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Arbuscular mycorrhizal fungi reduce nitrous oxide emissions from N_2O hotspots.

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Summary

- Nitrous oxide (N_2O) is a potent, globally important, greenhouse gas, predominantly released from agricultural soils during nitrogen (N) cycling. Arbuscular mycorrhizal fungi (AMF) form a mutualistic symbiosis with two-thirds of land plants, providing phosphorus and/or N in exchange for carbon. Since AMF acquire N, it was hypothesised that AMF hyphae may reduce N_2O production.
- AMF hyphae were either allowed (AMF) or prevented (non-AMF) access to a compartment containing an organic matter and soil patch in two independent microcosm experiments. Compartment and patch N_2O production was measured both before and after addition of ammonium and nitrate.
- In both experiments, N_2O production decreased when AMF hyphae were present prior to inorganic N addition. In the presence of AMF hyphae, N_2O production remained low following ammonium application, but increased in the non-AMF controls. In contrast, negligible N_2O was produced following nitrate application to either AMF treatment.
- Thus, the main N_2O source in this system appeared to be via nitrification and the production of N_2O was reduced in the presence of AMF hyphae. It is hypothesised that AMF hyphae may be out competing slow growing nitrifiers for ammonium. This has significant global implications for our understanding of soil N cycling pathways and N_2O production.

Keywords: agriculture, arbuscular mycorrhizal fungi (AMF), greenhouse gas, hyphosphere, N cycle, nitrification, nitrogen (N), nitrous oxide (N_2O)

Introduction

Agricultural soils are a major source of the globally important greenhouse gas, nitrous oxide (N_2O), a gaseous product of the nitrogen (N) cycle (Singh *et al*., 2010; Hartmann, 2013). In fact, the marked global N_2O atmospheric concentration increases between 1940 and 2005 were predominantly a result of increased use of N-based fertilisers in agricultural systems (S. Park *et al*., 2012). N_2O also has a long pertubation lifetime of 121 years (Hartmann, 2013), thus it is essential that we understand the soil derived fluxes of N_2O as, unlike shorter lived greenhouse gases (e.g. CH_4; Hartmann, 2013), any changes in the atmospheric concentration of N_2O will have long term effects. Consequently, N_2O is viewed as an immediate target to achieve greenhouse gas reductions (Wuebbles & Hayhoe, 2002; Reay *et al*., 2012). However, in order to achieve such reductions, an enhanced understanding of the major sources and sinks of N_2O is urgently required.

In recent years, our understanding of N_2O production in soil systems has significantly improved, mostly as a result of the development of isotopic methods for tracing the sources of N_2O (Baggs, 2008; Kool *et al*., 2011a; Ostrom & Ostrom, 2011). The rate of N_2O production is predominantly controlled by the availability of the inorganic N source (Hino *et al*., 2010), O_2 (Bollman & Conrad, 1998), and other factors that influence microbial activity (e.g. temperature, carbon (C) availability and pH (Bollman & Conrad, 1998; Prosser, 2007; Thomson *et al*., 2012)). In addition, recent evidence has revealed that N_2O reduction is not only confined to denitrifers. Other commonly occurring soil bacteria and archaea may also utilise exogenous N_2O, including under aerobic conditions, even though they lack the preceeding steps in the denitrification pathway (C.M. Jones *et al*., 2014; Sanford *et al*., 2012). Therefore, it follows that the net N_2O emitted from soils will be influenced by the presence of microorganisms.

Arbuscular mycorrhizal fungi (AMF) are a key group of soil microorganisms that form symbiotic associations with the majority of land plants (Smith & Read, 2008). Moreover, it is now widely acknowledged that these fungi play a previously unrecognised role in nitrogen (N) cycling, and can both aquire N for their host plant (Barrett *et al*., 2011; Herman *et al*., 2012) as well as having a substantial N requirement themselves (Hodge & Fitter 2010). There is also evidence for reduced nitrate (NO_3^-) leaching in the presence AMF (Asghari &

Cavagnaro, 2012; Cavagnaro *et al*., 2015; Köhl & van der Heijden 2016). Alongside NO_3^-, a major output of the N cycle is the potent greenhouse gas, N_2O. Therefore, it might be expected that these fungi may influence the availability of N substrates (ammonium (NH_4^+) and NO_3^-) for N_2O production. AMF have been shown to be able to acquire both NH_4^+ and NO_3^-, although it appears they may prefer the more energetically attractive NH_4^+ (Govindarajulu *et al*., 2005; Hodge & Storer, 2015). If these fungi compete effectively with other microorganisms for these inorganic N forms then this could reduce the availability of N substrates for N_2O producers, leading to a reduction in N_2O emissions. There is some circumstantial evidence to suggest this may be the case. For example, Bender *et al*. (2014) found a reduction in N_2O fluxes from soils influenced by AMF colonized roots when compared to soils influenced by roots alone. N_2O fluxes are also reduced when rice plants in draining paddies are arbuscular mycorrhizal (Zhang *et al*., 2015). Collectively, these studies suggest that AMF may alter N_2O emissions in conventional agricultural soils but, thus far, it has not been determined if this is mediated through physiological changes in the AMF colonised roots, or as a direct result of the AMF themselves. If AMF hyphae can directly reduce N_2O production, this could have significant implications for global N_2O production and our understanding of soil N cycling.

AMF hyphae have previously been demonstrated to proliferate in organic matter patches (e.g.; Hodge *et al*., 2001; Hodge, 2014; Barrett *et al*., 2014) and have been shown to take up and transfer N in the inorganic form from these patches to their host plant (Leigh *et al*., 2009; Hodge & Fitter, 2010). The two studies described here followed a similar experimental design to Hodge & Fitter (2010) using dried, milled *Zea mays* L. leaves mixed with an agricultural soil (which had a high N_2O production rate; Storer, 2013), to create organic matter 'patches'. These organic matter patches represent 'N_2O hotspots' which commonly occur in natural systems (Cowan *et al*., 2015). Both experiments tested the hypothesis that AMF hyphae would reduce N_2O production from the organic matter patches, while the second experiment further examined the hypothesis that a reduction in N_2O production was a consequence of reduced nitrification rates in the presence of AMF hyphae.

Materials and Methods

Microcosm design and growth media

To test the hypothesis that N_2O production was reduced in the presence of AMF hyphae, two experiments were established under glasshouse conditions using compartmented microcosm units. Experiment 1 was designed to determine the impact of AMF hyphae on N_2O production, whereas Experiment 2 was designed to determine whether AMF hyphae affected N_2O produced by nitrification and/or denitrification. Organic matter patches were used to create 'hotspots' of N_2O production; a commonly observed phenomenon under natural conditions.

Experiment 1

Microcosm units (Fig. 1a) were constructed by joining two 1 L (each 145 x 145 x 70 mm^3) plastic containers via a double-mesh membrane of either 20 µm (John Stanier & Co., Whitefield, Manchester, UK) or 0.45 µm (Osmonics Inc., Minnetonka, USA) pore size. These size membranes either allowed (AMF) or denied (non-AMF) AMF hyphal access between the two compartments. In all cases roots were prevented from passing between the compartments. There were three 6 mm drainage holes in the base of each compartment. In one compartment (the 'planted' compartment) a single *Z. mays* seedling (Incredible F1, Mr Fothergills, Newmarket, UK) inoculated with *Rhizophagus irregularis* (PlantWorks Ltd., Kent, UK) was placed, whereas the other compartment contained no plant (the 'unplanted' compartment).

Experiment 2

Three compartment microcosm units were used (Fig. 1b). Each microcosm consisted of a central 'planted' compartment (volume: 2 L, dimensions: 150 x 150 x 150 mm; Thumbs Up Ltd., Bury, UK), containing a single *Z. mays* plant inoculated with *R. irregularis*, and on either side of the central planted compartment, two unplanted compartments separated from the central compartment by a nylon mesh membrane as in Experiment 1 (volume: 2.6 L, dimensions: 140 x 140 x 160 mm; Lock & Lock, Australia PTY Ltd., Blacktown, NSW, Australia). The mesh window either allowed AMF hyphal access (AMF; 20 µm mesh) or prevented AMF hyphal access (non-AMF; 0.45 µm mesh) from the central planted to the outer unplanted compartments. A supporting stainless steel mesh (0.25 mm aperture; Mesh Direct, Hanscan Ltd., Burslem, UK) was placed inside the plant compartment over the nylon meshes (0.45 µm and 20 µm) as a precautionary measure to protect the finer meshes from possible root damage. Thus, each unit had one AMF outer compartment and one non-AMF outer

compartment, creating a paired design. The unplanted compartments were covered with a foil layer when the lids were not attached to prevent them from drying out.

Experiments 1 & 2: Growth media

In both Experiments, the planted and unplanted compartments contained a mix (1:1 v/v) of sand and Agsorb® (Agsorb®; Oil-Dri, USA; a calcined attapulgite clay soil conditioner) that had been rinsed thoroughly in deionised water to remove any excess soluble N and/or P. The planted compartments also had 50 g (Experiment 1) or 90 g (Experiment 2) of a fresh *R. irregularis* inoculum (Plantworks Ltd., Kent, UK) and 0.25 g L^{-1} bonemeal (a complex N and P source to encourage mycorrhizal development; 3.5% N, 8.7% P; Vitax, Leicestershire, UK). Three pre-germinated *Z. mays* seeds were added to each planted compartment for both Experiments on 25th June 2012 and thinned to one per pot after 11 d (Experiment 1) or 14 d (Experiment 2). A sterile centrifuge tube (Experiment 1: 15 cm^3; Experiment 2: 50 cm^3) was added to each of the unplanted compartments to create a hole into which the organic matter patches and gas probes could be added at a later date (see 'Organic matter patches and gas probes' section).

Growth conditions

Microcosm units were placed in a randomised block design in a heated, lit glasshouse. The Experiments ran for 78 days between 25th June – 10th Sept (Experiment 1) and 103 days between 25th June – 5th Oct 2012 (Experiment 2). Photosynthetically active radiation (PAR) was measured weekly for both Experiments at plant level in the centre of each block and averaged 141 (Standard Error of the Mean; SEM +/- 15) µmol m^{-2} s^{-1} (Experiment 1) and 251 (SEM +/- 45) µmol m^{-2} s^{-1} (Experiment 2). Overhead lights were used to extend the photoperiod to 16 h per day and the mean daily temperature over the experimental period was 21.9°C (SEM +/- 0.02) (Experiment 1) and 21.5°C (SEM +/- 0.3) (Experiment 2). The planted and unplanted compartments for all microcosm units were watered daily as required. After two weeks of plant growth, the planted compartments received 50 cm^3 of a reduced N and P nutrient solution as described by Leigh *et al*. (2009) once a week (Experiment 1 and 2). This was increased to twice weekly at 49 d after planting in Experiment 2 and to full N at 55 d after planting as the plants were starting to show symptoms of N deficiency. In Experiment 2, at 76 d the plants began to show P deficiency symptoms, so a 3/10th P, full N solution was used once a week in addition

169 to two 1/10th N and P additions. In total the plants received either 1.74 kg N ha^{-1} or 11.97 kg
170 N ha^{-1} in Experiments 1 and 2 respectively over the duration of the Experiments (11 weeks and
171 14 weeks respectively).

172

173 **Organic matter patches and gas probes**

174 *Organic matter patches*

175 Organic matter patch material comprised of 13 g dry weight (DW) equivalent agricultural soil
176 (sandy loam; 53˚92'N, -1˚00'E, pH 6.6 in 0.01M $CaCl_2$ (following Allen, 1974)) mixed with 2
177 g DW milled *Z. mays* leaves, all enclosed in a 20 µm mesh bag (70 mm x 60 mm). The mean
178 C and N content of the mixed organic patches were 1435 mg (SEM +/- 182) and 116 mg (SEM
179 +/- 15) (Experiment 1) or 1200 (SEM +/- 79) and 99 mg (SEM +/- 15) (Experiment 2)
180 respectively, with a C:N ratio of 12:1 in both Experiments. Each patch contained a gas probe
181 (described below) in the centre (Fig. 1c).

182

183 *Gas probes*

184 A stainless steel tube (9 cm long, outer diameter 1 cm, wall thickness 1 mm; Coopers Needle
185 Works Ltd., Birmingham, UK) was welded at the base to form an airtight seal (Fig. 1c). Two
186 diametrically opposed holes, 6 mm, were drilled through each tube 13 mm from the base. These
187 holes were covered in a PVDF membrane (0.2 µm, Bio-Rad Laboratories Inc., Hercules,
188 California, USA) that was air permeable but impermeable to water. This fine PVDF membrane
189 was then housed in a supporting silicone tube (wall thickness 0.8 mm, outer diameter 8 mm;
190 Silex Ltd., Hampshire, UK) with access holes exposing the membrane covering the holes. The
191 stainless steel tube was then sealed at the top with a white rubber Suba-Seal® (No. 13, Sigma-
192 Aldrich, Poole, UK) to form a gas sampling port. The total internal volume of the gas probe
193 was ca. 4.5 cm^3.

194

195 A single organic matter patch and gas probe was placed into the pre-formed holes in the
196 unplanted compartments 2 cm from the mesh window, 7 cm from the surface and covered with
197 sand and Agsorb® media at 29 or 28 d (Experiment 1 or 2 respectively) post-planting.

198

Inorganic nitrogen addition

In Experiment 1, half of the organic matter patches were injected with 7 cm^3 of 30 mM NH_4NO_3 and the other half with 7 cm^3 of deionised water ($n = 6$ in each case) at 44 d after patch addition. Consequently, the treatments were: AMF + NH_4NO_3, AMF + water, non-AMF + NH_4NO_3 and non-AMF + water. In Experiment 2, at 62 d after organic patch addition (90 d after planting) each patch was injected with one of 7 cm^3 of: 15 mM $(NH_4)_2SO_4$ (NH_4 treatment), 30 mM KNO_3 (NO_3 treatment), 15 mM K_2SO_4 (K_2SO_4 treatment) or deionised water (water treatment), where the N treatments were equivalent to 0.196 mg N g^{-1} DW patch ($n = 10$ in each case). In both experiments, two 3.5 cm^3 aliquots of solution were injected into each organic patch with an hour gap between each addition to reduce spread into the surrounding sand/Agsorb®.

Gas sampling and analysis

Experiment 1

The air in the gas probes was sampled before N addition at 44 d post-patch addition. The NH_4NO_3 and water addition treatments were then added and the gas probes were sampled again at 24, 48 and 96 h post-NH_4NO_3 addition. Before sample removal, 1 cm^3 of N_2 was added to the probe via the Suba-Seal®, taking care not to disturb the surrounding media. This was left for 10 s before a 1 cm^3 sample was slowly removed from the gas probe, waiting for a further 5 s to allow the sample to mix inside the syringes before removing the syringe. Each gas sample was then stored in a pre-filled 3 cm^3 Exetainer (with 6 cm^3 N_2), over-pressuring the sample to 7 cm^3 in total. All gas samples were analysed using a gas chromatograph (GC) which quantified the concentration of N_2O. The concentration (ppm) values for each sample were calculated by comparing to certified standards that were diluted in parallel in a 1 cm^3 standard: 6 cm^3 N_2 ratio and correcting for this dilution. The concentration values were also corrected for dilution from addition of N_2 to the gas probe just before gas sample removal.

Experiment 2

Gas sampling was carried out using both gas probes (as described above for Experiment 1) and continuous flow loop sampling with an attached Los Gatos Isotopic N_2O analyser (LGR N_2O; Los Gatos Research, Inc., California, USA) which provided an N_2O concentration once per

second. A gas-tight lid (Fig. 1b) was attached to each of the 80 unplanted compartments in block sequence for a minimum of 5 minutes, with a minimum of 2 min flushing the system with air between each compartment measurement. Gas sampling using both methods was carried out pre-N addition (58-59 and 61 d post-patch addition), and at 48, 96 and 192 h post-N addition (64, 66 and 70 d post-patch addition respectively).

When using the LGR N_2O analyser, the headspace in the microcosm unit (0.6 L), volume of connecting tubing (0.274 L) and internal volume of the N_2O analyser (0.850 L) along with the surface area of the soil sampled (0.024 m^{-2}) were used in the regression calculation of the N_2O flux rate in mg m^{-2} h^{-1}. These fluxes were calculated using values measured between 200 to 280 s after the cover-box lid was attached. All regressions were calculated using SAS (v9.3 SAS institute Inc., North Carolina, USA).

Post-harvest analyses

At harvest, aboveground material was removed at the soil surface and separated into stalk, flowers, ear, and leaf material. Roots were extracted from the sand/Agsorb® media, washed and fresh weight (FW) and DW of all plant material were recorded. In Experiment 1, the dried leaves (green leaves only, defined as > 50% green) were milled and analysed for C and N content using an elemental combustion system (Costech Analytical Technologies Inc., California, USA). The gravimetric water content (g g^{-1} DW) of soil, sand/Agsorb® and patches for each compartment were measured and the AMF extraradical mycorrhizal hyphae (ERM) were extracted from two 5 g (FW) samples from the organic patches and the surrounding growth medium in the unplanted compartments using a modified membrane filter technique (see Staddon *et al*., 2003) and acid fuchsin stain. Hyphal lengths were assessed using the gridline intercept method (Miller & Jastrow, 1992) for a minimum of 50 fields of view at x 125 magnification (using a 10 x 10 grid of 1 cm side lengths, Graticules Ltd. UK). These hyphal lengths were then converted to ERM length densities (m hyphae g^{-1} soil DW).

Data analysis

Data were first tested for normality and equality of variance using Kolmogorov-Smirnov and Levene's equality of variance tests respectively. Statistical analyses were carried out in either

SAS (v9.3 SAS institute Inc., North Carolina, USA) or Genstat (v16 VSN International Ltd., Hemel Hempstead, UK). The pre-N addition fluxes or concentrations were subtracted from the post-N addition fluxes or concentrations respectively to obtain the change in N_2O flux or concentration following N addition (referred to ΔN_2O).

In Experiment 1, where N_2O concentration and ERM length density data did not fulfil normality or equality of variance assumptions they were $\log_{10}$ transformed. All gas concentration, plant and AMF data were analysed using a two-way ANOVA including block, with Duncan's *post hoc* tests. However, transformations on changes in N_2O concentration following N addition failed to normalise the data, and non-parametric equivalent Friedman's two-way ANOVAs, including block, with Wilcoxon *post hoc* tests were used. Where N_2O concentrations were measured over time, repeated measures ANOVA including treatment and block was used on $\log_{10}$ transformed data. Pearson's product moment correlations were used to determine the relationship between variables. Where variables were not normally distributed, Spearman's rank order correlations were used. Untransformed data are presented in all figures.

In Experiment 2, differences among treatments were analysed using a two-way ANOVA including block with Duncan's *post hoc* tests. ERM length density data were $\log_{10}$ transformed before analysis. Where the data failed normality or equality of variance assumptions, non-parametric tests were used. A one-sample t-test or a Wilcoxon Signed Ranks test was used to compare absolute values or differences to zero.

In Experiment 2, the ΔN_2O data were not normally distributed and therefore a Friedman's non-parametric two-way ANOVA, controlling for block with Mann Whitney U (unpaired data) or Wilcoxon signed ranks (paired data) *post hoc* tests and an applied false discovery rate correction was used. Where comparisons in ΔN_2O flux or ΔN_2O concentration data were made over time, a non-parametric Friedman's repeated measures analysis was used. The relationship between the ΔN_2O flux and ΔN_2O concentration for each gas sample following N addition (48 h, 96 h and 192 h post-N addition) was determined using a Spearman's rank order correlation.

There was hyphal breakthrough in one of the non-AMF compartments (treatment: non-AMF, K_2SO_4) therefore this microcosm was excluded from the subsequent data analyses. In addition, the N_2O concentration for one experimental unit in the AMF treatment (treatment: $(NH_4)_2SO_4$) was out of range on the GC at the 48 h post N addition sample and therefore these AMF and non-AMF N_2O concentration values were also omitted.

Results

In the AMF treatments, *R. irregularis* colonized the organic matter patches successfully in both experiments with ERM length densities of 1.23 ± 0.25 m g^{-1} DW in Experiment 1 (non-AMF: 0.31 ± 0.05 m g^{-1} DW; $F_{1,12} = 30.77$, $P = 0.0001$) and 0.88 ± 0.08 m g^{-1} in Experiment 2 (non-AMF: 0.35 ± 0.04 m g^{-1} DW; $t_{39} = 8.993$, $P < 0.0001$).

Pre-N addition N_2O production

Prior to inorganic N addition there was a greater concentration of N_2O in the non-AMF patches than in the AMF patches in both experiments (Fig. 2; Experiment 1: $F_{1,12} = 6.46$, $P = 0.026$; Experiment 2: $S_{38} = -186$, $P = 0.0076$). A similar trend (at the $P < 0.1$ level) was found for the N_2O fluxes in Experiment 2, with greater N_2O fluxes measured from the non-AMF compartments than from the AMF compartments ($S_{38} = -128$, $P = 0.074$). In Experiment 2, N_2O fluxes measured by continuous flow loop sampling were positively correlated with the patch N_2O concentrations measured using gas probes ($r_s = 0.7495$, $P < 0.0001$). Since N_2O production is inherently variable, this level of consistency both between and within experiments is striking, particularly because it was observed in the absence of any additional applied inorganic N. In both experiments, there was no significant correlation between the pre-N addition AMF treatment N_2O concentration or fluxes and the ERM lengths ($P > 0.05$ in each case).

Post N-addition and harvest

Experiment 1

In Experiment 1 the highest patch N_2O concentrations were observed 24 h after the application of inorganic N or water in all treatments except AMF + water, demonstrating the

rapid response of N_2O producers to treatment application. The patch N_2O concentrations of the non-AMF and AMF + NH_4NO_3 treatments subsequently decreased over time. In contrast, the AMF + water patch N_2O concentration remained low. Consequently, there was a significant effect of both time and treatment on patch N_2O concentration in addition to a significant interaction between these two factors (Fig. 3; time: $F_{2,30} = 4.37$, $P = 0.023$; treatment: $F_{3,15} = 5.67$, $P = 0.0084$; time*treatment: $F_{6,30} = 3.23$, $P = 0.015$). These results therefore demonstrate how rapidly N_2O production rates can change over time and emphasise the requirement for repeated measurements following inorganic N application. Two-way ANOVAs at each time point showed that the N_2O concentration of the AMF + water treatment was lower than all other treatments at 24 h post-treatment application (Fig. 3; $F_{3,15} = 4.44$, $P = 0.020$). This effect decreased by the 48 h sample, although the non-AMF + water and non-AMF + NH_4NO_3 treatments still had a higher N_2O concentration than that of the AMF + water treatment ($F_{3,15} = 4.95$, $P = 0.014$). At 96 h post-treatment application, the AMF patch N_2O concentrations were not significantly different from each other but were significantly lower than those of the non-AMF patches ($F_{3,15} = 7.25$, $P = 0.0031$). At 24 h post-treatment application, the ΔN_2O concentration was higher in both the AMF + NH_4NO_3 and non-AMF + NH_4NO_3 treatments than the AMF + water treatment ($Q_3 = 8.2$, $P = 0.042$). However, the non-AMF + water treatment was not significantly different from the AMF + NH_4NO_3 treatment or non-AMF + NH_4NO_3 treatment.

There was no relationship between the AMF ERM length densities and N_2O concentration in the AMF patches at any point ($P > 0.05$ in each case) and the moisture contents of the organic patches did not differ among treatments at harvest ($Q_3 = 0.05$, $P = 0.827$). Additionally, there was no significant difference ($P > 0.05$) in total plant DW or the DW of the various plant tissues (i.e. leaf, total shoot, stalk, total root, root weight ratio, tassel) between the AMF and non-AMF treatments (see Supporting Information Table S1). Neither the addition of NH_4NO_3 or water had any effect on the leaf C and N content or concentrations or the C:N ratios ($P > 0.05$ in each case), therefore, these data were combined for comparison of the AMF versus the non-AMF treatments. Leaf C content did not differ between AMF and non-AMF plants (Table 1; $F_{1,12} = 0.30$, $P = 0.595$), although the leaf C concentrations were lower in the AMF compared to the non-AMF treatments (Table 1; $F_{1,12} = 5.37$, $P = 0.039$). Both the N content (Table 1; $F_{1,12} = 14{,}18$, $P = 0.0023$) and concentration ($F_{1,12} = 20.06$, $P = 0.0008$) of the leaves were higher in the AMF compared to the non-AMF treatments. Consequently, the C:N

353 ratio of the leaves was lower in the AMF compared to the non-AMF treatments (Table 1; $F_{1,12}$
354 = 18.51, P = 0.001). However, the organic patch N_2O concentration was not significantly
355 related to the leaf C or N content or concentration, or to the leaf C:N ratio, either before or
356 after N addition, for both the AMF and non-AMF treatments ($P > 0.05$ in each case).

357

358 ***Experiment 2***

359 There was a significant difference in ΔN_2O fluxes among the inorganic N and water
360 application treatments at 48 h post-application (Fig. 4; Q_7 = 44.85, $P < 0.0001$). In both the
361 AMF and non-AMF patches, more N_2O was produced following addition of NH_4^+ than any
362 other treatment. Strikingly, however, ca. 2.5 times more N_2O was produced from the non-
363 AMF than the AMF treatment (Fig. 4; S_9 = -26.5, P = 0. 0084). These differences then
364 declined by the 96 h sample and were no longer significant at the 192 h sample, again,
365 illustrating the transient nature of N_2O release and the importance of following the fluxes over
366 discrete timescales (Table 2). There was no significant difference in the % moisture content of
367 either the patch or sand/Agsorb® medium between the AMF and non-AMF treatments at
368 destructive harvest (patch: t_{39} = -0.26, P = 0.799; sand/Agsorb®: S_{39} = -47, P = 0.519).

369

370 **Discussion**

371 This is the first study to show that N_2O production is reduced as a direct consequence of the
372 presence of AMF hyphae. Moreover, this reduction was demonstrated in both the presence
373 and, notably, the absence of applied inorganic N, indicating that this is a persistent effect.
374 Studies to date have indicated that AMF may influence soil N_2O production, but this has
375 always been in the presence of plant roots and additional inorganic N (Lazcano *et al*., 2014;
376 Bender *et al*., 2015). Critically, the finding that N_2O production was reduced when AMF
377 hyphae, but not plant roots, were present was consistent between the two independent
378 experiments reported here.

379

380 Previous studies have applied inorganic N and assessed the N_2O flux from the
381 mycorrhizosphere (i.e. the soil influenced by AM colonised roots and AMF hyphae), often
382 only at a single time point after N application, potentially masking cumulative effects (Bender
383 *et al*., 2015). AMF hyphae can extend far beyond the plant roots alone, with the ERM being

10 times larger, in biomass terms, than the intraradical mycelium (Olsson *et al*., 1999). Thus, the influence of AMF hyphae on soils (in the 'hyphosphere') will extend beyond the zone of influence of roots alone, and studies to date have not explored this widespread zone of hyphal influence on N_2O production in isolation. Furthermore, since the mycorrhizosphere includes both AMF colonised plant roots and AMF hyphae, it is impossible to know whether any effect is a consequence of the AMF hyphae, roots, or both. Rhizodeposition differs between AM and non-AM plants (D.L. Jones *et al*., 2004), while C exudation from AMF hyphae may also result in quantitative and qualitative changes in the total C flux into the soil (Toljander *et al*., 2007). Moreover, AMF hyphae influence N cycling through the capture of N and subsequent transfer of at least some of this N to their associated host plant (Leigh *et al*., 2009; Thirkell *et al*., 2016). C and N are key controls of denitrification and nitrification rates (Bollman & Conrad, 1998; Hino *et al*., 2010). It is not possible, therefore, to separate AMF and root control of N_2O fluxes in the mycorrhizosphere without first separating the AMF hyphae from the plant roots.

Nevertheless, there is some evidence of AMF interacting with soil N_2O production in the mycorrhizosphere, although results have been inconsistent. Bender *et al*., (2015) found that the N_2O flux was lower following the application of NO_3^- in the AM mycorrhizosphere when compared to the rhizosphere of a non-AM control. In contrast, Cavagnaro *et al*., (2012) found no effect of AM plants on N_2O production, whereas Lazcano *et al*., (2014) found a reduction in N_2O in the mycorrhizosphere of AM plants. Thus, there is support for AMF resulting in reduced N_2O production in the mycorrhizosphere, but the cause of this reduction has so far been poorly understood, likely because of confounding effects of the host plant root system also being present. Hypotheses for the decreased N_2O production in the mycorrhizosphere included a reduction in denitrification (Bender *et al*., 2015), and increased water use by AM plants (Lazcano *et al*., 2014).

In this study, the finding of reduced N_2O production in the presence of AMF hyphae was evident even prior to inorganic N application. There was also evidence for an increase in both leaf N content and concentration when the AMF had access to the organic matter patches. This suggests that the AMF were supplying their host plant with additional N, presumably from the organic matter patch since all planted compartments received the same quantity of

nutrient solution. Whilst there is a wide range in reported contribution of AMF to plant N (reviewed by Hodge & Storer, 2015), the findings in this study are in agreement with previous investigations using ^{15}N that substantial quantities of N can be transferred from the patch to the plant via AMF hyphae (Leigh *et al*. 2009; Thirkell *et al*. 2016).

The inorganic N applications here were used as a tool to identify the pathway of N_2O production being influenced by the AMF hyphae. The addition of NO_3^- did not result in increased N_2O production from any treatment, suggesting that in this study denitrification was not a key factor in controlling N_2O production. There was also no significant difference in gravimetric water content of the organic matter patches, or the surrounding sand/Agsorb® medium at harvest. Thus, these factors were not important controls of N_2O production in the present study. Instead, we found direct evidence for a reduction in N_2O produced via nitrification in the presence of AMF hyphae. This is a critical finding and may help to explain variable N_2O fluxes under field conditions. Since one of the largest sources of N_2O are agricultural soils, it is highly relevant that the soil used here was agricultural in origin, and the plant material for the organic matter patches was *Z. mays*, a globally important crop (Leff *et al*., 2004).

The soil N_2O fluxes in this study were predominantly controlled by the availability of NH_4^+. These fluxes were monitored at intervals up to 192 hours after inorganic N application by which point the N_2O peak declined back to pre-N application levels, thus ensuring that the full response period was recorded. There was a significantly greater N_2O flux in response to NH_4^+ addition in the non-AMF than in the AMF treatment, indicating reduced N_2O production via nitrification in the presence of AMF hyphae. The current understanding of the main pathways of N_2O production in soils (as described in Baggs, 2011, Zhu *et al*., 2013), are shown in Fig. 5 together with the potential mechanisms by which AMF may interact with N_2O production. If NH_4^+ elicits N_2O production but NO_3^- application does not, by process of elimination the pathway involved in N_2O production must be a nitrification pathway.

The links between AMF presence and reduced nitrification rates are in broad agreement with a series of one field based and three mesocosm based studies by Veresoglou *et al*. (2011). The

potential nitrification rates (PNR) were lower in the mycorrhizospheres of AM plants compared to those from weakly-AM mycorrhizospheres (Veresoglou *et al*., 2011). The *nirK* gene, responsible for N_2O production has also been shown to be negatively correlated with AMF abundance (Bender *et al*., 2014). Thus, the presence of AM plants may reduce N_2O production by reducing nitrification rates. Our present study demonstrates, for the first time, that AMF hyphae have a direct and limiting influence on soil N_2O produced via nitrification, independent of any plant root influence.

The main 'nitrification' pathways in soil potentially resulting in N_2O release are nitrifier nitrification, and nitrifier denitrification. Nitrifier nitrification is an aerobic process and can be carried out by ammonia oxidising bacteria (AOB), archaea (AOA), and organsims capable of complete ammonia oxidation (comammox) (Daims *et al*., 2015; van Kessel *et al*., 2015). AOB and AOA have also been shown to produce N_2O (Jiang and Bakken, 1999; Jung *et al*., 2014). Nitrifier denitrification is also carried out by autotrophic nitrifiers, and can be a significant source of N_2O (Kool *et al*., 2011b; Wrage *et al*., 2001). Thus, there are various pathways by which the N_2O in this study may have been produced following the application of NH_4^+ and consequently reduced by the presence of AMF hyphae (Fig. 5).

Regardless of the process, the response to NH_4^+ application in the AMF treatments suggests that there was either a reduction in N_2O production, through reduced function or number of nitrifiers, or that nitrifier activity was masked by an increase in activity of N_2O reducers which can cause some soils to become N_2O sinks (Domeignoz-Horta *et al*., 2017). It's also feasible that the presence of AMF hyphae modified the microbial community shifting it away from N_2O producing nitrifiers or nitrifier denitrifiers, perhaps towards organisms capable of complete nitrification (van Kessel *et al*., 2015), or N_2O reduction (C.M. Jones *et al*., 2014; Sanford *et al*., 2012; Domeignoz-Horta *et al*., 2017).

Domeignoz-Horta *et al*. (2017) found that N_2O hotspots were predominantly controlled by changes in the microbial communities, whereas lower N_2O producing areas were more likely to be controlled by variation in soil properties. Using similar organic patches as in the present study, Nuccio *et al*. (2013) found that while there was no overall change in bacterial diversity,

the presence of AMF hyphae significantly modified the bacterial community. Interestingly, Gemmatimonadetes and Deltaproteobacteria were two of four bacterial phylum that had a higher relative abundance in response to the presence of AMF hyphae in the litter (Nuccio *et al*., 2013). Both the Gemmatimonadetes and Deltaproteobacteria have subsequently been found to posses *nosZ* genes, and can, thus, utilise exogenous N_2O as an electron acceptor (C.M. Jones *et al*., 2013; D. Park *et al*., 2017). AMF abundance has also been found to positively correlate with *nosZ* gene abundance (Bender *et al*., 2014). This together with the large export of N from the patch by the AMF hyphae and the resulting modifications in the physico-chemical environment in the decomposing litter patch may all contribute to a reduction in N_2O emissions.

Given the evidence that AMF are known to have a high N demand (Hodge & Fitter, 2010), one hypothesis may be that AMF hyphae were eliciting a longer term control on the nitrifying community as nitrifiers are inhereantly slow growing, taking from 8 h up to a number of days to double in number (Belser & Schmidt, 1980; Woldendorp & Laanbroek, 1989; Prosser, 2007; Prosser & Nicol, 2012). AMF hyphae are thought to predominantly take up inorganic N in the form of NH_4^+ (Govindarajulu *et al*., 2005; Tanaka & Yano, 2005), and AOB are generally thought to be poor competitors for NH_4^+ (Verhagen *et al*., 1995; Bollmann *et al*., 2002). The AMF hyphae may therefore have reduced the amount of available NH_4^+ in the hyphosphere resulting in a reduction in the population of active AOB. If AOB were the main N_2O producers, this may explain the reduced N_2O production prior to inorganic N application when the AMF hyphae were present. It may also explain the lack of N_2O production in the presence of AMF hyphae when NH_4^+ was applied, i.e. the AOB population may have been small and too slow-growing to respond to the inorganic NH_4^+ supplied, which may have, instead, been taken up by the N-rich AMF hyphae.

While AMF may increase or decrease the pH of surrounding media, thought to be a consequence of NO_3^- or NH_4^+ uptake respectively (Bago *et al*., 1996; Li *et al*., 1991), the relative importance of pH effects on N_2O production if C, NH_4^+ or NO_3^- are limiting are not clear (reviewed by Šimek & Cooper, 2002) with both increased and decreased nitrification-derived N_2O production reported under low pH conditions (Y. Cheng *et al*., 2013; Mørkved *et al*., 2007). The patch pH was not measured in this study, and potential changes in pH cannot

be fully discounted. However, the implications of N, and more importantly the form of N, exported by AMF on the local physico-chemical properties including pH, warrants more attention. This may also aid in explaining the differing impacts reported for AMF on decomposition processes, and their importance not only for N, but also C cycling and stabilization processes (Hodge, 2001; Hodge *et al*., 2001; L. Cheng *et al*., 2012).

In order to fully understand the mechanism for the reduction in N_2O production via nitrification observed in the presence of AMF hyphae found in this study, further research should focus upon gene expression and the responses of the microbial community including nitrifier communities, AOA, AOB and potential non-denitrifying N_2O reducers. Monitoring would also help to establish if nitrifier populations were suppressed by the presence of AMF hyphae, as we suggest. Furthermore, field based studies using a wider range of soil types and environmental conditions are an essential next step to determine the global scale and significance of this interaction in both natural and agricultural systems.

In conclusion, using two independent glasshouse based experiments, we have found that the presence of AMF hyphae reduced the production of the globally important greenhouse gas, N_2O. Cropped agricultural soils cover a significant proportion of land area, representing 28.4% of agricultural land, or 10.9% of the total global land area in 2011 (FAO, 2017). The diversity of AMF is reduced in agricultural soils (Helgason *et al*., 1998), and these soils are one of the largest contributors to N_2O emissions. This study suggests that a reduction in AMF presence may contribute to further increases in N_2O production. This could have significant implications for better management of agricultural soils in the future. Given the ubiquity of the AM association, including under agricultural situtations, these findings have global implications not only for our fundamental understanding of the mechanisms of soil N cycling, but also for greenhouse gas management and climate change mitigation.

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Author Contribution

K.S., P.I. and A.H. designed the research; K.S. performed the research and conducted all data analysis, A.C. performed practical work for Experiment 1. K.S. and A.H. wrote the manuscript.

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735 **Table 1.** Mean leaf N and C total content, concentration and C:N ratio of leaves from AMF and non-AMF
736 treatments in Experiment 1 ± standard error of the mean (n = 12). Different letters within rows represent
737 significant differences at P = 0.05 (in bold) as determined using two-way ANOVAs.

		AMF	Non-AMF
Leaf N	Total content (mg)	**13.8 ± 0.8^{a}**	**10.2 ± 0.9^{b}**
	Concentration (mg g^{-1} DW)	**11.3 ± 0.6^{f}**	**8.8 ± 0.5^{g}**
Leaf C	Total content (mg)	503.2 ± 19.9^{j}	488.1 ± 27.2^{j}
	Concentration (mg g^{-1} DW)	**413.4 ± 2.8^{m}**	**422.9 ± 3.7^{n}**
	Leaf C:N ratio	**37.6 ± 2.0^{x}**	**50.0 ± 3.0^{y}**

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Table 2. Experiment 2 Friedman's test statistics controlling for block comparing the post-N minus pre-N (61 days post-patch addition) patch N_2O concentrations (ΔN_2O concentrations) or compartment N_2O fluxes (ΔN_2O fluxes) among N addition treatments, for each of the gas sampling events.

	Time since N addition								
	48 h			96 h			192 h		
	Q	df	*P*	*Q*	df	*P*	*Q*	df	*P*
Patch ΔN_2O concentration	28.89	7	**0.0002*****	14.35	7	**0.045***	3.79	7	0.804
Compartment ΔN_2O flux	44.85	7	**<0.0001*****	25.63	7	**0.0006*****	4.80	7	0.684

Q = Friedman's test statistic, df = degrees of freedom, *n* = 10. Significant results are indicated in bold at *P* = 0.05 (**P* < 0.05, ***P* < 0.01, ****P* < 0.001).

Figure & Table Legends

Fig. 1 The microcosm units used in Experiments 1 (a) and Experiment 2 (b) and the organic matter patches and gas probes used in both experiments (c). In Experiment 1 the planted compartment was planted with a single *Zea mays* plant and contained the AMF inoculum whilst the unplanted compartment either allowed or prevented AMF hyphal access. In Experiment 2 the central compartment was also planted with a single *Z. mays* plant and contained the AMF inoculum. From the central, planted compartment, the AMF hyphae could access one outer, unplanted compartment (AMF) but not the other (non-AMF). The gas probe was placed within a mesh bag (the 'organic matter patch') which contained a mix of dried, milled *Z. mays* leaves and agricultural soil (c). The gas probe and organic matter patch designs were used in both experiments.

Fig. 2 Mean N_2O concentration (ppm) in AMF access (AMF) and no AMF access (non-AMF) organic matter patches (a) at 43 d post-patch addition in Experiment 1 and (b) at 58 d post-patch addition in Experiment 2. Error bars represent ± standard error of the mean (a: $n = 12$, b: $n = 39$). Different letters represent significant differences at $P < 0.05$ as determined using (a) two-way ANOVAs and (b) by comparing the ΔAMF value to zero (Wilcoxon Signed Rank test).

Fig. 3 Mean patch N_2O concentration at 24, 48 and 96 h following inorganic N (NH_4NO_3: filled symbols) or water (open symbols) addition for AMF access patches (AMF; solid lines) and no AMF access patches (non-AMF; dashed lines) shown over time. Error bars represent ± standard error of the mean ($n = 6$). Asterisks represent a significant difference among treatments within each sample period (*$P < 0.05$, **$P < 0.01$) as determined using a two-way ANOVA. Different letters within each sample timing represent significant differences between treatments for that sample timing ($P < 0.05$).

Fig. 4 Mean difference between 48 h post-N addition (64 d post-patch addition) and pre-N addition (61 days post-patch addition) N_2O flux (ΔN_2O flux) for AMF access (AMF; solid bars) and no AMF access (non-AMF; open bars) treatments, split by N addition treatment. The N addition treatments were: $(NH_4)_2SO_4$ (labelled as NH_4), KNO_3 (labelled as NO_3), K_2SO_4 or

778 water. Bars with different letters are significant at $P = 0.0018$ as determined using Mann
779 Whitney U or Wilcoxon signed rank *post hoc* tests with a False Discovery rate correction
780 applied. Asterisks below the bars indicate significant differences to zero (*$P < 0.05$, **$P <$
781 0.01). Error bars are ± standard error of the mean ($n = 10$).

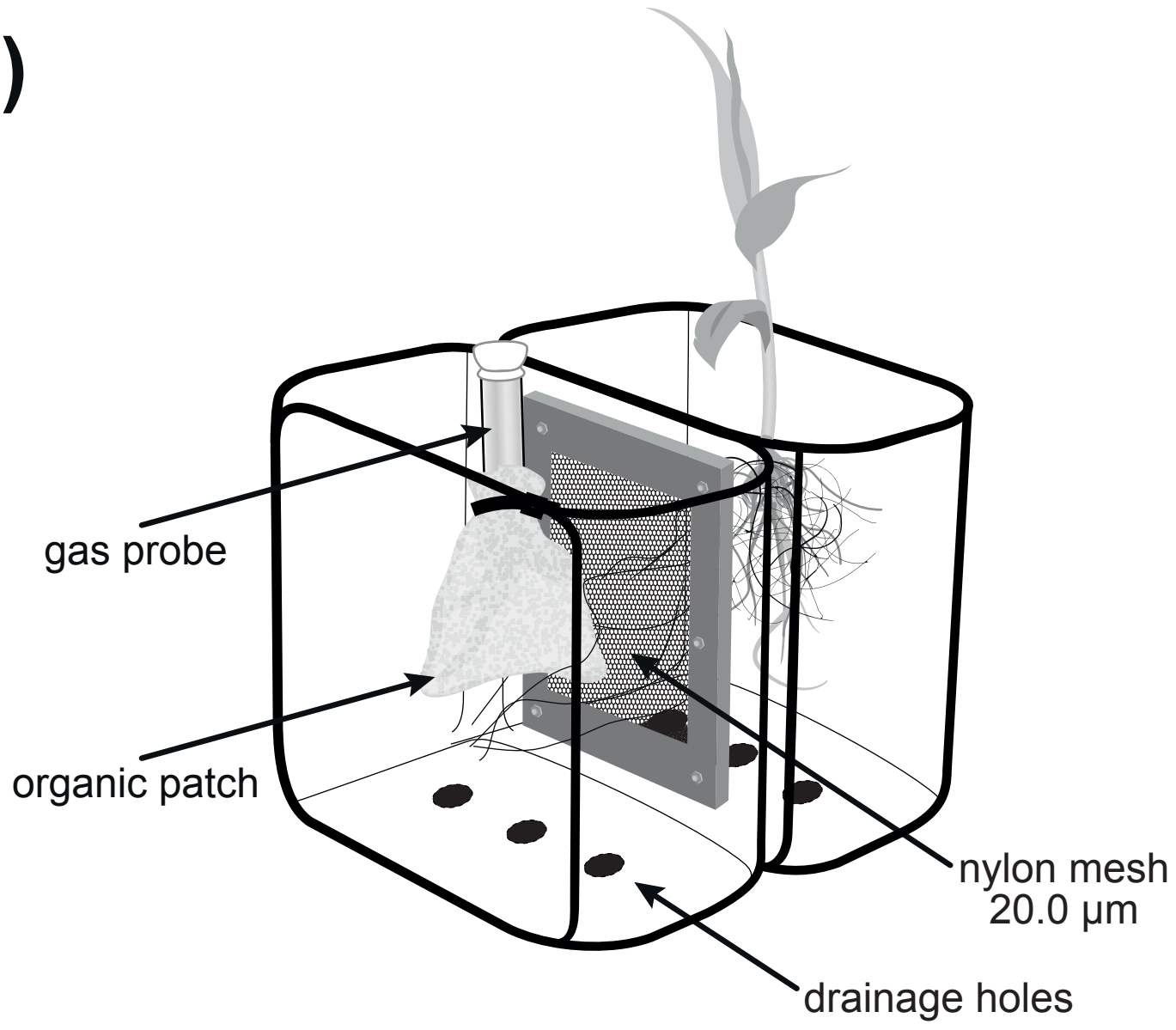
782

783 **Fig. 5** Summarized potential interactions between AMF hyphae and soil N_2O producing
784 processes as described in Baggs (2011) and Zhu *et al*. (2013). The solid and dashed bold lines
785 represent AMF effects that could result in an increase or decrease in N_2O production
786 respectively. AMF can affect the availability of nitrogen (N), phosphorus (P), copper (Cu)
787 and iron (Fe) in soils as well as potentially change soil pH. Nitrifier nitrification is generally
788 carried out by ammonia oxidising bacteria (AOB) and archaea (AOA). Dissimilatory
789 reduction of nitrate to ammonium (DNRA) may produce N_2O as a side product. DNRA is
790 also known as nitrate ammonification. There are various pathways and organisms capable of
791 carrying out these roles, but for simplicity, they are grouped by factors affecting the rate of
792 N_2O production (i.e. availability of O_2, or carbon (C)).

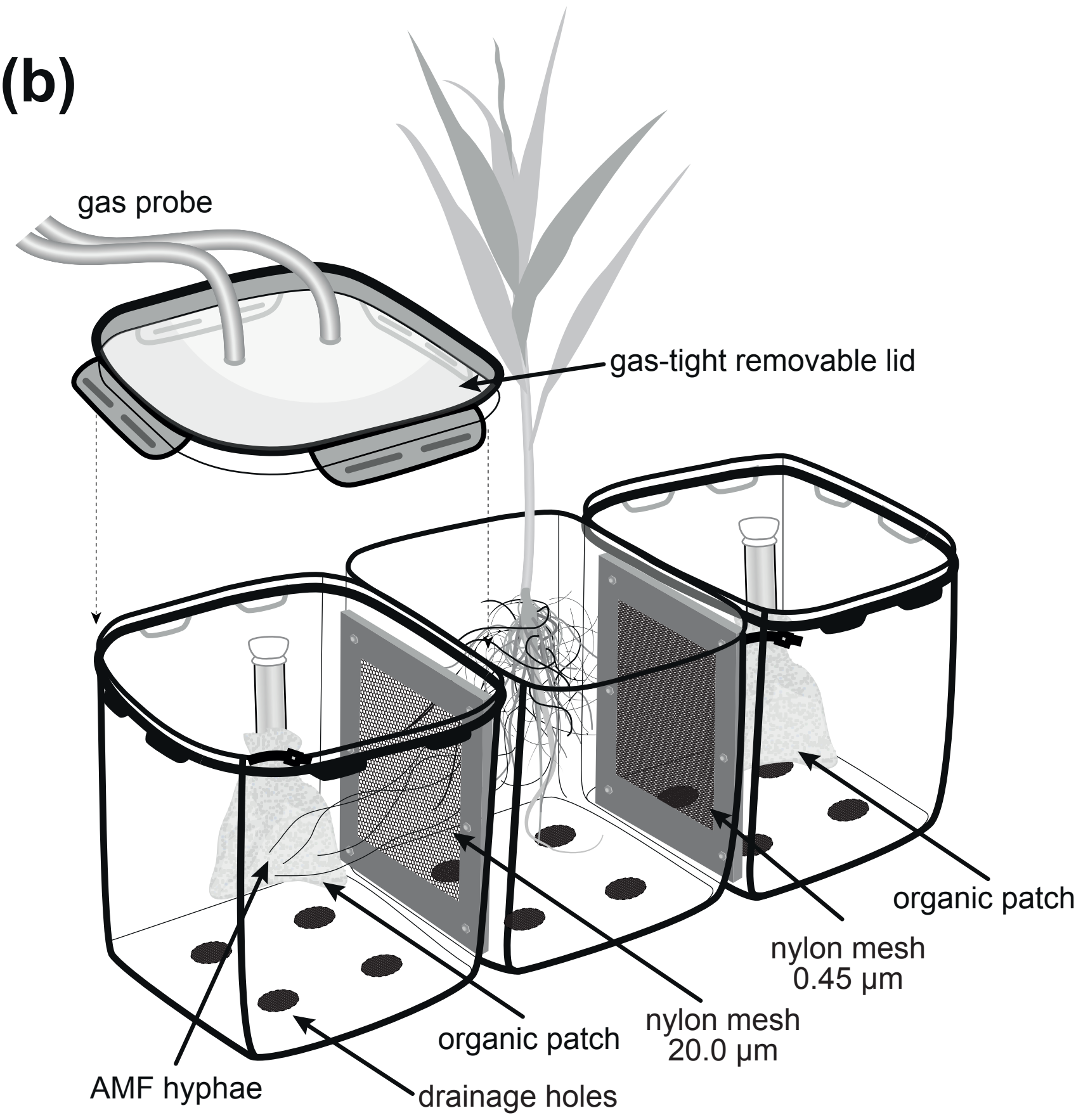
793

794 **Supporting Information Table S1.** Mean plant biomass parameters from AMF and non-AMF
795 treatments in Experiment 1.

(a)


gas probe
organic patch
nylon mesh
20.0 µm
drainage holes

(b)


gas probe
gas-tight removable lid
organic patch
nylon mesh
0.45 µm
nylon mesh
20.0 µm
organic patch
AMF hyphae
drainage holes

(c)

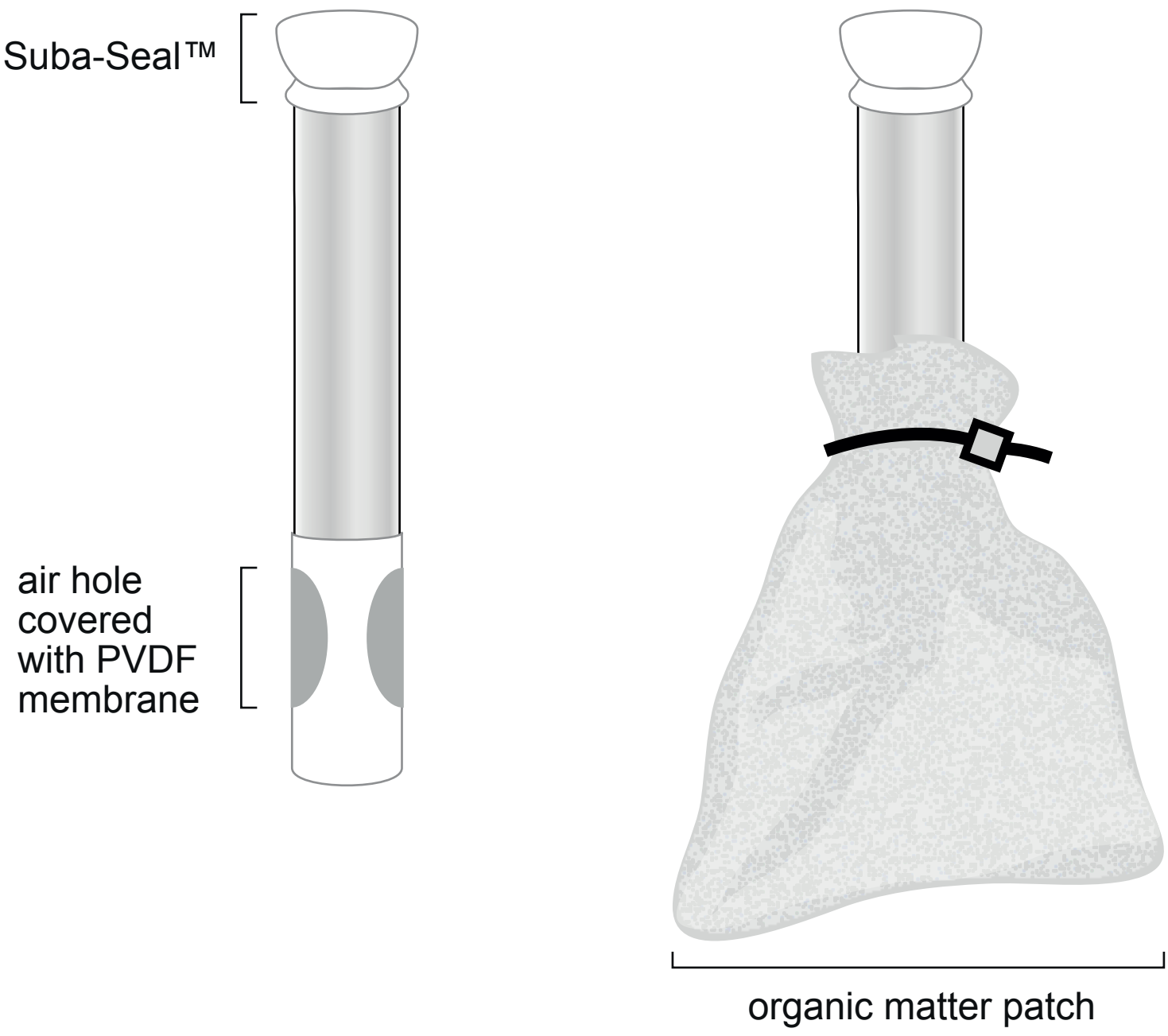

Suba-Seal™
air hole
covered
with PVDF
membrane
organic matter patch

Figure 2

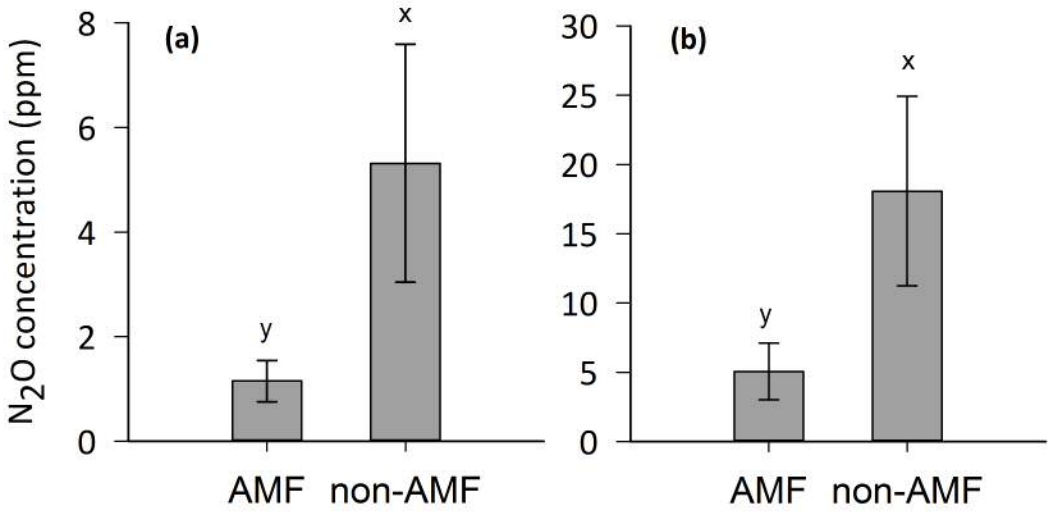

(a)
(b)
N_2O concentration (ppm)
8
6
4
2
0
30
25
20
15
10
5
0
x
y
x
y
AMF
non-AMF
AMF
non-AMF

Figure 3.

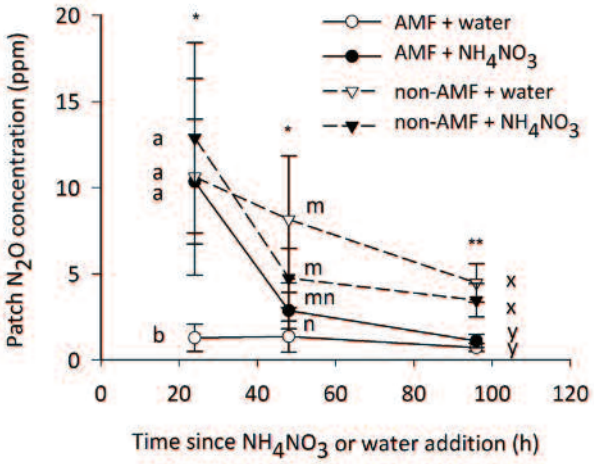

Patch N_2O concentration (ppm)
Time since NH_4NO_3 or water addition (h)
AMF + water
AMF + NH_4NO_3
non-AMF + water
non-AMF + NH_4NO_3
0
5
10
15
20
0
20
40
60
80
100
120
a
a
b
m
m
mn
n
x
x
y
y
*
*
**

Figure 4.

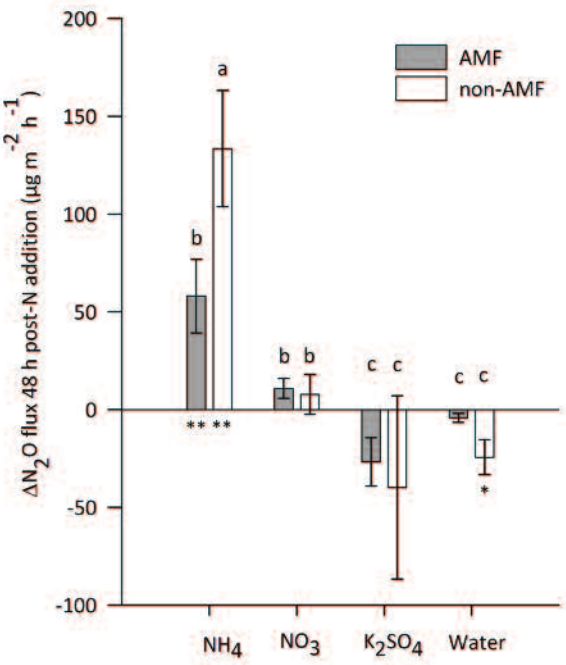
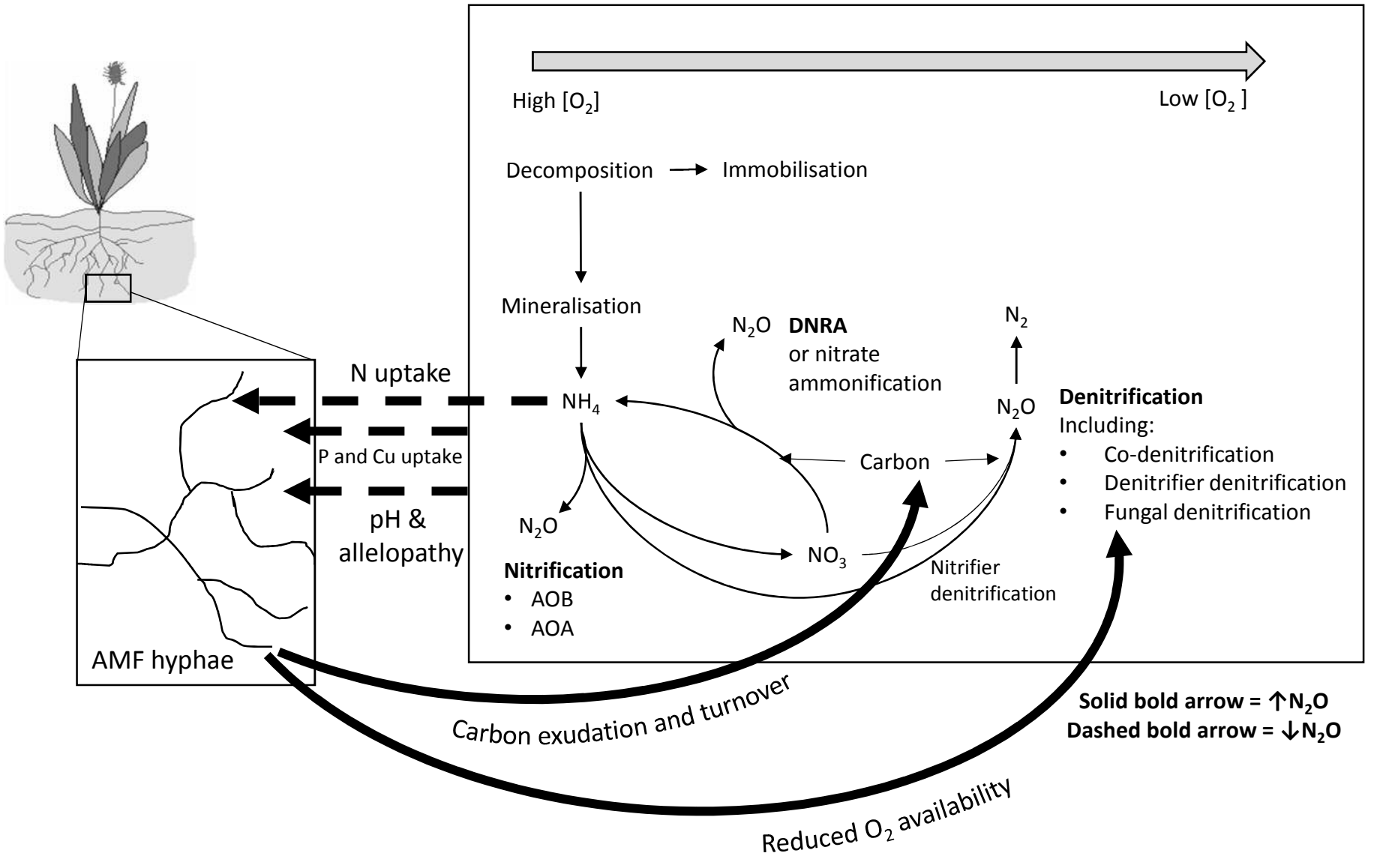

AMF
non-AMF
200
150
100
50
0
-50
-100
ΔN2O flux 48 h post-N addition (μg m-2 h-1)
a
b
b b
c c
c c
** **
*
NH4
NO3
K2SO4
Water

Figure 5


High [O2]
Low [O2]
Decomposition
Immobilisation
Mineralisation
N2O
DNRA
or nitrate
ammonification
N2
N uptake
NH4
N2O
Denitrification
Including:
• Co-denitrification
• Denitrifier denitrification
• Fungal denitrification
P and Cu uptake
Carbon
pH &
allelopathy
N2O
NO3
Nitrification
• AOB
• AOA
Nitrifier
denitrification
AMF hyphae
Carbon exudation and turnover
Solid bold arrow = ↑N2O
Dashed bold arrow = ↓N2O
Reduced O2 availability

***New Phytologist* Supporting Information**

Article title: **Arbuscular mycorrhizal fungi reduce nitrous oxide emissions via impacts on nitrification.**
Authors: Kate Storer, Aisha Coggan, Phil Ineson, Angela Hodge

The following Supporting Information is available for this article:

Table S1. Mean plant biomass parameters from AMF and non-AMF treatments in Experiment 1 ± standard error of the mean (n = 12). There were no significant differences between treatments for any of the parameters ($P > 0.05$ in each case) as determined using two-way ANOVAs.

	AMF	Non-AMF
Leaf DW (g)	$1.22^{a} \pm 0.05$	$1.16^{a} \pm 0.07$
Stalk DW (g)	$2.08^{b} \pm 0.08$	$1.97^{b} \pm 0.11$
Tassel DW (g)	$0.03^{c} \pm 0.01$	$0.02^{c} \pm 0.003$
Total shoot DW (g)	$3.89^{d} \pm 0.32$	$3.82^{d} \pm 0.33$
Root DW (g)	$1.24^{e} \pm 0.29$	$1.68^{e} \pm 0.45$
Total plant DW (g)	$5.13^{f} \pm 0.41$	$5.50^{f} \pm 0.57$
Root weight ratio	$0.23^{g} \pm 0.03$	$0.27^{g} \pm 0.05$