4.21, 0.18, 0.18, 3.51, 3.51, 0.21 and 0.07 kg ha^{-1}, respectively) to prevent limitation by other soil nutrients than N. The small amount of N as urea was added to stimulate the N cycling. The first fertilization was performed on May 17th (day 83 of the plant growth) and the second one on May 26th (day 92 of the plant growth), which was the day of the fertilizer treatment application.

2.4. Fertilization treatments

Two months after AMF inoculation, which corresponds to day 92 after planting, urea solution (0.117 g N pot^{-1} corresponding to 82 kg ha^{-1}) was applied to eight +M and eight -M pots (-M/N and +M/N, respectively). The recommended amount of N applied to *Brachiaria decumbens* pastures ranges between 50 and 150 kg N ha^{-1} (Alvim et al., 1990; De Morais et al., 2006). Similarly, to another eight +M pots and eight -M pots, the same amount of urea together with DCD (10% of applied N) were added (-M/DCD and +M/DCD). The remaining 16 pots (eight +M and eight -M) were watered with the same amount of water (-M/Ctr; +M/Ctr). Four repetitions of each treatment were sampled seven days after fertilization (day 99 of plant growth) and another four repetitions at the end of the experiment (14 days after fertilization, day 106 of plant growth) for the soil analysis, plant biomass and N uptake. The application of nitrification inhibitors, such as DCD in order to suppress NH_4^+ oxidation has been successfully used in pot and field experiments (Tao et al., 2018) and can provide an important insight into the N_2O releasing pathways. Nitrification inhibiting substances selectively target AOs by

deactivating the ammonium monooxygenase enzyme so that NH_4^+ remains available for AMF, other soil heterotrophs and plants. The reduced mobility of N resulting from suppressed NH_4^+ oxidation could have implications for the relationship between AMF and the host plant towards higher dependency on the symbiosis.

2.5. Measurement of greenhouse gases

The emissions of N_2O and CO_2 were measured one day before fertilization treatment application and periodically (1-2 measurements per day) for two weeks since the application of the fertilization treatments using portable Fourier Transform Infrared Spectroscopy (FTIR) Gas Analyzer (Gasmet DX4040, USA). After this period, N_2O emissions were stable and close to zero. During each measurement, pots were covered with a non-transparent plastic chamber (2.5 l volume), sealed and the gas concentration was determined every 20 seconds for ten minutes in order to obtain a linear regression of N_2O (and CO_2) concentration with time.

2.6. Plant growth and nitrogen uptake

Plant aboveground biomass was cut twice before the fertilization treatments, and again right before the fertilization treatments were applied (day 92 of plant growth), and nine days later (day 101 of plant growth). All biomass was dried at 70°C until constant weight and weighed. Cut biomass was collected during the whole experiment and pooled with the shoot biomass obtained during destructive sampling of the same pot. Seven and 14 days after fertilization, four pots from each treatment were destructively sampled and plant roots were manually removed from the soil, dried and weighed. The total N content in both shoot and root biomass was determined using Kjeldahl digestion followed by steam distillation (Bremner and Mulvaney, 1982).

223 **2.7. Mycorrhizal parameters**

224 Mycorrhizal root colonization was determined in all destructively sampled pots (day
225 seven and day 14 after fertilization) from 100 views as a percentage of colonized root segments
226 of total segments observed. Fresh fine roots were hand-picked from the soils, washed and
227 cleared with 10% KOH for 30 minutes in water-bath at 85°C. Cleared roots were stained during
228 heating (5 min, 80°C) with 5% ink-vinegar solution (Vierheilig et al., 1998). AMF spores were
229 extracted from the soil using wet-sieving and decanting method followed by sucrose
230 centrifugation (Sieverding, 1991) and counted under stereo-microscope. The roots used for
231 mycorrhizal colonization were weighed at the fresh state and a subsample was dried to
232 determine the moisture content. The biomass of roots used for mycorrhizal colonization
233 quantification was summed up to the total root biomass.

234

235 **2.8. Soil carbon and nitrogen pools, potential urease activity and nitrification rate**

236 In all destructively sampled pots, mineral N was extracted with 1M KCl solution (1:10
237 w/v) and NH_4^+-N and NO_3^--N contents were determined colorimetrically using the sodium
238 salicylate method (Forster JC, 1995) and sulphanilamide and N-(-naphthyl) ethylendiamine
239 dihydrochloride method (Miranda et al., 2001), respectively. For the potential urease activity
240 determination, a method proposed by Kandeler and Gerber 1988) and modified by Kandeler *et*
241 *al.* (1999) was used and the activity was determined as NH_4^+ produced during the incubation
242 with urea solution as a substrate. The rest of the soil was stored (not more than 14 days) at 4
243 °C until the analysis of microbial biomass C (MBC) and N (MBN), by fumigating 15 g of fresh
244 soil with ethanol-free chloroform followed by extraction with 0.5M K_2SO_4 (1:4 w/v) (Vance et
245 al., 1987). The concentration of microbial biomass N was determined by Kjeldahl digestion and
246 steam distillation (Bremner and Mulvaney, 1982) while MBC was determined colorimetrically
247 (578 nm) by quantification of Cr^{3+} produced by reduction of Cr^{6+} after microwave digestion

(Speedwave four, Berghog, Eningen, Germany) at 135°C for 30 min. Microbial biomass C and N were calculated as the difference between the C and N contents in fumigated and non-fumigated samples, divided by 0.38 (Joergensen, 1996) and 0.54 (Brookes et al., 1985), respectively. Potential nitrification rate (PNR) was determined with the modified shaken-slurry method (Hart et al., 1994) using 5 g of fresh sieved soil. Duplicates of soil samples were mixed with 50 ml of nitrification potential solution (1 mM potassium phosphate pH 7.2; 0.5 mM ammonium sulphate) and agitated at 200 rpm in orbital shaker. One set of samples was taken immediately after buffer addition while the other set was agitated for 24 hours before the quantification of NO_3^- using the same method as in soil extracts.

2.9. DNA extraction and real-time PCR quantification

Soil DNA was extracted from 0.25 g of fresh soil from destructively sampled pots using DNeasy PowerSoil DNA isolation kit (QUIAGEN, Hilden, Germany) according to the manufacturer's instructions. The quantity and purity of the obtained DNA was determined by 260/280 nm and 260/230 nm measurements using a Nanodrop spectrophotometer (DeNovix, Wilmington, DE, USA).

Quantitative PCR (qPCR) was performed to assess the abundance of the *amoA* gene of both ammonia oxidizing archaea (AOA) and ammonia oxidizing bacteria (AOB) (Supplementary materia Table S1). The qPCR was performed in 10-µL reaction mixtures containing the following components: 5 µL of iTaq™ Universal SYBR® Green Supermix (Bio-Rad, BioRad Laboratories, Inc., Hercules, CA), 0.5 µM of each primer (Supplementary material Table S1) and 1 µL of diluted DNA extracts. The optimal dilution of DNA extracts was tested to compensate any reaction inhibition by humic acids co-extracted during DNA isolation (data not shown). All qPCR assays were run on an Applied Biosystems ABI 7300 (Applied Biosystems, NJ, USA) sequence detection system starting with the initial denaturation step at 95 ºC for 10

minutes, followed by amplification cycles specific for each target gene (Supplementary material Table S1). A melting curve analysis was performed after each assay to ensure that only the products of the desired melting temperature were generated. The standard curves for quantifying gene copy numbers were determined by cloning the PCR products in a plasmid using the procedures reported by (Okano et al., 2004). The population sizes of AOA and AOB were estimated as the normalized copies per gram of dry soil.

2.10. Calculations and statistical analysis

The mycorrhizal dependence of selected parameters was calculated according to Hetrick *et al.* (1992). The plant or soil trait for which the effect size is calculated is the percentage increase of +M respect to the mean of –M treatments.

The repeated measurements of GHG were analyzed using SPSS 22.0 program (IBM SPSS, Inc., Chicago, USA) using Linear Mixed Model. The presence of AMF, the type of fertilization (control, urea or urea+DCD) and time of measurement were used as fixed factors, while each pot was considered as a random factor in which time was nested as a repeated measurement. Several models with different covariance structure were carried out and Linear Mixed Model was selected according to the lowest Akaike's information criterion. When a significant single or interaction effect was detected ($p<0.05$), the LSD post-hoc tests ($p<0.05$) were used to test the differences between fertilization treatments. The cumulative GHG emissions were calculated by linear interpolation between measurements. The measured soil properties, AMF parameters, plant biomass and biomass N content were analysed using a Linear Mixed Model with presence of AMF, the type of fertilization (control, urea or urea+DCD) and time of measurement as fixed and the block as a random factor. Normality and homogeneity of the variance were tested using Shapiro-Wilk and Levene's tests, respectively,

297 and when necessary, the values were log-transformed to meet the normality and homogeneity
298 criteria.

299 **3. Results**

300 The native AMF successfully colonized all inoculated plants with a mean root
301 colonization of 71%, while all the -M pots remained without colonization (Supplementary
302 material Table S2). Similarly, the spore density was high in all +M treatments (3600 spores 100
303 g^{-1} soil) and negligible in -M pots (2.8 spores 100 g^{-1} soil). No significant differences in
304 mycorrhizal parameters (root colonization and AMF spore density) were found between the
305 three fertilizer treatments applied (data not shown). Pots were periodically weighted during the
306 experiment in order to detect possible difference in evapotranspiration between mycorrhizal
307 and non-mycorrhizal treatments and no differences were detected. Therefore, the same amount
308 of water was applied to all pots in order to reach the same moisture content.
309

310 **3.1. GHG emissions**

311 The N_2O emission rates during the first 14 days after fertilization were affected by
312 fertilization ($F=43.57$, $p<0.001$) and reduced by the inoculation with AMF ($F=8.736$, $P<0.01$)
313 (Fig. 1, Supplementary material Table S3). However, the differences between the +M and -M
314 treatments were significant only in case of urea application without DCD, with N_2O emissions
315 being 46% lower in the +M than in the -M pots (Fig 1; Supplementary material Table S3). The
316 highest N_2O emission rates and the highest differences between -M and +M treatments were
317 observed between 36 and 84 hours after initiation of the fertilization treatments (Fig. 1;
318 Supplementary material Fig. S2 and Table S3). The emissions of N_2O were increased by AMF
319 in the control treatment, but decreased in the case of urea addition when compared to –M
320 (Supplementary material Table S3). Unlike the N_2O, the CO_2 emissions were not affected by
321 the AMF treatment (Fig. 1) and were increased by both urea and urea+DCD application

322 (F=40.65, p<0.001).The cumulative N_2O and CO_2 emissions can be found in Supplementary
323 material Fig. S2.
324
325 **3.2. Plant growth and N uptake**
326 The inoculation with AMF reduced the shoot biomass but increased the root biomass of
327 the *Brachiaria decumbens* Stapf. plants (Table 1). Furthermore, the biomass was significantly
328 higher in the treatments fertilized with urea and urea+DCD (Table 1). The positive effect of
329 AMF on root biomass was detected only when plants were fertilized with urea or urea+DCD
330 (Table 1). The N content of plant shoots was significantly lower in the plants colonized by AMF
331 while the N content of plant roots was increased by the AMF root colonization (Table 1). The
332 fertilizer application increased significantly the shoot N content without differences between
333 urea and urea+DCD (LSD, p<0.05). The negative impact of AMF on the shoot N content was
334 only evident when urea or urea+DCD were applied.
335 The total amount of N in the shoot biomass was higher in the -M pots, particularly when
336 plants were fertilized with urea or urea+DCD (Table 1). However, the root N uptake was higher
337 in the +M pots (Table 1). In addition, no significant differences between urea or urea+DCD
338 (LSD, p<0.05) were found neither in the shoot nor in the root N uptake.
339
340 **3.3. Soil properties and microbial parameters**
341 The presence of AMF did not affect the NH_4^+ in soil, but reduced the content of NO_3^-
342 (Table 2). However, the mineral N contents were strongly affected by the type of fertilizer: the
343 highest content of NH_4^+ was found when urea was applied in combination with DCD while the
344 highest concentration of NO_3^- was detected when urea was applied without nitrification
345 inhibitor. In both cases, higher contents were found after seven days than after 14 days.
346 Furthermore, reduced amount of NO_3- was found in mycorrhizal treatment amended with DCD

when compared the control pots with DCD (Table 2). Urease activity was only affected by sampling time with higher activity after 14 days than after seven days (Table 2).

The MBN was higher in +M than in the -M pots while the MBC was not affected by the presence of AMF (Table 2). However, in urea-amended pots, the MBC was higher in the +M/N than in –M/N. Urea application increased the PNR in the –M/N treatment respect to +M/N (Fig. 2, Supplementary material Table S3).

3.4. Functional genes abundance

The quantification of *amoA* gene copies revealed a higher abundance of AOA than AOB ($5.4x10^5$ number of copies g^{-1} soil of AOB *vs.* $2.87x10^9$ number of copies g^{-1} soil of AOA). The *amoA*-AOA copy numbers were unaffected by the three studied factors (AMF, fertilization and time) (Supplementary material Table S4), while the abundance of *amoA*-AOB was significantly increased by the AMF inoculation and by the application of urea (LSD, $p<0.05$).

4. Discussion

In this study, we demonstrated that the presence of AMF can have a substantial impact on N_2O emissions from tropical grasslands at least shortly after fertilization. Although the effect of AMF on N_2O emissions has been addressed in earlier studies, the majority of researchers focused on N_2O production under rather anaerobic conditions (Bender et al., 2014; Lazcano et al., 2014) while few suggested that the reduced N_2O production rates could be related to the out-competition of slow-growing nitrifiers by AMF hyphae (Storer et al., 2017). In this study, we evaluated the impact of native AMF of tropical grass on N_2O emissions after the application of urea. We hypothesized that the inoculation of *B. decumbens* by AMF would reduce the N_2O release from soil due to reduced amounts of NH_4^+ directly available to AOs and consequent reduced growth of both AOA and AOB.

4.1. Nitrous oxide production pathways

The majority of N_2O is released during nitrification and denitrification processes (Butterbach-Bahl et al., 2013). While denitrification is the dominant N_2O-producing process under oxygen-limited condition (i.e. high moisture content), nitrification can be highly relevant under aerobic conditions (Dobbie et al., 1999). Furthermore, two nitrification-related pathways could be responsible for N_2O emissions: (i) the ammonia oxidation with the importance increasing with raising O_2 concentrations, and (ii) nitrifier denitrification taking place under lower O_2 concentrations (Zhu et al., 2013). Nevertheless, the N_2O production in soil is subject to fluctuations and spatial variability and both processes likely occur simultaneously. Under some circumstances, both nitrification-related pathways can account for important amounts of produced N_2O. For example, nitrification produced between 83 and 95% of total released N_2O in soils ranging between 45 and 50% of WFPS (Huang et al., 2014) while nitrifier denitrification

was responsible for 34-50% of total N_2O at lower O_2 concentrations (Zhu et al., 2013), despite being not considered a strictly anaerobic process (Shaw et al., 2006).

The application of urea had the strongest effect on N_2O production rates whereas the N_2O production after urea+DCD application remained comparable to N_2O production rates in control soil without fertilization. Furthermore, in the urea-amended pots, the AMF strongly suppressed the cumulative N_2O emissions (by 46%). Several studies have demonstrated that AMF interact with soil biota and can influence the N_2O production in the soil (Bender et al., 2014; Lazcano et al., 2014; Storer et al., 2017) and reduce leaching losses (Martínez-García et al., 2017). Under elevated water content, N_2O emission rates were reduced by AMF (when compared to non-mycorrhizal plants) in the study of Bender et al. (2014) and of Lazcano et al. (2014), which the authors related to the reduced abundance of denitrifiers and denitrification rates, and to higher water uptake of AMF plants, respectively. On the other hand, Storer et al. (2017) did not observe any effect of NO_3^- application on N_2O production and strong effect of NH_4^+, indicating that N_2O emissions were released during nitrification, rather than denitrification. In the present study, we observed increased emissions after fertilization with urea and no increase of N_2O production when urea was applied together with DCD, indicating that DCD-suppressed nitrification was the direct reason of lower N_2O emissions, or that lack of NO_3^- in soil solution prevented N_2O release by soil denitrifiers. Storer et al. (2017) suggested that the reduction of N_2O emissions is linked to the reduced abundance of AOs resulting from the superiority of AMF in NH_4^+ uptake and low competitive capacity of AOB (Verhagen et al., 1995).

4.2. The abundance of ammonium oxidizers

Contrary to our hypothesis, the number of *amoA*-AOB gene copies increased in the presence of AMF after urea application (Table 2) while no change was observed in case of AOA, which, however, outnumbered the abundance of AOB by three orders. Similar positive

effect of AMF on AOs has been observed also by Amora-Lazcano et al. (1998). Although AOA and AOB share the *amoA* gene responsible for oxidation of ammonia, it remains unclear whether AOA share the genes required for nitrifier denitrification as in case of AOB (Stieglmeier et al., 2014). Furthermore, it should be pointed out that soil N transformation processes can occur simultaneously and functionally different microbial groups can share by-products (Hu et al., 2015), which makes separation between N_2O-forming pathways challenging. Thus, the increased AOB abundance could lead to increased nitrifier denitrification followed by N_2O reduction by heterotrophic microbes in anaerobic microsites releasing N_2 rather than N_2O. Such situation may occur inside of soil aggregates with high microbial activity resulting from input of high amount of easily decomposable C originated from hyphae exudation or decomposing senescence AMF hyphae. A similar mechanism has also been proposed by Storer *et al.* (2017) suggesting that reduced N_2O emissions are not necessarily reflected in reduced abundance of AOs or PNR activity, but could be caused by increased N_2O consumption as indicated by Domeignoz-Horta *et al.* (2017). Furthermore, the variations of the N_2O emissions has been identified to be dependent on both the activity and diversity of one clade of nosZ (nosZII) encoding the nitrous oxide reductase, the only known N_2O consuming mechanism (Domeignoz-Horta et al., 2017). Thus, especially in a short-term experiment such as the present one, the total abundance of genes is less relevant in N_2O production when compared to the gene expression and the abundance of active nitrifying and denitrifying populations.

4.3. Soil moisture content and nitrification rate

Mycorrhizal hyphae are well known to play a pivotal role in stabilization of soil aggregates (Rillig, 2004) which in turn affects soil-water relations. AMF thin and dense mycelium can penetrate to smaller soil pores which can substantially increase the water uptake of the host plants which can reduce anaerobic conditions (Ruiz-Lozano and Azcon, 1995). Furthermore,

433 in case of positive mycorrhizal effect, plant water uptake can be increased due to higher biomass
434 production. Mycorrhizal effect on water removal from the pots and increasing availability of
435 O_2 was suggested by Bender *et al.* (2014). Also in the study of Lazcano et al. (2014) the
436 emissions of N_2O were decreased by AMF and this drop seemed to be more related to increased
437 use of water than improved N uptake, as AMF plants showed higher photosynthesis and
438 stomatal conductance compared to non-mycorrhizal tomatoes. The changes in soil moisture
439 have substantial effect on greenhouse gases emissions especially at high soil-water content and
440 low O_2 concentrations (Hu et al., 2015). Nevertheless, we did not observe any difference
441 between the moisture of +M and -M pots, possibly due to lower biomass production of AMF-
442 infected plants (Supplementary material Table S5).
443
444 **4.4. Short-term plant-microbe competition and nitrogen immobilization**
445 Nitrification, and, consequently, the N_2O production, depend not only on the abundance
446 and activity of AOs, but also on the supply of NH_4^+ which can be reduced as a result of the
447 AMF and plant N uptake. The role of AMF in plant nutrition has been repeatedly demonstrated
448 (Hodge et al., 2010; Hodge and Storer, 2014) and seems to be dependent on the amount of soil
449 N content. Thus, the presence of the extensive extraradical mycelium of AMF can reduce N_2O
450 emissions by direct immobilization of N within AMF biomass as well as by improved plant N
451 nutrition resulting from N transfer from fungus to the host root. The increased content of MBN
452 in AMF pots and its role in N_2O emissions reduction indicate that short-term immobilization
453 could be the key mechanism of the suppression of N_2O production. Most of the plant
454 growth/nutrient parameters were negatively affected by AMF presence, suggesting that reduced
455 N_2O emissions could be caused by N immobilization in the AMF mycelium rather than in plant
456 biomass, especially during the first week after the fertilization, when the majority of N_2O was
457 released. The competition-related stress could also explain the plant biomass partitioning

towards increased production of roots when compared to aboveground biomass. Furthermore, the percentage of the root colonization with mycorrhizal arbuscules, which are the exchange sites between the host plants and AMF (Gianinazzi et al., 1979) and indicators of actively functioning mycorrhizae, was rather low accounting only for 8.5% of the root area, while the percentage of root colonization by AMF vesicles was 29.7% (Supporting Information Table S2). Vesicles serve as storage organs where AMF accumulate lipids and glycolipids, which could be considered beneficial only to the fungal partner. This high occurrence of vesicles and rather low abundance of arbuscules within the host plant roots can indicate unidirectional benefits, confirming the stress of plants resulting from the competition for N. Although the ability of both plant and fungal partner to up- or down-regulate the intensity of the symbiosis has been observed *in vitro* (Kiers et al., 2011), our understanding of such regulation under realistic field conditions remains limited. Nevertheless, plants may have gained other benefits from the symbiosis even at the cost of slightly reduced biomass production. Long-term studies are required in order to understand the outcome of symbiosis and the implications of the benefits gained by both partners in the long-run for the plant production, N use efficiency and N_2O emissions, as short-term immobilization could result in enhanced N_2O emissions after the senescence of the hyphae.

The allometric biomass allocation has been observed in several studies to be affected by AMF infection, especially in non-stressed plants grown from seeds (Veresoglou et al., 2012) where increased allocation to shoots can be viewed as a sign of improved nutrition resulting from improved nutrient uptake by AMF mycelium. In our case, on the contrary, only root biomass production was increased by AMF infection, suggesting that plants were submitted to stress originating from increased N competition after N amendment, regardless the DCD application. On the other hand, a positive AMF effect (difference in plant N uptake by AMF-plants compared to non-AMF plants) could be expected when AMF are C-limited and plant is

N- (or other nutrient) limited (Corrêa et al., 2015). Thus, after N application to N-limited soils, rapid uptake of N by AMF and growth of mycelium could be expected, but only until C-limitation of fungi. We did not observed any effect of on AMF on root biomass in control treatments (without N fertilization) probably because AMF growth remained N limited and did not induce physiological changes in plants.

Nevertheless, besides microbial N immobilization, the increased root N uptake seemed to be an important driver of N_2O production mitigation. Nevertheless, it has been observed that while plant roots are more successful in acquisition of mineral N in the long-term, soil microorganisms, including AMF, often outcompete plants in the short-term (Kuzyakov and Xu, 2013). Thus, the N immobilization by microbes and AMF may be temporal and plant roots may gain the advantage in the long-run as they compete for the same N each time the microbes and fungi die. Furthermore, the potential improvement of plant nutrition by AMF in the long-term may result in enhanced N uptake by plants and increased biomass production which can contribute to the reduction of N_2O emissions.

5. Conclusions

We investigated the interactive effect of native arbuscular mycorrhizal fungi and urea application on N_2O emissions, plant growth and the abundance of AOs. Furthermore, using DCD nitrification inhibitor we could identify nitrification-related pathway as the source of N_2O emissions in this experiment. The production of N_2O was increased by urea application without DCD and AM pots released only 54% of the N_2O produced in non-AMF pots over a period of two weeks. The negative plant growth response to AMF presence indicated the competition between plants and AMF, which is further confirmed by higher MBN content in AMF pots. Nevertheless, the abundance of *amoA* gene of AOB was higher in mycorrhizal pots when compared to control. Such a short-term immobilization of N in the AMF mycelium and other soil biota can clearly reduce the N supply available for nitrification and subsequent N_2O

production. Nevertheless, this N will likely be released after the senescence of the AMF hyphae and become remobilized and potentially taken up by plants or used by soil microorganisms. Considering the increase of abundance of AOB in AMF-inoculated treatments, the possibility of increased N_2O emissions and the possible changes in competitive ability of mycorrhizal plants in later stages after fertilization or after repeated N addition deserve attention in future experiments.

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Conflict of interest

No conflict of interest has been declared.

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708 **Table 1** Plant biomass production, N content and N uptake. Means are followed by standard
709 error between parentheses (n=4). The outputs Linear Mixed Model are shown at the bottom of
710 the table.

	Shoot biomass	Root biomass	Shoot N content	Root N content	Shoot N uptake	Root N uptake
	(g pot^{-1})		(%)		(mg pot $^{-1}$)	
day 7						
-M/Ctr	15.10 (0.40)	1.67 (0.14)	1.16 (0.04)	1.35 (0.18)	173.9 (2.8)	22.31 (3.20)
-M/N	15.11 (0.50)	1.56 (0.22)	2.37 (0.09)	1.24 (0.06)	357.3 (15.1)	19.33 (3.04)
-M/DCD	14.20 (0.18)	1.23 (0.13)	2.23 (0.11)	1.38 (0.03)	315.2 (12.4)	17.07 (1.84)
+M/Ctr	13.16 (0.10)	1.16 (0.14)	1.24 (0.04)	1.57 (0.08)	163.1 (5.9)	18.37 (2.90)
+M/N	12.50 (1.09)	1.70 (0.12)	2.07 (0.08)	1.44 (0.07)	259.0 (29.1)	24.35 (1.68)
+M/DCD	13.81 (1.07)	1.94 (0.12)	1.89 (0.17)	1.63 (0.07)	264.7 (41.8)	31.37 (0.72)
day 14						
-M/Ctr	15.96 (0.12)	1.67 (0.15)	1.05 (0.05)	1.10 (0.09)	167.1 (7.7)	17.99 (0.35)
-M/N	16.31 (0.42)	1.40 (0.11)	2.29 (0.07)	1.22 (0.08)	372.8 (7.9)	17.12 (1.75)
-M/DCD	16.55 (0.99)	1.80 (0.17)	2.36 (0.05)	1.15 (0.03)	390.2 (22.2)	20.51 (1.93)
+M/Ctr	13.24 (0.48)	1.38 (0.12)	1.31 (0.11)	1.36 (0.03)	171.4 (9.6)	18.83 (1.60)
+M/N	16.08 (0.55)	2.15 (0.20)	2.14 (0.12)	1.42 (0.11)	343.5 (23.0)	30.26 (2.77)
+M/DCD	14.52 (0.31)	1.96 (0.28)	2.03 (0.02)	1.41 (0.14)	294.7 (8.8)	27.31 (3.57)
Effects						
F-value *(p-value)*						
M	21.48 (***)	4.641 (*)	6.673 (*)	18.51 (***)	18.26 (***)	27.47 (***)
F	1.068 (n.s.)	4.771 (*)	167.6 (***)	0.490 (n.s.)	91.23 (***)	5.578 (**)
T	16.85 (***)	5.874 (*)	0.607 (n.s.)	8.647 (**)	9.949 (**)	0.020 (n.s.)
MxF	0.926 (n.s.)	13.63 (***)	9.252 (***)	0.105 (n.s.)	4.016 (*)	11.59 (***)
MxT	0.000 (n.s.)	0.401 (n.s.)	1.154 (n.s.)	0.019 (n.s.)	0.355 (n.s)	0.392 (n.s.)
FxT	2.425 (n.s.)	0.527 (n.s.)	1.037 (n.s.)	1.710 (n.s.)	2.379 (n.s.)	0.518 (n.s.)
MxFxT	2.924 (n.s.)	4.975 (*)	0.267 (n.s.)	0.011 (n.s.)	2.273 (n.s.)	4.425 (*)

711 -M/Ctr no mycorrhiza control; -M/N no mycorrhiza and urea application; -M/DCD no
712 mycorrhiza and urea with DCD application; +M/Ctr arbuscular mycorrhiza control; +M/N
713 arbuscular mycorrhiza and urea application; +M/DCD arbuscular mycorrhiza and urea with
714 DCD application. M mycorrhiza; F fertilizer; T time
715 *, **, *** indicate $p<0.05$, $p<0.01$ and $p<0.001$, respectively
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717

718 **Table 2** Potential urease activity, NH_4^+-N and NO_3^--N content, microbial biomass C (MBC)
719 and microbial biomass N (MBN). Means are followed by standard error between parentheses
720 (n=4). The outputs of general linear model are shown at the bottom of the table.

	Urease*	NH_4^+-N	NO_3^--N	N_{min}	MBC	MBN
				(mg kg^{-1})		
day 7						
-M/Ctr	7.09 (1.08)	1.81 (0.54)	1.04 (0.60)	2.85 (0.78)	90.56 (20.53)	17.32 (2.73)
-M/N	9.24 (0.30)	1.97 (0.64)	18.19 (2.73)	20.16 (2.11)	76.87 (19.77)	16.51 (4.93)
-M/DCD	11.49 (1.65)	15.77 (1.20)	2.04 (0.84)	17.81 (1.67)	101.67 (8.06)	27.16 (3.32)
+M/Ctr	7.81 (0.62)	2.43 (0.84)	1.02 (0.25)	3.45 (0.93)	73.14 (9.71)	25.18 (5.52)
+M/N	8.34 (1.14)	7.39 (2.63)	19.78 (3.63)	27.16 (4.39)	109.0 (17.83)	25.80 (0.27)
+M/DCD	6.66 (1.90)	21.57 (5.34)	0.02 (0.01)	21.60 (5.33)	82.27 (15.75)	28.53 (3.86)
day 14						
-M/Ctr	10.08 (1.25)	2.24 (0.53)	2.59 (1.15)	4.84 (1.39)	95.83 (0.83)	16.47 (6.70)
-M/N	12.03 (2.59)	0.80 (0.36)	5.36 (2.29)	6.16 (2.11)	70.73 (17.46)	20.04 (4.08)
-M/DCD	8.90 (0.47)	5.71 (2.87)	4.41 (0.07)	10.12 (2.90)	103.4 (20.56)	27.31 (5.89)
+M/Ctr	9.76 (0.22)	4.57 (2.62)	0.23 (0.16)	4.80 (2.61)	90.62 (16.47)	24.90 (5.86)
+M/N	12.05 (1.19)	1.74 (0.37)	9.06 (1.78)	10.79 (2.03)	139.17 (8.00)	39.16 (10.78)
+M/DCD	13.22 (2.61)	2.98 (1.21)	1.30 (0.38)	4.28 (1.47)	150.3 (25.28)	18.29 (5.62)
Factor						
F-value *(p-value)*						
M	0.037 (n.s.)	2.371 (n.s.)	6.195 (*)	0.000 (n.s.)	3.432 (n.s)	4.294 (*)
F	1.549 (n.s.)	13.82 (***)	73.38 (***)	21.16 (***)	1.770 (n.s.)	1.286 (n.s.)
T	9.129 (***)	13.55 (***)	0.410 (n.s.)	12.87 (***)	4.187 (*)	0.176 (n.s.)
MxF	0.051 (n.s.)	1.428 (n.s.)	7.997 (***)	2.058 (n.s.)	3.553 (*)	3.126 (n.s.)
MxT	3.138 (n.s.)	0.531 (n.s.)	0.250 (n.s.)	1.080 (n.s.)	4.073 (*)	0.051 (n.s.)
FxT	0.189 (n.s.)	8.243 (***)	14.88 (***)	7.514 (**)	0.661 (n.s.)	1.705 (n.s.)
MxFxT	3.366 (*)	0.443 (n.s.)	3.378 (n.s.)	1.399 (n.s.)	0.681 (n.s.)	0.536 (n.s.)

721 * Potential urease activity (mg NH_4^+-N g^{-1} h^{-1})
722 -M/Ctr no mycorrhiza control; -M/N no mycorrhiza and urea application; -M/DCD no
723 mycorrhiza and urea with DCD application; +M/Ctr arbuscular mycorrhiza control; +M/N
724 arbuscular mycorrhiza and urea application; +M/DCD arbuscular mycorrhiza and urea with
725 DCD application. M mycorrhiza; F fertilizer; T time
726 *, **, *** indicate $p<0.05$, $p<0.01$ and $p<0.001$, respectively
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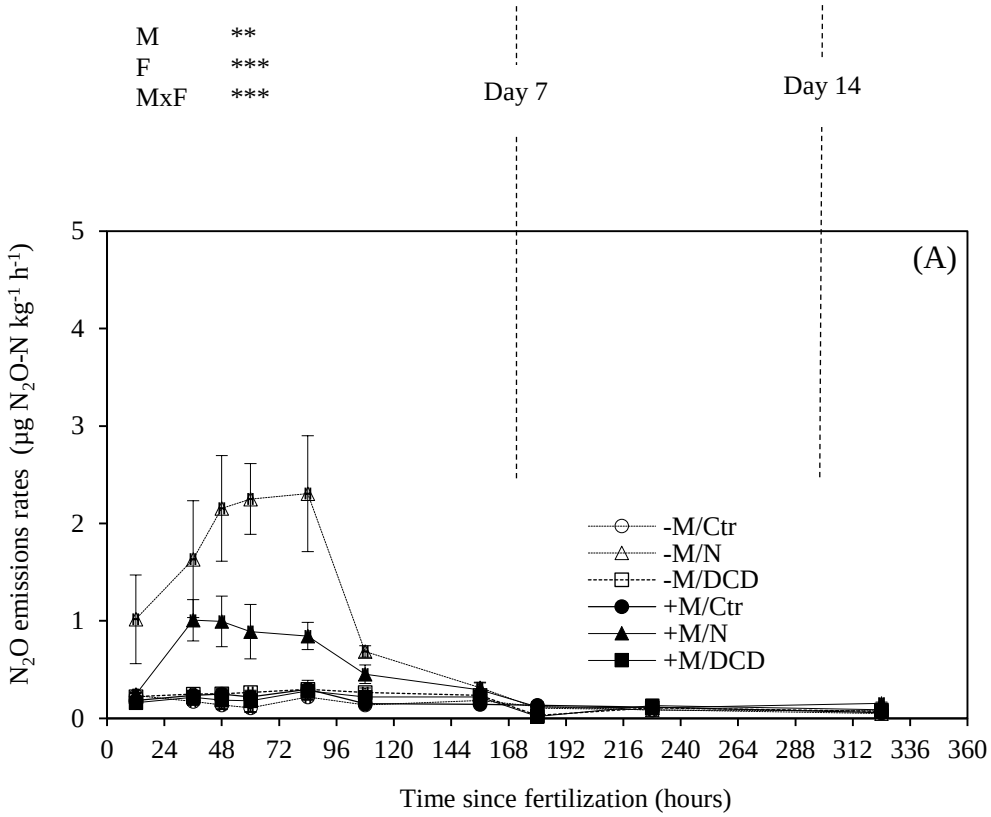

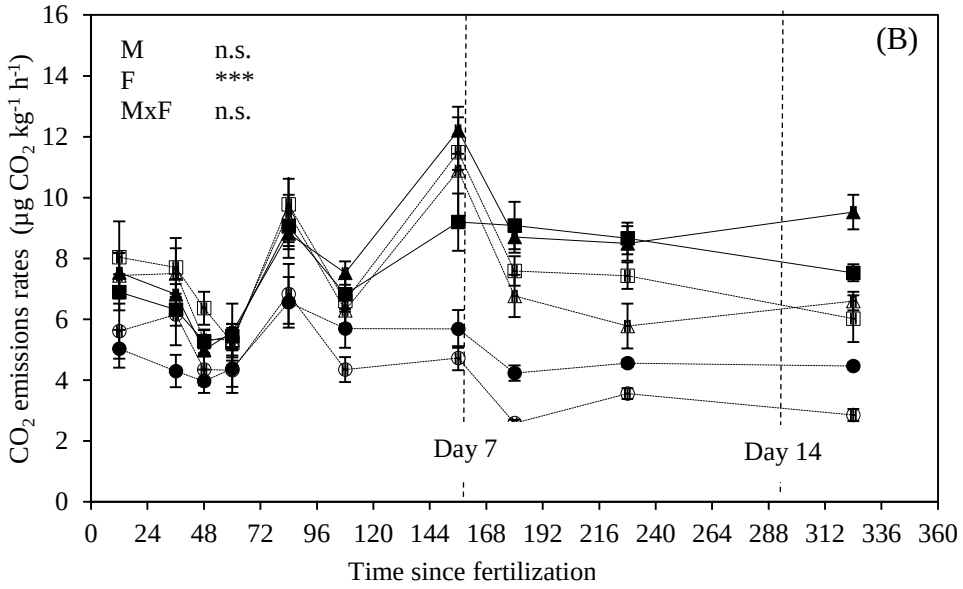

Figure 1 N_2O (A) and CO_2 (B) emission rates from pots planted with *B.decumbens*. Vertical dotted lines indicate times of destructive sampling. Error bars indicate standard errors (n=6). – M/Ctr no mycorrhiza control; -M/N no mycorrhiza and urea application; -M/DCD no mycorrhiza and urea with DCD application; +M/Ctr arbuscular mycorrhiza control; +M/N arbuscular mycorrhiza and urea application; +M/DCD arbuscular mycorrhiza and urea with DCD application. M effect of mycorrhizal inoculation; F effect of fertilizer. *,**,*** indicate statistically significant differences (Linear Mixed Model) at $p<0.05$, $p<0.01$ and $p<0.001$, respectivelly, n.s. not significant.

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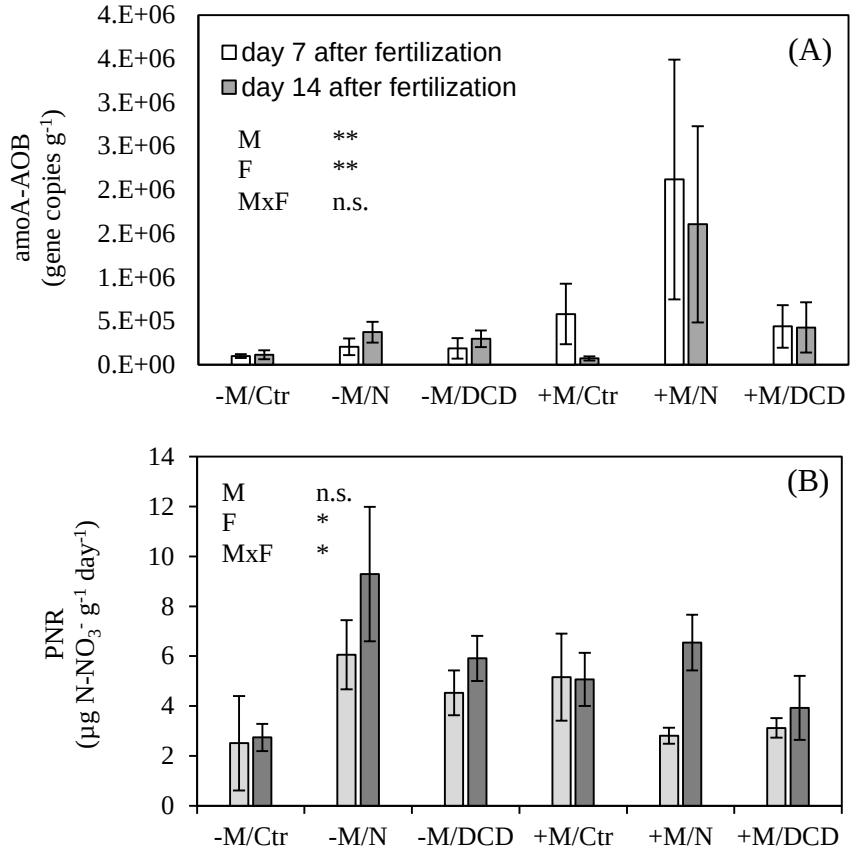

Figure 2 The *amoA* gene abundance of AOB (A) and the potential nitrification rate (PNR) (B). Bars represent standard error of the mean (n=4). –M/Ctr no mycorrhiza control; -M/N no mycorrhiza and urea application; -M/DCD no mycorrhiza and urea with DCD application; +M/Ctr arbuscular mycorrhiza without fertilization (control); +M/N arbuscular mycorrhiza and urea application; +M/DCD arbuscular mycorrhiza and urea with DCD application. M effect of mycorrhizal inoculation; F effect of fertilizer. *,**,*** inidicate statistically significant differences (Linear Mixed Model) at $p<0.05$, $p<0.01$ and $p<0.001$, respectivelly, n.s. not significant.

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