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**Arbuscular mycorrhizal fungi reduce nitrous oxide emissions from N$_2$O hotspots.**

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Summary

- Nitrous oxide (N\textsubscript{2}O) 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\textsubscript{2}O 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\textsubscript{2}O production was measured both before and after addition of ammonium and nitrate.

- In both experiments, N\textsubscript{2}O production decreased when AMF hyphae were present prior to inorganic N addition. In the presence of AMF hyphae, N\textsubscript{2}O production remained low following ammonium application, but increased in the non-AMF controls. In contrast, negligible N\textsubscript{2}O was produced following nitrate application to either AMF treatment.

- Thus, the main N\textsubscript{2}O source in this system appeared to be via nitrification and the production of N\textsubscript{2}O 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\textsubscript{2}O production.

Keywords: agriculture, arbuscular mycorrhizal fungi (AMF), greenhouse gas, hyphosphere, N cycle, nitrification, nitrogen (N), nitrous oxide (N\textsubscript{2}O)
Introduction

Agricultural soils are a major source of the globally important greenhouse gas, nitrous oxide (N\(_2\)O), a gaseous product of the nitrogen (N) cycle (Singh et al., 2010; Hartmann, 2013). In fact, the marked global N\(_2\)O 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\(_2\)O also has a long perturbation lifetime of 121 years (Hartmann, 2013), thus it is essential that we understand the soil derived fluxes of N\(_2\)O as, unlike shorter lived greenhouse gases (e.g. CH\(_4\); Hartmann, 2013), any changes in the atmospheric concentration of N\(_2\)O will have long term effects. Consequently, N\(_2\)O 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\(_2\)O is urgently required.

In recent years, our understanding of N\(_2\)O production in soil systems has significantly improved, mostly as a result of the development of isotopic methods for tracing the sources of N\(_2\)O (Baggs, 2008; Kool et al., 2011a; Ostrom & Ostrom, 2011). The rate of N\(_2\)O 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\(_2\)O reduction is not only confined to denitrifiers. Other commonly occurring soil bacteria and archaea may also utilise exogenous N\(_2\)O, 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\(_2\)O 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 &
Alongside NO$_3^-$, a major output of the N cycle is the potent greenhouse gas, N$_2$O. Therefore, it might be expected that these fungi may influence the availability of N substrates (ammonium (NH$_4^+$) and NO$_3^-$) for N$_2$O 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$_2$O producers, leading to a reduction in N$_2$O emissions. There is some circumstantial evidence to suggest this may be the case. For example, Bender et al. (2014) found a reduction in N$_2$O fluxes from soils influenced by AMF colonized roots when compared to soils influenced by roots alone. N$_2$O 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$_2$O 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$_2$O production, this could have significant implications for global N$_2$O 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$_2$O production rate; Storer, 2013), to create organic matter ‘patches’. These organic matter patches represent ‘N$_2$O hotspots’ which commonly occur in natural systems (Cowan et al., 2015). Both experiments tested the hypothesis that AMF hyphae would reduce N$_2$O production from the organic matter patches, while the second experiment further examined the hypothesis that a reduction in N$_2$O 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$_2$O 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$_2$O production, whereas Experiment 2 was designed to determine whether AMF hyphae affected N$_2$O produced by nitrification and/or denitrification. Organic matter patches were used to create ‘hotspots’ of N$_2$O 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 25\(^{th}\) 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 25\(^{th}\) June – 10\(^{th}\) Sept (Experiment 1) and 103 days between 25\(^{th}\) June – 5\(^{th}\) 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) \(\mu\text{mol m}^{-2} \text{s}^{-1}\) (Experiment 1) and 251 (SEM +/- 45) \(\mu\text{mol m}^{-2} \text{s}^{-1}\) (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
to two 1/10th N and P additions. In total the plants received either 1.74 kg N ha\(^{-1}\) or 11.97 kg N ha\(^{-1}\) in Experiments 1 and 2 respectively over the duration of the Experiments (11 weeks and 14 weeks respectively).

**Organic matter patches and gas probes**

*Organic matter patches*

Organic matter patch material comprised of 13 g dry weight (DW) equivalent agricultural soil (sandy loam; 53°92'N, -1°00'E, pH 6.6 in 0.01M CaCl\(_2\) (following Allen, 1974)) mixed with 2 g DW milled Z. mays leaves, all enclosed in a 20 µm mesh bag (70 mm x 60 mm). The mean C and N content of the mixed organic patches were 1435 mg (SEM +/- 182) and 116 mg (SEM +/- 15) (Experiment 1) or 1200 (SEM +/- 79) and 99 mg (SEM +/- 15) (Experiment 2) respectively, with a C:N ratio of 12:1 in both Experiments. Each patch contained a gas probe (described below) in the centre (Fig. 1c).

*Gas probes*

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

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

In Experiment 1, half of the organic matter patches were injected with 7 cm$^3$ of 30 mM NH$_4$NO$_3$ 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$_4$NO$_3$, AMF + water, non-AMF + NH$_4$NO$_3$ 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$_4$)$_2$SO$_4$ (NH$_4$ treatment), 30 mM KNO$_3$ (NO$_3$ treatment), 15 mM K$_2$SO$_4$ (K$_2$SO$_4$ 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$_4$NO$_3$ and water addition treatments were then added and the gas probes were sampled again at 24, 48 and 96 h post-NH$_4$NO$_3$ 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$_2$O. 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$_2$O analyser (LGR N$_2$O; Los Gatos Research, Inc., California, USA) which provided an N$_2$O 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\textsubscript{2}O analyser, the headspace in the microcosm unit (0.6 L), volume of connecting tubing (0.274 L) and internal volume of the N\textsubscript{2}O analyser (0.850 L) along with the surface area of the soil sampled (0.024 m\textsuperscript{-2}) were used in the regression calculation of the N\textsubscript{2}O flux rate in mg m\textsuperscript{-2} h\textsuperscript{-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\textsuperscript{®} 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\textsuperscript{-1} DW) of soil, sand/Agsorb\textsuperscript{®} 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\textsuperscript{-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$_2$O flux or concentration following N addition (referred to $\Delta$N$_2$O).

In Experiment 1, where N$_2$O 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$_2$O 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$_2$O 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 $\Delta$N$_2$O 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 $\Delta$N$_2$O flux or $\Delta$N$_2$O concentration data were made over time, a non-parametric Friedman’s repeated measures analysis was used. The relationship between the $\Delta$N$_2$O flux and $\Delta$N$_2$O 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, K₂SO₄) therefore this microcosm was excluded from the subsequent data analyses. In addition, the N₂O concentration for one experimental unit in the AMF treatment (treatment: (NH₄)₂SO₄) was out of range on the GC at the 48 h post N addition sample and therefore these AMF and non-AMF N₂O 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 N₂O production

Prior to inorganic N addition there was a greater concentration of N₂O 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 N₂O fluxes in Experiment 2, with greater N₂O fluxes measured from the non-AMF compartments than from the AMF compartments (\( S_{38} = -128, P = 0.074 \)). In Experiment 2, N₂O fluxes measured by continuous flow loop sampling were positively correlated with the patch N₂O concentrations measured using gas probes (\( r_{5} = 0.7495, P < 0.0001 \)). Since N₂O 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 N₂O 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 N₂O 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\(_2\)O producers to treatment application. The patch N\(_2\)O concentrations of
the non-AMF and AMF + NH\(_4\)NO\(_3\) treatments subsequently decreased over time. In contrast,
the AMF + water patch N\(_2\)O concentration remained low. Consequently, there was a
significant effect of both time and treatment on patch N\(_2\)O 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\(_2\)O production rates can change over time and emphasise
the requirement for repeated measurements following inorganic N application. Two-way
ANOVA at each time point showed that the N\(_2\)O 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\(_4\)NO\(_3\) treatments still had a higher N\(_2\)O 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\(_2\)O 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 ∆N\(_2\)O concentration was higher in both the AMF + NH\(_4\)NO\(_3\)
and non-AMF + NH\(_4\)NO\(_3\) 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\(_4\)NO\(_3\) treatment or non-AMF + NH\(_4\)NO\(_3\) treatment.

There was no relationship between the AMF ERM length densities and N\(_2\)O 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\(_4\)NO\(_3\)
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
ratio of the leaves was lower in the AMF compared to the non-AMF treatments (Table 1; $F_{1,12} = 18.51$, $P = 0.001$). However, the organic patch $\text{N}_2\text{O}$ concentration was not significantly related to the leaf C or N content or concentration, or to the leaf C:N ratio, either before or after N addition, for both the AMF and non-AMF treatments ($P > 0.05$ in each case).

**Experiment 2**

There was a significant difference in $\Delta\text{N}_2\text{O}$ fluxes among the inorganic N and water application treatments at 48 h post-application (Fig. 4; $Q_7 = 44.85$, $P < 0.0001$). In both the AMF and non-AMF patches, more $\text{N}_2\text{O}$ was produced following addition of $\text{NH}_4^+$ than any other treatment. Strikingly, however, ca. 2.5 times more $\text{N}_2\text{O}$ was produced from the non-AMF than the AMF treatment (Fig. 4; $S_9 = -26.5$, $P = 0.0084$). These differences then declined by the 96 h sample and were no longer significant at the 192 h sample, again, illustrating the transient nature of $\text{N}_2\text{O}$ release and the importance of following the fluxes over discrete timescales (Table 2). There was no significant difference in the % moisture content of either the patch or sand/Agsorb® medium between the AMF and non-AMF treatments at destructive harvest (patch: $t_{39} = -0.26$, $P = 0.799$; sand/Agsorb®: $S_{39} = -47$, $P = 0.519$).

**Discussion**

This is the first study to show that $\text{N}_2\text{O}$ production is reduced as a direct consequence of the presence of AMF hyphae. Moreover, this reduction was demonstrated in both the presence and, notably, the absence of applied inorganic N, indicating that this is a persistent effect. Studies to date have indicated that AMF may influence soil $\text{N}_2\text{O}$ production, but this has always been in the presence of plant roots and additional inorganic N (Lazcano *et al.*, 2014; Bender *et al.*, 2015). Critically, the finding that $\text{N}_2\text{O}$ production was reduced when AMF hyphae, but not plant roots, were present was consistent between the two independent experiments reported here.

Previous studies have applied inorganic N and assessed the $\text{N}_2\text{O}$ flux from the mycorrhizosphere (i.e. the soil influenced by AM colonised roots and AMF hyphae), often only at a single time point after N application, potentially masking cumulative effects (Bender *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 N₂O 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 N₂O fluxes in the mycorrhizosphere without first separating the AMF hyphae from the plant roots.

Nevertheless, there is some evidence of AMF interacting with soil N₂O production in the mycorrhizosphere, although results have been inconsistent. Bender et al., (2015) found that the N₂O flux was lower following the application of NO₃⁻ 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 N₂O production, whereas Lazcano et al., (2014) found a reduction in N₂O in the mycorrhizosphere of AM plants. Thus, there is support for AMF resulting in reduced N₂O 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 N₂O 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 N₂O 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$_2$O
production being influenced by the AMF hyphae. The addition of NO$_3^-$ did not result in
increased N$_2$O production from any treatment, suggesting that in this study denitrification was
not a key factor in controlling N$_2$O 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$_2$O production in the
present study. Instead, we found direct evidence for a reduction in N$_2$O produced via
nitrification in the presence of AMF hyphae. This is a critical finding and may help to explain
variable N$_2$O fluxes under field conditions. Since one of the largest sources of N$_2$O 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$_2$O 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$_2$O peak declined back to pre-N application levels, thus ensuring that the full
response period was recorded. There was a significantly greater N$_2$O flux in response to NH$_4^+$
addition in the non-AMF than in the AMF treatment, indicating reduced N$_2$O production via
nitrification in the presence of AMF hyphae. The current understanding of the main pathways
of N$_2$O 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$_2$O production.

If NH$_4^+$ elicits N$_2$O production but NO$_3^-$ application does not, by process of elimination the
pathway involved in N$_2$O 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 N₂O production has also been shown to be negatively correlated with AMF abundance (Bender et al., 2014). Thus, the presence of AM plants may reduce N₂O production by reducing nitrification rates. Our present study demonstrates, for the first time, that AMF hyphae have a direct and limiting influence on soil N₂O produced via nitrification, independent of any plant root influence.

The main ‘nitrification’ pathways in soil potentially resulting in N₂O 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 organisms capable of complete ammonia oxidation (comammox) (Daims et al., 2015; van Kessel et al., 2015). AOB and AOA have also been shown to produce N₂O (Jiang and Bakken, 1999; Jung et al., 2014). Nitrifier denitrification is also carried out by autotrophic nitrifiers, and can be a significant source of N₂O (Kool et al., 2011b; Wrage et al., 2001). Thus, there are various pathways by which the N₂O in this study may have been produced following the application of NH₄⁺ and consequently reduced by the presence of AMF hyphae (Fig. 5).

Regardless of the process, the response to NH₄⁺ application in the AMF treatments suggests that there was either a reduction in N₂O production, through reduced function or number of nitrifiers, or that nitrifier activity was masked by an increase in activity of N₂O reducers which can cause some soils to become N₂O sinks (Domeignoz-Horta et al., 2017). It’s also feasible that the presence of AMF hyphae modified the microbial community shifting it away from N₂O producing nitrifiers or nitrifier denitrifiers, perhaps towards organisms capable of complete nitrification (van Kessel et al., 2015), or N₂O reduction (C.M. Jones et al., 2014; Sanford et al., 2012; Domeignoz-Horta et al., 2017).

Domeignoz-Horta et al. (2017) found that N₂O hotspots were predominantly controlled by changes in the microbial communities, whereas lower N₂O 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 N₂O 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 N₂O 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 inherently 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 NH₄⁺ (Govindarajulu et al., 2005; Tanaka & Yano, 2005), and AOB are generally thought to be poor competitors for NH₄⁺ (Verhagen et al., 1995; Bollmann et al., 2002). The AMF hyphae may therefore have reduced the amount of available NH₄⁺ in the hyphosphere resulting in a reduction in the population of active AOB. If AOB were the main N₂O producers, this may explain the reduced N₂O production prior to inorganic N application when the AMF hyphae were present. It may also explain the lack of N₂O production in the presence of AMF hyphae when NH₄⁺ was applied, i.e. the AOB population may have been small and too slow-growing to respond to the inorganic NH₄⁺ 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 NO₃⁻ or NH₄⁺ uptake respectively (Bago et al., 1996; Li et al., 1991), the relative importance of pH effects on N₂O production if C, NH₄⁺ or NO₃⁻ are limiting are not clear (reviewed by Šimek & Cooper, 2002) with both increased and decreased nitrification-derived N₂O 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$_2$O 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$_2$O 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$_2$O. 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$_2$O emissions. This study suggests that a reduction in AMF presence may contribute to further increases in N$_2$O 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 situations, 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.

Acknowledgements

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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.

References


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

<table>
<thead>
<tr>
<th></th>
<th>AMF</th>
<th>Non-AMF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leaf N</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total content (mg)</td>
<td>13.8 ± 0.8$^a$</td>
<td>10.2 ± 0.9$^b$</td>
</tr>
<tr>
<td>Concentration (mg g$^{-1}$ DW)</td>
<td>11.3 ± 0.6$^f$</td>
<td>8.8 ± 0.5$^g$</td>
</tr>
<tr>
<td>Leaf C</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total content (mg)</td>
<td>503.2 ± 19.9$^i$</td>
<td>488.1 ± 27.2$^j$</td>
</tr>
<tr>
<td>Concentration (mg g$^{-1}$ DW)</td>
<td>413.4 ± 2.8$^m$</td>
<td>422.9 ± 3.7$^n$</td>
</tr>
<tr>
<td>Leaf C:N ratio</td>
<td>37.6 ± 2.0$^x$</td>
<td>50.0 ± 3.0$^y$</td>
</tr>
</tbody>
</table>
Table 2. Experiment 2 Friedman’s test statistics controlling for block comparing the post-N minus pre-N (61 days post-patch addition) patch N₂O concentrations (ΔN₂O concentrations) or compartment N₂O fluxes (ΔN₂O fluxes) among N addition treatments, for each of the gas sampling events.

<table>
<thead>
<tr>
<th>Time since N addition</th>
<th>48 h</th>
<th>96 h</th>
<th>192 h</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Q</td>
<td>df</td>
<td>P</td>
</tr>
<tr>
<td>Patch ΔN₂O concentration</td>
<td>28.89</td>
<td>7</td>
<td>0.0002***</td>
</tr>
<tr>
<td>Compartment ΔN₂O flux</td>
<td>44.85</td>
<td>7</td>
<td>&lt;0.0001***</td>
</tr>
</tbody>
</table>

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\(_2\)O 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 ∆AMF value to zero (Wilcoxon Signed Rank test).

**Fig. 3** Mean patch N\(_2\)O concentration at 24, 48 and 96 h following inorganic N (NH\(_4\)NO\(_3\): 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\(_2\)O flux (∆N\(_2\)O 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\(_4\))\(_2\)SO\(_4\) (labelled as NH\(_4\)), KNO\(_3\) (labelled as NO\(_3\)), K\(_2\)SO\(_4\) or...
Bars with different letters are significant at $P = 0.0018$ as determined using Mann Whitney U or Wilcoxon signed rank post hoc tests with a False Discovery rate correction applied. Asterisks below the bars indicate significant differences to zero (*$P < 0.05$, **$P < 0.01$). Error bars are ± standard error of the mean ($n = 10$).

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

**Supporting Information Table S1.** Mean plant biomass parameters from AMF and non-AMF treatments in Experiment 1.
nylon mesh 20.0 µm

organic patch
gas probe

(a)

nylon mesh 0.45 µm

organic patch
AMF hyphae
drainage holes

gas probe
gas-tight removable lid

(b)

Suba-Seal™

air hole covered with PVDF membrane

organic matter patch

c)
Figure 3.
Figure 5

Diagram showing the nitrogen cycle with processes such as immobilisation, decomposition, mineralisation, nitrification, denitrification, and the roles of AMF hyphae, pH, and allelopathy. The diagram illustrates the movement of nitrogen through the cycle and the interaction with oxygen levels (High [O₂] and Low [O₂]).

Key processes include:
- **Nitrification**:
  - AOB (Nitrosomonas)
  - AOA (Nitroso bipolaris)
- **Denitrification**:
  - Co-denitrification
  - Denitrifier denitrification
  - Fungal denitrification
- **Immobilisation**
- **Mineralisation**
- **DNRA** (dissimilatory nitrate reduction to ammonia)

Arrows indicate the direction of nitrogen movement and include symbols for carbon exudation and turnover, reduced O₂ availability, and P and Cu uptake.
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.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>AMF</th>
<th>Non-AMF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leaf DW (g)</td>
<td>1.22a ± 0.05</td>
<td>1.16a ± 0.07</td>
</tr>
<tr>
<td>Stalk DW (g)</td>
<td>2.08b ± 0.08</td>
<td>1.97b ± 0.11</td>
</tr>
<tr>
<td>Tassel DW (g)</td>
<td>0.03c ± 0.01</td>
<td>0.02c ± 0.003</td>
</tr>
<tr>
<td>Total shoot DW (g)</td>
<td>3.89d ± 0.32</td>
<td>3.82d ± 0.33</td>
</tr>
<tr>
<td>Root DW (g)</td>
<td>1.24e ± 0.29</td>
<td>1.68e ± 0.45</td>
</tr>
<tr>
<td>Total plant DW (g)</td>
<td>5.13f ± 0.41</td>
<td>5.50f ± 0.57</td>
</tr>
<tr>
<td>Root weight ratio</td>
<td>0.23g ± 0.03</td>
<td>0.27g ± 0.05</td>
</tr>
</tbody>
</table>