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Arbuscular mycorrhizal fungi reduce nitrous oxide emissions from N₂O hotspots

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Summary

- Nitrous oxide (N₂O) 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. As AMF acquire N, it was hypothesized that AMF hyphae may reduce N₂O production.
- AMF hyphae were either allowed (AMF) or prevented (nonAMF) access to a compartment containing an organic matter and soil patch in two independent microcosm experiments. Compartment and patch N₂O production was measured both before and after addition of ammonium and nitrate.
- In both experiments, N₂O production decreased when AMF hyphae were present before inorganic N addition. In the presence of AMF hyphae, N₂O production remained low following ammonium application, but increased in the nonAMF controls. By contrast, negligible N₂O was produced following nitrate application to either AMF treatment.
- Thus, the main N₂O source in this system appeared to be via nitrification, and the production of N₂O was reduced in the presence of AMF hyphae. It is hypothesized that AMF hyphae may be outcompeting slow-growing nitrifiers for ammonium. This has significant global implications for our understanding of soil N cycling pathways and N₂O production.

Introduction

Agricultural soils are a major source of the globally important greenhouse gas, nitrous oxide (N₂O), a gaseous product of the nitrogen (N) cycle (Singh et al., 2010; Hartmann et al., 2013). In fact, the marked global N₂O atmospheric concentration increases between 1940 and 2005 were predominantly a result of increased use of N-based fertilizers in agricultural systems (Park et al., 2012). N₂O also has a long perturbation lifetime of 121 yr (Hartmann et al., 2013), and thus it is essential that we understand the soil-derived fluxes of N₂O, as, unlike shorter-lived greenhouse gases (e.g. CH₄; Hartmann et al., 2013), any changes in the atmospheric concentration of N₂O will have long-term effects. Consequently, N₂O 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 N₂O is urgently required.

In recent years, our understanding of N₂O production in soil systems has significantly improved, mostly as a result of the development of isotopic methods for tracing the sources of N₂O (Baggs, 2008; Kool et al., 2011a; Ostrom & Ostrom, 2011). The rate of N₂O production is predominantly controlled by the availability of the inorganic N source (Hino et al., 2010), O₂ (Bollmann & Conrad, 1998), and other factors that influence microbial activity (e.g. temperature, carbon (C) availability and pH; Bollmann & Conrad, 1998; Prosser, 2007; Thomson et al., 2012). In addition, recent evidence has revealed that N₂O reduction is not only confined to denitrifiers. Other commonly occurring soil bacteria and archaea may also utilize exogenous N₂O, including under aerobic conditions, even though they lack the preceding steps in the denitrification pathway (Sanford et al., 2012; Jones et al., 2014). Therefore, it follows that the net N₂O 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 unrecognized role in nitrogen (N) cycling, and can both aquire N for their host plant (Barrett et al., 2011; Herman et al., 2012) and have a substantial N requirement themselves (Hodge & Fitter, 2010). There is also evidence of reduced nitrate (NO₃⁻) leaching in the presence AMF (Asghari & Cavagnaro, 2012; Cavagnaro et al., 2015; Köhl & van der Heijden, 2016). Alongside NO₃⁻, a major output of the N cycle is the potent greenhouse gas, N₂O. Therefore, it might be expected that these fungi might influence the availability of N substrates (ammonium (NH₄⁺) and NO₃⁻)
for N\textsubscript{2}O production. AMF have been shown to be able to acquire both NH\textsubscript{4}\textsuperscript{+} and NO\textsubscript{3}\textsuperscript{−}, although it appears they may prefer the more energetically attractive NH\textsubscript{4}\textsuperscript{+} (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\textsubscript{2}O producers, leading to a reduction in N\textsubscript{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\textsubscript{2}O fluxes from soils influenced by AMF-colonized roots when compared with soils influenced by roots alone. N\textsubscript{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\textsubscript{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-colonized roots, or as a direct result of the AMF themselves. If AMF hyphae can directly reduce N\textsubscript{2}O production, this could have significant implications for global N\textsubscript{2}O production and our understanding of soil N cycling.

Arbuscular mycorrhizal fungi hyphae have previously been demonstrated to proliferate in organic matter patches (e.g. Hodge et al., 2001; Barrett et al., 2014; Hodge, 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 that of Hodge & Fitter (2010) using dried, milled Zea mays L. leaves mixed with an agricultural soil (which had a high N\textsubscript{2}O production rate; Storer, 2013), to create organic matter ‘patches’. These organic matter patches represent ‘N\textsubscript{2}O hotspots’ which commonly occur in natural systems (Cowan et al., 2015). Both experiments tested the hypothesis that AMF hyphae would reduce N\textsubscript{2}O production from the organic matter patches, while the second experiment further examined the hypothesis that a reduction in N\textsubscript{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\textsubscript{2}O production was reduced in the presence of AMF hyphae, two experiments were established under glasshouse conditions using compartmented microcosm units. Expt 1 was designed to determine the impact of AMF hyphae on N\textsubscript{2}O production, whereas Expt 2 was designed to determine whether AMF hyphae affected N\textsubscript{2}O produced by nitrification and/or denitrification. Organic matter patches were used to create ‘hotspots’ of N\textsubscript{2}O production, a commonly observed phenomenon under natural conditions.

Expt 1 Microcosm units (Fig. 1a) were constructed by joining two 11 plastic containers (each 145 × 145 × 70 mm\textsuperscript{3}) via a double-mesh membrane of either 20 μm (John Stanier & Co., Whitefield, Manchester, UK) or 0.45 μm pore size (Osmonics Inc., Minnetonka, MN, USA). These size membranes either allowed (AMF) or denied (nonAMF) 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 Rhizopagus irregularis (PlantWorks Ltd, Kent, UK) was placed, whereas the other compartment contained no plant (the ‘unplanted’ compartment).

Expt 2 Three compartment microcosm units were used (Fig. 1b). Each microcosm consisted of a central ‘planted’ compartment (volume, 2 l; dimensions, 150 × 150 × 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 Expt 1 (volume, 2.6 l; dimensions, 140 × 140 × 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 (nonAMF; 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 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 nonAMF 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.

Expts 1 and 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, Chicago, IL, USA; a calcined attapulgite clay soil conditioner) that had been rinsed thoroughly in deionized water to remove any excess soluble N and/or P. The planted compartments also had 50 g (Expt 1) or 90 g (Expt 2) of a fresh R. irregularis inoculum (Plantworks Ltd, Sittingbourne, Kent, UK) and 0.25 g l\textsuperscript{−1} bionemal (a complex N and P source to encourage mycorrhizal development; 3.5% N, 8.7% P; Vitax, Coalville, Leicestershire, UK). Three pregerminated Z. mays seeds were added to each planted compartment for both experiments on 25 June 2012 and thinned to one per pot after 11 d (Expt 1) or 14 d (Expt 2). A sterile centrifuge tube (Expt 1, 15 cm\textsuperscript{3}; Expt 2, 50 cm\textsuperscript{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 randomized block design in a heated, lit glasshouse. The Experiments ran for 78 d between 25 June and 10 September (Expt 1), and 103 d between 25 June and 10 October 2012 (Expt 2). Photosynthetically active radiation (PAR) was measured weekly for both experiments at plant level
in the centre of each block and averaged (± SEM) 141 ± 15 (Expt 1) and 251 ± 45 μmol m⁻² s⁻¹ (Expt 2). Overhead lights were used to extend the photoperiod to 16 h d⁻¹ and the mean daily temperatures over the experimental period were 21.9 ± 0.02°C (Expt 1) and 21.5 ± 0.3°C (Expt 2). The planted and unplanted compartments for all microcosm units were watered daily as required. After 2 wk of plant growth, the planted compartments received 50 cm³ of a reduced N and P nutrient solution as described by Leigh et al. (2009) once a wk (Expts 1 and 2). This was increased to twice weekly at 49 d after planting in Expt 2 and to full N at 55 d after planting as the plants were starting to show symptoms of N deficiency. In Expt 2, at 76 d, the plants began to show P-deficiency symptoms, so a 3/10 P, full N solution was used once a wk in addition to two 1/10 N and P additions. In total the plants received either 1.74 or 11.97 kg N ha⁻¹ in Expts 1 and 2, respectively, over the duration of the experiments (11 and 14 wk, respectively).

Organic matter patches and gas probes

Organic matter patches Organic matter patch material comprised 13 g DW equivalent agricultural soil (sandy loam; 53°92′ N, −1°00′ E, pH 6.6 in 0.01 M CaCl₂ (following Allen, 1974)) mixed with 2 g DW milled Z. mays leaves, all enclosed in a 20 μm mesh bag (70 × 60 mm). The mean (± SEM) C and N contents of the mixed organic patches were 1435 ± 182 and 116 ± 15 mg (Expt 1) or 1200 ± 79 and 99 ± 15 mg (Expt 2), respectively, with a C : N ratio of 12 : 1 in both experiments. Each patch contained a gas probe (described in the next section) 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 air-tight seal (Fig. 1c). Two diametrically opposed holes, of 6 mm, were drilled through each tube 13 mm from the base. These holes were covered in a polyvinylidene difluoride (PVDF) membrane (0.2 μm; Bio-Rad) 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, Lindford, Bordon, 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) to form a gas sampling port. The total internal volume of the gas probe was c. 4.5 cm³.

A single organic matter patch and gas probe were placed into the preformed 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 (Expt 1 or 2, respectively) post-planting.

Inorganic nitrogen addition
In Expt 1, half of the organic matter patches were injected with 7 cm³ of 30 mM NH₄NO₃ and the other half with 7 cm³ of deionized water (n = 6 in each case) at 44 d after patch addition. Consequently, the treatments were: AMF + NH₄NO₃, AMF + water, nonAMF + NH₄NO₃ and nonAMF + water. In Expt 2, at 62 d after organic patch addition (90 d after planting) each patch was injected with one of 7 cm³ of 15 mM (NH₄)₂SO₄ (NH₄ treatment), 30 mM KNO₃ (NO₃ treatment), 15 mM K₂SO₄ (K₂SO₄ treatment) or deionized 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 cm³ aliquots of solution were injected into each organic patch with a 1 h gap between each addition to reduce spread into the surrounding sand/Agsorb®.

Gas sampling and analysis
Expt 1 The air in the gas probes was sampled before N addition at 44 d after patch addition. The NH₄NO₃ and water addition treatments were then added and the gas probes were sampled again at 24, 48 and 96 h after NH₄NO₃ addition. Before sample removal, 1 cm² of N₂ 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 cm³ 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 prefilled 3 cm³ Exsyringe (Labco Ltd, Lampeter, Ceredigion, UK) (with 6 cm³ N₂), overpressurizing the sample to 7 cm³ in total. All gas samples were analysed using a gas chromatograph (GC) which quantified the concentration of N₂O. The concentration (ppm) values for each sample were calculated by comparing with certified standards that were diluted in parallel in a 1 cm³ standard: 6 cm³ N₂ ratio and correcting for this dilution. The concentration values were also corrected for dilution from addition of N₂ to the gas probe just before gas sample removal.

Expt 2 Gas sampling was carried out using both gas probes (as described for Expt 1) and continuous flow loop sampling with an attached Los Gatos Isotopic N₂O analyser (LGR N₂O, Los Gatos Research Inc., San Jose, CA, USA) which provided an N₂O 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 min, with a minimum of 2 min flushing the system with air between each compartment measurement. Gas sampling using both methods was carried out before N addition (58–59 and 61 d after patch addition), and at 48, 96 and 192 h after N addition (64, 66 and 70 d after patch addition, respectively).

When using the LGR N₂O analyser, the headspace in the microcosm unit (0.6 L), volume of connecting tubing (0.274 L) and internal volume of the N₂O 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 N₂O flux rate in mg m⁻² h⁻¹. These fluxes were calculated using values measured between 200 and 280 s after the cover-box lid was attached. All regressions were calculated using SAS v.9.3 (SAS institute Inc., Cary, NC, USA).

Post-harvest analyses
At harvest, above-ground material was removed at the soil surface and separated into stalk, flowers, ear, and leaf material. Roots were extracted from the sand/Agsorb® media and washed, and FW and DW of all plant material were recorded. In Expt 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., Valencia, CA, 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., 1999) 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 ×125 magnification (using a square grid of 1 cm side length split into 10 × 10 grid sections; Graticules Ltd, Tonbridge, 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 v.9.3 or GENSTAT v.16 (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 N₂O flux or concentration following N addition (referred to ΔN₂O).

In Expt 1, where N₂O concentration and ERM length density data did not fulfill normality or equality of variance assumptions, they were log₁₀-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 N₂O concentration following N addition failed to normalize the data, and nonparametric equivalent Friedman’s two-way ANOVAs, including block, with Wilcoxon post hoc tests were used. Where N₂O concentrations were measured over time, repeated-measures ANOVA, including treatment and block, was used on log₁₀-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 Expt 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, nonparametric tests were used. A one-sample $t$-test or a Wilcoxon signed-rank test was used to compare absolute values or differences to zero.

In Expt 2, the $\Delta$N$_2$O data were not normally distributed and therefore a Friedman’s nonparametric two-way ANOVA, controlling for block, with Mann–Whitney $U$-test (unpaired data) or Wilcoxon signed rank (paired data) post hoc test and an applied false discovery rate correction were used. Where comparisons in $\Delta$N$_2$O flux or $\Delta$N$_2$O concentration data were made over time, a nonparametric Friedman’s repeated-measures analysis was used. The relationships between the $\Delta$N$_2$O flux and $\Delta$N$_2$O concentration for each gas sample following N addition (48, 96 and 192 h post-N addition) were determined using a Spearman rank order correlation.

There was hyphal breakthrough in one of the nonAMF compartments (treatment: nonAMF, K$_2$SO$_4$) and therefore this microcosm was excluded from the subsequent data analyses. In addition, the N$_2$O concentration for one experimental unit in the AMF treatment (treatment: (NH$_4$)$_2$SO$_4$) was out of range on the GC for the sample taken 48 h post-N addition and therefore these AMF and nonAMF N$_2$O 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 Expt 1 (nonAMF, 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 Expt 2 (nonAMF, 0.35±0.04 m g$^{-1}$ DW; $t_{39} = 8.993$, $P < 0.0001$).

Pre-N addition N$_2$O production

Before inorganic N addition there was a greater concentration of N$_2$O in the nonAMF patches than in the AMF patches in both experiments (Fig. 2; Expt 1, $F_{1,12} = 6.46$, $P = 0.026$; Expt 2, $S_{38} = -186$, $P = 0.0076$). A similar trend (at the $P < 0.1$ level) was found for the N$_2$O fluxes in Expt 2, with greater N$_2$O fluxes measured from the nonAMF compartments than from the AMF compartments ($S_{38} = -128$, $P = 0.074$). In Expt 2, N$_2$O fluxes measured by continuous flow loop sampling were positively correlated with the patch N$_2$O concentrations measured using gas probes ($r = 0.7495$, $P < 0.0001$). As N$_2$O production is inherently variable, this degree 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$_2$O concentration or fluxes and the ERM lengths ($P > 0.05$ in each case).

Post-N addition and harvest

Expt 1 In Expt 1 the highest patch N$_2$O 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 nonAMF and AMF + NH$_4$NO$_3$ treatments subsequently decreased over time. By 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 emphasize the requirement for repeated measurements.

Fig. 2 Mean N$_2$O concentration (ppm) in arbuscular mycorrhizal fungal access (AMF) and no AMF access (nonAMF) organic matter patches at 43 d after patch addition in Expt 1 (a) and at 98 d after patch addition in Expt 2 (b). Error bars are ± SEM ($n = 12$; b, $n = 39$). Different letters represent significant differences at $P < 0.05$ as determined using: (a) two-way ANOVAs; and (b) by comparing the AAMF value with zero (Wilcoxon signed-rank test).

Fig. 3 Mean patch nitrous oxide (N$_2$O) concentration at 24, 48 and 96 h after addition of inorganic nitrogen (N) (NH$_4$NO$_3$: closed symbols) or water (open symbols) for arbuscular mycorrhizal fungal access patches (AMF; solid lines) and no AMF access patches (nonAMF; dashed lines) shown over time. Error bars are ± SEM ($n = 6$). 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 ($P < 0.05$).
following inorganic N application. Two-way ANOVAs 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 nonAMF + water and nonAMF + 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 nonAMF patches ($F_{3,15} = 7.25, P = 0.0031$). At 24 h post-treatment application, the $\Delta$N$_2$O concentration was higher in both the AMF + NH$_4$NO$_3$ and nonAMF + NH$_4$NO$_3$ treatments than in the AMF + water treatment ($Q = 8.2, P = 0.042$). However, the nonAMF + water treatment was not significantly different from the AMF + NH$_4$NO$_3$ treatment or nonAMF + 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 ($Q = 0.707, P = 0.871$). 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 nonAMF treatments (see Supporting Information Table S1). The addition of NH$_4$NO$_3$ or water had no effect on the leaf C and N content or concentrations or on the C : N ratios ($P > 0.05$ in each case), and therefore these data were combined for comparison of the AMF with the nonAMF treatments. Leaf C content did not differ between AMF and nonAMF plants (Table 1; $F_{1,12} = 0.30, P = 0.595$), although the leaf C concentrations were lower in the AMF than in the nonAMF 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 than in the nonAMF treatments. Consequently, the C : N ratio of the leaves was lower in the AMF than in the nonAMF treatments (Table 1; $F_{1,12} = 18.51, P = 0.001$). However, the organic patch N$_2$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 nonAMF treatments ($P > 0.05$ in each case).

Table 1 Mean (± SEM) leaf nitrogen (N) and carbon (C) total content and concentration, and C : N ratio of Zea mays leaves from arbuscular mycorrhizal fungi (AMF) and nonAMF treatments in Expt 1 (n = 12)

<table>
<thead>
<tr>
<th></th>
<th>AMF</th>
<th>NonAMF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leaf N</td>
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<tr>
<td>Total content</td>
<td>13.8 ± 0.8 a</td>
<td>10.2 ± 0.9 b</td>
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<tr>
<td>Concentration</td>
<td>11.3 ± 0.6 f</td>
<td>8.8 ± 0.5 g</td>
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<tr>
<td>Leaf C</td>
<td></td>
<td></td>
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<tr>
<td>Total content</td>
<td>503.2 ± 19.9 j</td>
<td>488.1 ± 27.2 j</td>
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<tr>
<td>Concentration</td>
<td>413.4 ± 2.8 m</td>
<td>422.9 ± 3.7 n</td>
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<tr>
<td>Leaf C : N</td>
<td>37.6 ± 2.0 x</td>
<td>50.0 ± 3.0 y</td>
</tr>
</tbody>
</table>

Different letters within rows represent significant differences at $P = 0.05$ (in bold) as determined using two-way ANOVAs.

Fig. 4 Mean difference between 48 h post-nitrogen (N) addition (64 d after patch addition) and pre-N addition (64 d after patch addition) nitrous oxide (N$_2$O) fluxes ($\Delta$N$_2$O flux) for arbuscular mycorrhizal fungal access (AMF; closed bars) and no AMF access (nonAMF; open bars) treatments, split by N-addition treatment. The N-addition treatments were NH$_4$SO$_4$ (labelled as NH$_4$), KNO$_3$ (labelled as NO$_3$), K$_2$SO$_4$ or water. Bars with different letters are significant at $P = 0.0018$ as determined by Mann–Whitney U or Wilcoxon signed-rank post hoc tests with a false discovery rate correction applied. Asterisks below the bars indicate significant differences from zero (*, $P < 0.05$; **, $P < 0.01$). Error bars are ± SEM (n = 10).

Expt 2 There was a significant difference in $\Delta$N$_2$O fluxes between the inorganic N and water application treatments at 48 h post-application (Fig. 4; $Q = 44.85, P < 0.0001$). In both the AMF and nonAMF patches, more N$_2$O was produced following addition of NH$_4^+$ than for any other treatment. Strikingly, however, c. 2.5 times more N$_2$O was produced from the nonAMF than from the AMF treatment (Fig. 4; $S_H = -26.5, P = 0.0084$). 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 N$_2$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 nonAMF 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 N$_2$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 N$_2$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 N$_2$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 N$_2$O flux from the mycorrhizosphere (i.e. the soil influenced by AM colonized 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 N$_2$O production in isolation. Furthermore, as the mycorrhizosphere includes both AMF-colonized plant roots and AMF hyphae, it is impossible to know whether any effect is a consequence of the AMF hyphae or roots, or both. Rhizodeposition differs between AM and nonAM plants (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 (Bollmann & Conrad, 1998; Hino et al., 2010). It is not possible, therefore, to separate AMF and root control of N$_2$O 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$_2$O production in the mycorrhizosphere, although results have been inconsistent. Bender et al. (2015) found that the N$_2$O flux was lower following the application of NO$_3^-$ in the AM mycorrhizosphere when compared with the rhizosphere of a nonAM control. By contrast, Cavagnaro et al. (2012) found no effect of AM plants on N$_2$O production, whereas Lazcano et al. (2014) found a reduction in N$_2$O in the mycorrhizosphere of AM plants. Thus, there is support for AMF resulting in reduced N$_2$O production in the mycorrhizosphere, but the cause of this reduction has so far been poorly understood, probably because of confounding effects of the host plant root system also being present. Hypotheses for the decreased N$_2$O 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$_2$O production in the presence of AMF hyphae was evident even before 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, as 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. As agricultural soils are one of the largest sources of N$_2$O, 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 h after inorganic N application, by which point the N$_2$O peak declined back to pre-N application values, thus ensuring that the full response period was recorded. There was a significantly greater N$_2$O flux in response to NH$_4^+$ addition

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### Table 2

<table>
<thead>
<tr>
<th>Time since N addition</th>
<th>Patch $\Delta$N$_2$O concentration</th>
<th>Compartment $\Delta$N$_2$O flux</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Q</td>
<td>d.f.</td>
</tr>
<tr>
<td>48 h</td>
<td>28.89</td>
<td>7</td>
</tr>
<tr>
<td>96 h</td>
<td>44.85</td>
<td>7</td>
</tr>
<tr>
<td>192 h</td>
<td>7.39</td>
<td>7</td>
</tr>
</tbody>
</table>

Q, Friedman’s test statistic; d.f., degrees of freedom; n = 10. Significant results are indicated in bold at $P = 0.05$ (*), $P < 0.05$; ***, $P < 0.001$. 

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in the nonAMF 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 were lower in the mycorrhizospheres of AM plants than in those from weakly AM mycorrhizospheres (Veresoglou et al., 2011). The nirK gene, responsible for N$_2$O 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$_2$O 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$_2$O produced via nitrification, independent of any plant root influence.

The main ‘nitrification’ pathways in soil potentially resulting in N$_2$O release are nitrifier nitrification, and nitrifier denitrification. Nitrifier nitrification is an aerobic process and can be carried out by ammonia-oxidizing 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 N$_2$O (Jiang & Bakken, 1999; Jung et al., 2014). Nitrifier denitrification is also carried out by autotrophic nitrifiers, and can be a significant source of N$_2$O (Wrage et al., 2001; Kool et al., 2011b). Thus, there are various pathways by which the N$_2$O 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$_2$O production, through reduced function or number of nitrifiers, or that nitrifier activity was masked by an increase in activity of N$_2$O reducers, which can cause some soils to become N$_2$O sinks (Domeignoz-Horta et al., 2017). It is also feasible that the presence of AMF hyphae modified the microbial community, shifting it away from N$_2$O-producing nitrifiers or nitrifier denitrifiers, perhaps towards organisms capable of complete

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**Fig. 5** Summarized potential interactions between arbuscular mycorrhizal fungal (AMF) hyphae and soil nitrous oxide (N$_2$O)-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 and decrease in N$_2$O production, respectively. AMF can affect the availability of nitrogen (N), phosphorus (P), copper (Cu) and iron (Fe) in soils, as well as potentially changing soil pH. Nitrifier nitrification is generally carried out by ammonia-oxidizing bacteria (AOB) and archaea (AOA). Dissimilatory reduction of nitrate to ammonium (DNRA) may produce N$_2$O 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$_2$O production (i.e. availability of O$_2$, or carbon).
nitrification (van Kessel et al., 2015) or N₂O reduction (Sanford et al., 2012; Jones et al., 2014; 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 possess nosZ genes, and can thus utilize exogenous N₂O as an electron acceptor (Jones et al., 2013; 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 physicochemical environment in the decomposing litter patch, may 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 could 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 predominantly to take up inorganic N in the form of NH₄⁺ (Govindaraju 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 before 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 (Li et al., 1991; Bago et al., 1996), the relative importance of pH effects on N₂O production if C, NH₄⁺ or NO₃⁻ are limiting is not clear (reviewed by Šimek & Cooper, 2002), with both increased and decreased nitrification-derived N₂O production reported under low-pH conditions (Morkved et al., 2007; Cheng et al., 2013). 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 physicochemical properties, including pH, warrant further 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 for C cycling and stabilization processes (Hodge, 2001; Hodge et al., 2001; Cheng et al., 2012).

In order to fully understand the mechanism for the reduction in N₂O 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 nondenitrifying N₂O 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, N₂O. 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 N₂O emissions. This study suggests that a reduction in the presence of AMF may contribute to further increases in N₂O 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.

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Author contributions

K.S., P.I. and A.H. designed the research; K.S. performed the data creation during this research are available by request from the University of York Data Catalogue. https://doi.org/10.15124/67decab3-9ea6-4cde-812e-3c762eba2ec6.

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