

This is a repository copy of *Arbuscular mycorrhizal fungi reduce nitrous oxide emissions from N2O hotspots.*

White Rose Research Online URL for this paper:

<https://eprints.whiterose.ac.uk/125152/>

Version: Accepted Version

Article:

Storer, Kate Elizabeth, Coggan, Aisha, Ineson, Philip orcid.org/0000-0002-5194-0022 et al. (1 more author) (2018) Arbuscular mycorrhizal fungi reduce nitrous oxide emissions from N2O hotspots. *New Phytologist*. pp. 1285-1295. ISSN 1469-8137

<https://doi.org/10.1111/nph.14931>

Reuse

This article is distributed under the terms of the Creative Commons Attribution (CC BY) licence. This licence allows you to distribute, remix, tweak, and build upon the work, even commercially, as long as you credit the authors for the original work. More information and the full terms of the licence here:

<https://creativecommons.org/licenses/>

Takedown

If you consider content in White Rose Research Online to be in breach of UK law, please notify us by emailing eprints@whiterose.ac.uk including the URL of the record and the reason for the withdrawal request.

1 **Arbuscular mycorrhizal fungi reduce nitrous oxide emissions from N₂O hotspots.**

2 Kate Storer^{1,2}, Aisha Coggan^{1,3}, Phil Ineson¹, Angela Hodge¹

3 ¹Department of Biology, University of York, Heslington, York, YO10 5DD.

4 ²Author for correspondence and present address: ADAS High Mowthorpe, Duggleby, Malton,
5 North Yorkshire YO17 8BP, Kate.Storer@adas.co.uk, Tel: +44 (0)1944 738646

6 ³Deceased

7 Word counts:

8 Main text word count (excluding summary, references and legends): 6247

9 Summary: 200

10 Introduction: 788

11 Materials and Methods: 2331

12 Results: 971

13 Discussion: 2125

14 Acknowledgements: 32

15 Number of Figures: 5

16 Number of tables: 2

17 Number of Supporting Information files: 1 (Table S1)

18

19

20 **Summary**

- 21 • Nitrous oxide (N₂O) is a potent, globally important, greenhouse gas, predominantly
22 released from agricultural soils during nitrogen (N) cycling. Arbuscular mycorrhizal
23 fungi (AMF) form a mutualistic symbiosis with two-thirds of land plants, providing
24 phosphorus and/or N in exchange for carbon. Since AMF acquire N, it was
25 hypothesised that AMF hyphae may reduce N₂O production.
- 26 • AMF hyphae were either allowed (AMF) or prevented (non-AMF) access to a
27 compartment containing an organic matter and soil patch in two independent
28 microcosm experiments. Compartment and patch N₂O production was measured both
29 before and after addition of ammonium and nitrate.
- 30 • In both experiments, N₂O production decreased when AMF hyphae were present prior
31 to inorganic N addition. In the presence of AMF hyphae, N₂O production remained
32 low following ammonium application, but increased in the non-AMF controls. In
33 contrast, negligible N₂O was produced following nitrate application to either AMF
34 treatment.
- 35 • Thus, the main N₂O source in this system appeared to be via nitrification and the
36 production of N₂O was reduced in the presence of AMF hyphae. It is hypothesised
37 that AMF hyphae may be out competing slow growing nitrifiers for ammonium. This
38 has significant global implications for our understanding of soil N cycling pathways
39 and N₂O production.

40

41 **Keywords:** agriculture, arbuscular mycorrhizal fungi (AMF), greenhouse gas, hyphosphere, N
42 cycle, nitrification, nitrogen (N), nitrous oxide (N₂O)

43 **Introduction**

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

55

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

68

69 Arbuscular mycorrhizal fungi (AMF) are a key group of soil microorganisms that form
70 symbiotic associations with the majority of land plants (Smith & Read, 2008). Moreover, it is
71 now widely acknowledged that these fungi play a previously unrecognised role in nitrogen
72 (N) cycling, and can both acquire N for their host plant (Barrett *et al.*, 2011; Herman *et al.*,
73 2012) as well as having a substantial N requirement themselves (Hodge & Fitter 2010). There
74 is also evidence for reduced nitrate (NO_3^-) leaching in the presence AMF (Asghari &

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

92

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

104

105 **Materials and Methods**

106 **Microcosm design and growth media**

107 To test the hypothesis that N₂O production was reduced in the presence of AMF hyphae, two
108 experiments were established under glasshouse conditions using compartmented microcosm
109 units. Experiment 1 was designed to determine the impact of AMF hyphae on N₂O production,
110 whereas Experiment 2 was designed to determine whether AMF hyphae affected N₂O produced
111 by nitrification and/or denitrification. Organic matter patches were used to create ‘hotspots’ of
112 N₂O production; a commonly observed phenomenon under natural conditions.

113

114 *Experiment 1*

115 Microcosm units (Fig. 1a) were constructed by joining two 1 L (each 145 x 145 x 70 mm³)
116 plastic containers via a double-mesh membrane of either 20 µm (John Stanier & Co.,
117 Whitefield, Manchester, UK) or 0.45 µm (Osmonics Inc., Minnetonka, USA) pore size. These
118 size membranes either allowed (AMF) or denied (non-AMF) AMF hyphal access between the
119 two compartments. In all cases roots were prevented from passing between the compartments.
120 There were three 6 mm drainage holes in the base of each compartment. In one compartment
121 (the ‘planted’ compartment) a single *Z. mays* seedling (Incredible F1, Mr Fothergills,
122 Newmarket, UK) inoculated with *Rhizophagus irregularis* (PlantWorks Ltd., Kent, UK) was
123 placed, whereas the other compartment contained no plant (the ‘unplanted’ compartment).

124

125 *Experiment 2*

126 Three compartment microcosm units were used (Fig. 1b). Each microcosm consisted of a
127 central ‘planted’ compartment (volume: 2 L, dimensions: 150 x 150 x 150 mm; Thumbs Up
128 Ltd., Bury, UK), containing a single *Z. mays* plant inoculated with *R. irregularis*, and on either
129 side of the central planted compartment, two unplanted compartments separated from the
130 central compartment by a nylon mesh membrane as in Experiment 1 (volume: 2.6 L,
131 dimensions: 140 x 140 x 160 mm; Lock & Lock, Australia PTY Ltd., Blacktown, NSW,
132 Australia). The mesh window either allowed AMF hyphal access (AMF; 20 µm mesh) or
133 prevented AMF hyphal access (non-AMF; 0.45 µm mesh) from the central planted to the outer
134 unplanted compartments. A supporting stainless steel mesh (0.25 mm aperture; Mesh Direct,
135 Hanscan Ltd., Burslem, UK) was placed inside the plant compartment over the nylon meshes
136 (0.45 µm and 20 µm) as a precautionary measure to protect the finer meshes from possible root
137 damage. Thus, each unit had one AMF outer compartment and one non-AMF outer

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

140

141 *Experiments 1 & 2: Growth media*

142 In both Experiments, the planted and unplanted compartments contained a mix (1:1 v/v) of sand
143 and Agsorb® (Agsorb®; Oil-Dri, USA; a calcined attapulgite clay soil conditioner) that had
144 been rinsed thoroughly in deionised water to remove any excess soluble N and/or P. The planted
145 compartments also had 50 g (Experiment 1) or 90 g (Experiment 2) of a fresh *R. irregularis*
146 inoculum (Plantworks Ltd., Kent, UK) and 0.25 g L⁻¹ bonemeal (a complex N and P source to
147 encourage mycorrhizal development; 3.5% N, 8.7% P; Vitax, Leicestershire, UK). Three pre-
148 germinated *Z. mays* seeds were added to each planted compartment for both Experiments on
149 25th June 2012 and thinned to one per pot after 11 d (Experiment 1) or 14 d (Experiment 2). A
150 sterile centrifuge tube (Experiment 1: 15 cm³; Experiment 2: 50 cm³) was added to each of the
151 unplanted compartments to create a hole into which the organic matter patches and gas probes
152 could be added at a later date (see ‘Organic matter patches and gas probes’ section).

153

154 **Growth conditions**

155 Microcosm units were placed in a randomised block design in a heated, lit glasshouse. The
156 Experiments ran for 78 days between 25th June – 10th Sept (Experiment 1) and 103 days between
157 25th June – 5th Oct 2012 (Experiment 2). Photosynthetically active radiation (PAR) was
158 measured weekly for both Experiments at plant level in the centre of each block and averaged
159 141 (Standard Error of the Mean; SEM +/- 15) $\mu\text{mol m}^{-2} \text{s}^{-1}$ (Experiment 1) and 251 (SEM +/-
160 45) $\mu\text{mol m}^{-2} \text{s}^{-1}$ (Experiment 2). Overhead lights were used to extend the photoperiod to 16 h
161 per day and the mean daily temperature over the experimental period was 21.9°C (SEM +/-
162 0.02) (Experiment 1) and 21.5°C (SEM +/- 0.3) (Experiment 2). The planted and unplanted
163 compartments for all microcosm units were watered daily as required. After two weeks of plant
164 growth, the planted compartments received 50 cm³ of a reduced N and P nutrient solution as
165 described by Leigh *et al.* (2009) once a week (Experiment 1 and 2). This was increased to twice
166 weekly at 49 d after planting in Experiment 2 and to full N at 55 d after planting as the plants
167 were starting to show symptoms of N deficiency. In Experiment 2, at 76 d the plants began to
168 show P deficiency symptoms, so a 3/10th P, full N solution was used once a week in addition

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

172

173 **Organic matter patches and gas probes**

174 *Organic matter patches*

175 Organic matter patch material comprised of 13 g dry weight (DW) equivalent agricultural soil
176 (sandy loam; 53°92'N, -1°00'E, pH 6.6 in 0.01M CaCl₂ (following Allen, 1974)) mixed with 2
177 g DW milled *Z. mays* leaves, all enclosed in a 20 µm mesh bag (70 mm x 60 mm). The mean
178 C and N content of the mixed organic patches were 1435 mg (SEM +/- 182) and 116 mg (SEM
179 +/- 15) (Experiment 1) or 1200 (SEM +/- 79) and 99 mg (SEM +/- 15) (Experiment 2)
180 respectively, with a C:N ratio of 12:1 in both Experiments. Each patch contained a gas probe
181 (described below) in the centre (Fig. 1c).

182

183 *Gas probes*

184 A stainless steel tube (9 cm long, outer diameter 1 cm, wall thickness 1 mm; Coopers Needle
185 Works Ltd., Birmingham, UK) was welded at the base to form an airtight seal (Fig. 1c). Two
186 diametrically opposed holes, 6 mm, were drilled through each tube 13 mm from the base. These
187 holes were covered in a PVDF membrane (0.2 µm, Bio-Rad Laboratories Inc., Hercules,
188 California, USA) that was air permeable but impermeable to water. This fine PVDF membrane
189 was then housed in a supporting silicone tube (wall thickness 0.8 mm, outer diameter 8 mm;
190 Silex Ltd., Hampshire, UK) with access holes exposing the membrane covering the holes. The
191 stainless steel tube was then sealed at the top with a white rubber Suba-Seal® (No. 13, Sigma-
192 Aldrich, Poole, UK) to form a gas sampling port. The total internal volume of the gas probe
193 was ca. 4.5 cm³.

194

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

198

199 **Inorganic nitrogen addition**

200 In Experiment 1, half of the organic matter patches were injected with 7 cm³ of 30 mM NH₄NO₃
201 and the other half with 7 cm³ of deionised water ($n = 6$ in each case) at 44 d after patch addition.
202 Consequently, the treatments were: AMF + NH₄NO₃, AMF + water, non-AMF + NH₄NO₃ and
203 non-AMF + water. In Experiment 2, at 62 d after organic patch addition (90 d after planting)
204 each patch was injected with one of 7 cm³ of: 15 mM (NH₄)₂SO₄ (NH₄ treatment), 30 mM
205 KNO₃ (NO₃ treatment), 15 mM K₂SO₄ (K₂SO₄ treatment) or deionised water (water treatment),
206 where the N treatments were equivalent to 0.196 mg N g⁻¹ DW patch ($n = 10$ in each case). In
207 both experiments, two 3.5 cm³ aliquots of solution were injected into each organic patch with
208 an hour gap between each addition to reduce spread into the surrounding sand/Agsorb®.

209

210 **Gas sampling and analysis**

211 *Experiment 1*

212 The air in the gas probes was sampled before N addition at 44 d post-patch addition. The
213 NH₄NO₃ and water addition treatments were then added and the gas probes were sampled again
214 at 24, 48 and 96 h post-NH₄NO₃ addition. Before sample removal, 1 cm³ of N₂ was added to
215 the probe via the Suba-Seal®, taking care not to disturb the surrounding media. This was left
216 for 10 s before a 1 cm³ sample was slowly removed from the gas probe, waiting for a further 5
217 s to allow the sample to mix inside the syringes before removing the syringe. Each gas sample
218 was then stored in a pre-filled 3 cm³ Exetainer (with 6 cm³ N₂), over-pressuring the sample to
219 7 cm³ in total. All gas samples were analysed using a gas chromatograph (GC) which quantified
220 the concentration of N₂O. The concentration (ppm) values for each sample were calculated by
221 comparing to certified standards that were diluted in parallel in a 1 cm³ standard: 6 cm³ N₂ ratio
222 and correcting for this dilution. The concentration values were also corrected for dilution from
223 addition of N₂ to the gas probe just before gas sample removal.

224

225 *Experiment 2*

226 Gas sampling was carried out using both gas probes (as described above for Experiment 1) and
227 continuous flow loop sampling with an attached Los Gatos Isotopic N₂O analyser (LGR N₂O;
228 Los Gatos Research, Inc., California, USA) which provided an N₂O concentration once per

229 second. A gas-tight lid (Fig. 1b) was attached to each of the 80 unplanted compartments in
230 block sequence for a minimum of 5 minutes, with a minimum of 2 min flushing the system with
231 air between each compartment measurement. Gas sampling using both methods was carried out
232 pre-N addition (58-59 and 61 d post-patch addition), and at 48, 96 and 192 h post-N addition
233 (64, 66 and 70 d post-patch addition respectively).

234

235 When using the LGR N₂O analyser, the headspace in the microcosm unit (0.6 L), volume of
236 connecting tubing (0.274 L) and internal volume of the N₂O analyser (0.850 L) along with the
237 surface area of the soil sampled (0.024 m²) were used in the regression calculation of the N₂O
238 flux rate in mg m⁻² h⁻¹. These fluxes were calculated using values measured between 200 to 280
239 s after the cover-box lid was attached. All regressions were calculated using SAS (v9.3 SAS
240 institute Inc., North Carolina, USA).

241

242 **Post-harvest analyses**

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

256

257 **Data analysis**

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

260 SAS (v9.3 SAS institute Inc., North Carolina, USA) or Genstat (v16 VSN International Ltd.,
261 Hemel Hempstead, UK). The pre-N addition fluxes or concentrations were subtracted from the
262 post-N addition fluxes or concentrations respectively to obtain the change in N₂O flux or
263 concentration following N addition (referred to Δ N₂O).

264

265 In Experiment 1, where N₂O concentration and ERM length density data did not fulfil normality
266 or equality of variance assumptions they were log₁₀ transformed. All gas concentration, plant
267 and AMF data were analysed using a two-way ANOVA including block, with Duncan's *post*
268 *hoc* tests. However, transformations on changes in N₂O concentration following N addition
269 failed to normalise the data, and non-parametric equivalent Friedman's two-way ANOVAs,
270 including block, with Wilcoxon *post hoc* tests were used. Where N₂O concentrations were
271 measured over time, repeated measures ANOVA including treatment and block was used on
272 log₁₀ transformed data. Pearson's product moment correlations were used to determine the
273 relationship between variables. Where variables were not normally distributed, Spearman's
274 rank order correlations were used. Untransformed data are presented in all figures.

275

276 In Experiment 2, differences among treatments were analysed using a two-way ANOVA
277 including block with Duncan's *post hoc* tests. ERM length density data were log₁₀ transformed
278 before analysis. Where the data failed normality or equality of variance assumptions, non-
279 parametric tests were used. A one-sample t-test or a Wilcoxon Signed Ranks test was used to
280 compare absolute values or differences to zero.

281

282 In Experiment 2, the Δ N₂O data were not normally distributed and therefore a Friedman's non-
283 parametric two-way ANOVA, controlling for block with Mann Whitney U (unpaired data) or
284 Wilcoxon signed ranks (paired data) *post hoc* tests and an applied false discovery rate correction
285 was used. Where comparisons in Δ N₂O flux or Δ N₂O concentration data were made over time,
286 a non-parametric Friedman's repeated measures analysis was used. The relationship between
287 the Δ N₂O flux and Δ N₂O concentration for each gas sample following N addition (48 h, 96 h
288 and 192 h post-N addition) was determined using a Spearman's rank order correlation.

289

290 There was hyphal breakthrough in one of the non-AMF compartments (treatment: non-AMF,
291 K₂SO₄) therefore this microcosm was excluded from the subsequent data analyses. In
292 addition, the N₂O concentration for one experimental unit in the AMF treatment (treatment:
293 (NH₄)₂SO₄) was out of range on the GC at the 48 h post N addition sample and therefore these
294 AMF and non-AMF N₂O concentration values were also omitted.

295

296 **Results**

297 In the AMF treatments, *R. irregularis* colonized the organic matter patches successfully in
298 both experiments with ERM length densities of $1.23 \pm 0.25 \text{ m g}^{-1} \text{ DW}$ in Experiment 1 (non-
299 AMF: $0.31 \pm 0.05 \text{ m g}^{-1} \text{ DW}$; $F_{1,12} = 30.77$, $P = 0.0001$) and $0.88 \pm 0.08 \text{ m g}^{-1}$ in Experiment
300 2 (non-AMF: $0.35 \pm 0.04 \text{ m g}^{-1} \text{ DW}$; $t_{39} = 8.993$, $P < 0.0001$).

301

302 ***Pre-N addition N₂O production***

303 Prior to inorganic N addition there was a greater concentration of N₂O in the non-AMF
304 patches than in the AMF patches in both experiments (Fig. 2; Experiment 1: $F_{1,12} = 6.46$, $P =$
305 0.026 ; Experiment 2: $S_{38} = -186$, $P = 0.0076$). A similar trend (at the $P < 0.1$ level) was found
306 for the N₂O fluxes in Experiment 2, with greater N₂O fluxes measured from the non-AMF
307 compartments than from the AMF compartments ($S_{38} = -128$, $P = 0.074$). In Experiment 2,
308 N₂O fluxes measured by continuous flow loop sampling were positively correlated with the
309 patch N₂O concentrations measured using gas probes ($r_s = 0.7495$, $P < 0.0001$). Since N₂O
310 production is inherently variable, this level of consistency both between and within
311 experiments is striking, particularly because it was observed in the absence of any additional
312 applied inorganic N. In both experiments, there was no significant correlation between the
313 pre-N addition AMF treatment N₂O concentration or fluxes and the ERM lengths ($P > 0.05$ in
314 each case).

315

316 ***Post N-addition and harvest***

317 ***Experiment 1***

318 In Experiment 1 the highest patch N₂O concentrations were observed 24 h after the
319 application of inorganic N or water in all treatments except AMF + water, demonstrating the

320 rapid response of N₂O producers to treatment application. The patch N₂O concentrations of
321 the non-AMF and AMF + NH₄NO₃ treatments subsequently decreased over time. In contrast,
322 the AMF + water patch N₂O concentration remained low. Consequently, there was a
323 significant effect of both time and treatment on patch N₂O concentration in addition to a
324 significant interaction between these two factors (Fig. 3; time: $F_{2,30} = 4.37$, $P = 0.023$;
325 treatment: $F_{3,15} = 5.67$, $P = 0.0084$; time*treatment: $F_{6,30} = 3.23$, $P = 0.015$). These results
326 therefore demonstrate how rapidly N₂O production rates can change over time and emphasise
327 the requirement for repeated measurements following inorganic N application. Two-way
328 ANOVAs at each time point showed that the N₂O concentration of the AMF + water
329 treatment was lower than all other treatments at 24 h post-treatment application (Fig. 3; $F_{3,15} =$
330 4.44 , $P = 0.020$). This effect decreased by the 48 h sample, although the non-AMF + water
331 and non-AMF + NH₄NO₃ treatments still had a higher N₂O concentration than that of the
332 AMF + water treatment ($F_{3,15} = 4.95$, $P = 0.014$). At 96 h post-treatment application, the AMF
333 patch N₂O concentrations were not significantly different from each other but were
334 significantly lower than those of the non-AMF patches ($F_{3,15} = 7.25$, $P = 0.0031$). At 24 h
335 post-treatment application, the Δ N₂O concentration was higher in both the AMF + NH₄NO₃
336 and non-AMF + NH₄NO₃ treatments than the AMF + water treatment ($Q_3 = 8.2$, $P = 0.042$).
337 However, the non-AMF + water treatment was not significantly different from the AMF +
338 NH₄NO₃ treatment or non-AMF + NH₄NO₃ treatment.

339

340 There was no relationship between the AMF ERM length densities and N₂O concentration in
341 the AMF patches at any point ($P > 0.05$ in each case) and the moisture contents of the organic
342 patches did not differ among treatments at harvest ($Q_3 = 0.05$, $P = 0.827$). Additionally, there
343 was no significant difference ($P > 0.05$) in total plant DW or the DW of the various plant
344 tissues (i.e. leaf, total shoot, stalk, total root, root weight ratio, tassel) between the AMF and
345 non-AMF treatments (see Supporting Information Table S1). Neither the addition of NH₄NO₃
346 or water had any effect on the leaf C and N content or concentrations or the C:N ratios ($P >$
347 0.05 in each case), therefore, these data were combined for comparison of the AMF versus the
348 non-AMF treatments. Leaf C content did not differ between AMF and non-AMF plants
349 (Table 1; $F_{1,12} = 0.30$, $P = 0.595$), although the leaf C concentrations were lower in the AMF
350 compared to the non-AMF treatments (Table 1; $F_{1,12} = 5.37$, $P = 0.039$). Both the N content
351 (Table 1; $F_{1,12} = 14.18$, $P = 0.0023$) and concentration ($F_{1,12} = 20.06$, $P = 0.0008$) of the
352 leaves were higher in the AMF compared to the non-AMF treatments. Consequently, the C:N

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

357

358 ***Experiment 2***

359 There was a significant difference in ΔN_2O fluxes among the inorganic N and water
360 application treatments at 48 h post-application (Fig. 4; $Q_7 = 44.85$, $P < 0.0001$). In both the
361 AMF and non-AMF patches, more N_2O was produced following addition of NH_4^+ than any
362 other treatment. Strikingly, however, ca. 2.5 times more N_2O was produced from the non-
363 AMF than the AMF treatment (Fig. 4; $S_9 = -26.5$, $P = 0.0084$). These differences then
364 declined by the 96 h sample and were no longer significant at the 192 h sample, again,
365 illustrating the transient nature of N_2O release and the importance of following the fluxes over
366 discrete timescales (Table 2). There was no significant difference in the % moisture content of
367 either the patch or sand/Agsorb® medium between the AMF and non-AMF treatments at
368 destructive harvest (patch: $t_{39} = -0.26$, $P = 0.799$; sand/Agsorb®: $S_{39} = -47$, $P = 0.519$).

369

370 **Discussion**

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

379

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

384 10 times larger, in biomass terms, than the intraradical mycelium (Olsson *et al.*, 1999). Thus,
385 the influence of AMF hyphae on soils (in the 'hyphosphere') will extend beyond the zone of
386 influence of roots alone, and studies to date have not explored this widespread zone of hyphal
387 influence on N₂O production in isolation. Furthermore, since the mycorrhizosphere includes
388 both AMF colonised plant roots and AMF hyphae, it is impossible to know whether any effect
389 is a consequence of the AMF hyphae, roots, or both. Rhizodeposition differs between AM and
390 non-AM plants (D.L. Jones *et al.*, 2004), while C exudation from AMF hyphae may also
391 result in quantitative and qualitative changes in the total C flux into the soil (Toljander *et al.*,
392 2007). Moreover, AMF hyphae influence N cycling through the capture of N and subsequent
393 transfer of at least some of this N to their associated host plant (Leigh *et al.*, 2009; Thirkell *et*
394 *al.*, 2016). C and N are key controls of denitrification and nitrification rates (Bollman &
395 Conrad, 1998; Hino *et al.*, 2010). It is not possible, therefore, to separate AMF and root
396 control of N₂O fluxes in the mycorrhizosphere without first separating the AMF hyphae from
397 the plant roots.

398

399 Nevertheless, there is some evidence of AMF interacting with soil N₂O production in the
400 mycorrhizosphere, although results have been inconsistent. Bender *et al.*, (2015) found that
401 the N₂O flux was lower following the application of NO₃⁻ in the AM mycorrhizosphere when
402 compared to the rhizosphere of a non-AM control. In contrast, Cavagnaro *et al.*, (2012) found
403 no effect of AM plants on N₂O production, whereas Lazcano *et al.*, (2014) found a reduction
404 in N₂O in the mycorrhizosphere of AM plants. Thus, there is support for AMF resulting in
405 reduced N₂O production in the mycorrhizosphere, but the cause of this reduction has so far
406 been poorly understood, likely because of confounding effects of the host plant root system
407 also being present. Hypotheses for the decreased N₂O production in the mycorrhizosphere
408 included a reduction in denitrification (Bender *et al.*, 2015), and increased water use by AM
409 plants (Lazcano *et al.*, 2014).

410

411 In this study, the finding of reduced N₂O production in the presence of AMF hyphae was
412 evident even prior to inorganic N application. There was also evidence for an increase in both
413 leaf N content and concentration when the AMF had access to the organic matter patches.
414 This suggests that the AMF were supplying their host plant with additional N, presumably
415 from the organic matter patch since all planted compartments received the same quantity of

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

420

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

433

434 The soil N_2O fluxes in this study were predominantly controlled by the availability of NH_4^+ .
435 These fluxes were monitored at intervals up to 192 hours after inorganic N application by
436 which point the N_2O peak declined back to pre-N application levels, thus ensuring that the full
437 response period was recorded. There was a significantly greater N_2O flux in response to NH_4^+
438 addition in the non-AMF than in the AMF treatment, indicating reduced N_2O production via
439 nitrification in the presence of AMF hyphae. The current understanding of the main pathways
440 of N_2O production in soils (as described in Baggs, 2011, Zhu *et al.*, 2013), are shown in Fig.
441 5 together with the potential mechanisms by which AMF may interact with N_2O production.
442 If NH_4^+ elicits N_2O production but NO_3^- application does not, by process of elimination the
443 pathway involved in N_2O production must be a nitrification pathway.

444

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

447 potential nitrification rates (PNR) were lower in the mycorrhizospheres of AM plants
448 compared to those from weakly-AM mycorrhizospheres (Veresoglou *et al.*, 2011). The *nirK*
449 gene, responsible for N₂O production has also been shown to be negatively correlated with
450 AMF abundance (Bender *et al.*, 2014). Thus, the presence of AM plants may reduce N₂O
451 production by reducing nitrification rates. Our present study demonstrates, for the first time,
452 that AMF hyphae have a direct and limiting influence on soil N₂O produced via nitrification,
453 independent of any plant root influence.

454

455 The main ‘nitrification’ pathways in soil potentially resulting in N₂O release are nitrifier
456 nitrification, and nitrifier denitrification. Nitrifier nitrification is an aerobic process and can be
457 carried out by ammonia oxidising bacteria (AOB), archaea (AOA), and organisms capable of
458 complete ammonia oxidation (comammox) (Daims *et al.*, 2015; van Kessel *et al.*, 2015).
459 AOB and AOA have also been shown to produce N₂O (Jiang and Bakken, 1999; Jung *et al.*,
460 2014). Nitrifier denitrification is also carried out by autotrophic nitrifiers, and can be a
461 significant source of N₂O (Kool *et al.*, 2011b; Wrage *et al.*, 2001). Thus, there are various
462 pathways by which the N₂O in this study may have been produced following the application
463 of NH₄⁺ and consequently reduced by the presence of AMF hyphae (Fig. 5).

464

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

473

474 Domeignoz-Horta *et al.* (2017) found that N₂O hotspots were predominantly controlled by
475 changes in the microbial communities, whereas lower N₂O producing areas were more likely
476 to be controlled by variation in soil properties. Using similar organic patches as in the present
477 study, Nuccio *et al.* (2013) found that while there was no overall change in bacterial diversity,

478 the presence of AMF hyphae significantly modified the bacterial community. Interestingly,
479 Gemmatimonadetes and Deltaproteobacteria were two of four bacterial phylum that had a
480 higher relative abundance in response to the presence of AMF hyphae in the litter (Nuccio *et*
481 *al.*, 2013). Both the Gemmatimonadetes and Deltaproteobacteria have subsequently been
482 found to possess *nosZ* genes, and can, thus, utilise exogenous N₂O as an electron acceptor
483 (C.M. Jones *et al.*, 2013; D. Park *et al.*, 2017). AMF abundance has also been found to
484 positively correlate with *nosZ* gene abundance (Bender *et al.*, 2014). This together with the
485 large export of N from the patch by the AMF hyphae and the resulting modifications in the
486 physico-chemical environment in the decomposing litter patch may all contribute to a
487 reduction in N₂O emissions.

488

489 Given the evidence that AMF are known to have a high N demand (Hodge & Fitter, 2010),
490 one hypothesis may be that AMF hyphae were eliciting a longer term control on the nitrifying
491 community as nitrifiers are inherently slow growing, taking from 8 h up to a number of days
492 to double in number (Belser & Schmidt, 1980; Woldendorp & Laanbroek, 1989; Prosser,
493 2007; Prosser & Nicol, 2012). AMF hyphae are thought to predominantly take up inorganic N
494 in the form of NH₄⁺ (Govindarajulu *et al.*, 2005; Tanaka & Yano, 2005), and AOB are
495 generally thought to be poor competitors for NH₄⁺ (Verhagen *et al.*, 1995; Bollmann *et al.*,
496 2002). The AMF hyphae may therefore have reduced the amount of available NH₄⁺ in the
497 rhizosphere resulting in a reduction in the population of active AOB. If AOB were the main
498 N₂O producers, this may explain the reduced N₂O production prior to inorganic N application
499 when the AMF hyphae were present. It may also explain the lack of N₂O production in the
500 presence of AMF hyphae when NH₄⁺ was applied, i.e. the AOB population may have been
501 small and too slow-growing to respond to the inorganic NH₄⁺ supplied, which may have,
502 instead, been taken up by the N-rich AMF hyphae.

503

504 While AMF may increase or decrease the pH of surrounding media, thought to be a
505 consequence of NO₃⁻ or NH₄⁺ uptake respectively (Bago *et al.*, 1996; Li *et al.*, 1991), the
506 relative importance of pH effects on N₂O production if C, NH₄⁺ or NO₃⁻ are limiting are not
507 clear (reviewed by Šimek & Cooper, 2002) with both increased and decreased nitrification-
508 derived N₂O production reported under low pH conditions (Y. Cheng *et al.*, 2013; Mørkved *et*
509 *al.*, 2007). The patch pH was not measured in this study, and potential changes in pH cannot

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

515

516 In order to fully understand the mechanism for the reduction in N₂O production via
517 nitrification observed in the presence of AMF hyphae found in this study, further research
518 should focus upon gene expression and the responses of the microbial community including
519 nitrifier communities, AOA, AOB and potential non-denitrifying N₂O reducers. Monitoring
520 would also help to establish if nitrifier populations were suppressed by the presence of AMF
521 hyphae, as we suggest. Furthermore, field based studies using a wider range of soil types and
522 environmental conditions are an essential next step to determine the global scale and
523 significance of this interaction in both natural and agricultural systems.

524

525 In conclusion, using two independent glasshouse based experiments, we have found that the
526 presence of AMF hyphae reduced the production of the globally important greenhouse gas,
527 N₂O. Cropped agricultural soils cover a significant proportion of land area, representing
528 28.4% of agricultural land, or 10.9% of the total global land area in 2011 (FAO, 2017). The
529 diversity of AMF is reduced in agricultural soils (Helgason *et al.*, 1998), and these soils are
530 one of the largest contributors to N₂O emissions. This study suggests that a reduction in AMF
531 presence may contribute to further increases in N₂O production. This could have significant
532 implications for better management of agricultural soils in the future. Given the ubiquity of
533 the AM association, including under agricultural situations, these findings have global
534 implications not only for our fundamental understanding of the mechanisms of soil N cycling,
535 but also for greenhouse gas management and climate change mitigation.

536

537 **Acknowledgements**

538 KS was supported by a PhD studentship funded by the Biotechnology and Biological
539 Sciences Research Council (BBSRC), UK, grant BB/GO16801/1, and AC's student placement
540 was supported by the British Mycological Society.

541

542 **Author Contribution**

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

546

547 **References**

548 **Allen SE. 1974.** *Chemical analysis of ecological materials*. Oxford, UK: Blackwell Scientific
549 Publications.

550 **Asghari HR, Cavagnaro TR. 2012.** Arbuscular mycorrhizas reduce nitrogen loss via
551 leaching. *PLoS ONE* **7**: e29825.

552 **Baggs EM. 2008.** A review of stable isotope techniques for N₂O source partitioning in soils,
553 recent progress, remaining challenges and future considerations. *Rapid Communications in*
554 *Mass Spectrometry* **22**: 1664-1672.

555 **Baggs EM. 2011.** Soil microbial sources of nitrous oxide, recent advances in knowledge,
556 emerging challenges and future direction. *Current Opinion in Environmental Sustainability* **3**:
557 321-327.

558 **Bago B, Vierheilig H, Piché Y, Azcón-Aguilar C. 1996.** Nitrate depletion and pH changes
559 induced by the extraradical mycelium of the arbuscular mycorrhizal fungus *Glomus*
560 *intraradices* grown in monoxenic culture. *New Phytologist* **133**: 273-280.

561 **Barrett G, Campbell CD, Fitter AH, Hodge A. 2011.** The arbuscular mycorrhizal fungus
562 *Glomus hoi* can capture and transfer nitrogen from organic patches to its associated host plant
563 at low temperature. *Applied Soil Ecology* **48**: 102-105.

564 **Barrett G, Campbell CD, Hodge A. 2014.** The direct response of the external mycelium of
565 arbuscular mycorrhizal fungi to temperature and the implications for nutrient transfer. *Soil*
566 *Biology & Biochemistry* **78**: 109-117.

567 **Belser LW, Schmidt EL. 1980.** Growth and oxidation kinetics of three genera of ammonia
568 oxidising nitrifiers. *FEMS Microbiology Letters* **7**: 213-216.

569 **Bender SF, Plantenga F, Neftel A, Jocher M, Oberholzer HR, Köhl L, Giles M, Daniell**
570 **TJ, van der Heijden MGA. 2014.** Symbiotic relationships between soil fungi and plants
571 reduce N₂O emissions from soil. *The ISME Journal* **8**: 1336-1345.

572 **Bender SF, Conen F, van der Heijden MGA. 2015.** Mycorrhizal effects on nutrient cycling,
573 nutrient leaching and N₂O production in experimental grassland. *Soil Biology and*
574 *Biochemistry* **80**: 283-292.

575 **Bollmann A, Bär-Gilissen MJ, Laanbroek HJ. 2002.** Growth at low ammonium
576 concentrations and starvation response as potential factors involved in niche differentiation
577 among ammonia-oxidizing bacteria. *Applied and Environmental Microbiology* **68**: 4751-
578 4757.

579 **Bollmann A, Conrad R. 1998.** Influence of O₂ availability on NO and N₂O release by
580 nitrification and denitrification in soils. *Global Change Biology* **4**: 387-396.

581 **Cavagnaro TR, Barrios-Masias FH, Jackson LE. 2012.** Arbuscular mycorrhizas and their
582 role in plant growth, nitrogen interception and soil gas efflux in an organic production system.
583 *Plant and Soil* **353**: 181-194.

584 **Cavagnaro TR, Bender SF, Asghari HR, van der Heijden MGA. 2015.** The role of
585 arbuscular mycorrhizas in reducing soil nutrient loss. *Trends in Plant Science* **20**: 283-290.

586 **Cheng L, Booker FL, Tu C, Burkey KO, Zhou L, Shaw HD, Ruffy TW, Hu S. 2012.**
587 Arbuscular mycorrhizal fungi increase organic carbon decomposition under elevated CO₂.
588 *Science* **337**: 1084-1087.

589 **Cheng Y, Wang J, Mary B, Zhang JB, Cai ZC, Chang SX. 2013.** Soil pH has contrasting
590 effects on gross and net nitrogen mineralizations in adjacent forest and grassland soils in
591 central Alberta, Canada. *Soil Biology and Biochemistry* **57**: 848-857.

592 **Cowan NJ, Norman P, Famulari D, Levy PE, Reay DS, Skiba UM. 2015.** Spatial
593 variability and hotspots of soil N₂O fluxes from intensively grazed grassland. *Biogeosciences*
594 **12**: 1585-1596.

595 **Daims H, Lebedeva EV, Pjevac P, Han P, Herbold C, Albertsen M, Jehmlich N,**
596 **Palatinszky M, Vierheilig J, Bulaev A, et al. 2015.** Complete nitrification by *Nitrospira*
597 bacteria. *Nature* **528**: 504-509.

598 **Domeignoz-Horta LA, Philippot L, Peyrard C, Bru D, Breuil MC, Bizouard F, Justes E,**
599 **Mary B, Léonard J, Spor A. 2017.** Peaks of in situ N₂O emissions are influenced by N₂O –
600 producing and reducing microbial communities across arable soils. *Global Change Biology*
601 **00:** 1-11.

602 **FAO. 2017.** FAOSTAT Agri-Environmental Indicators: Land. Accessed January 2017.

603 **Govindarajulu M, Pfeffer PE, Jin HR, Abubaker J, Douds DD, Allen JW, Bücking H,**
604 **Lammers PJ, Shachar-Hill Y. 2005.** Nitrogen transfer in the arbuscular mycorrhizal
605 symbiosis. *Nature* **435:** 819-823.

606 **Hartmann DL, Klein Tank AMG, Rusticucci M, Alexander LV, Brönnimann S, Charabi**
607 **Y, Dentener FJ, Dlugokencky EJ, Easterling DR, Kaplan A, et al. 2013.** Observations:
608 Atmosphere and Surface. In: Stocker TF, Qin D, Plattner, G-K, Tignor M, Allen SK,
609 Boschung J, Nauels A, Xia Y, Bex V, Midgley PM, eds. *Climate Change 2013: The Physical*
610 *Science Basis. Contribution of Working Group I to the Fifth Assessment Report of the*
611 *Intergovernmental Panel on Climate Change.* Cambridge, UK and NY, USA: Cambridge
612 University Press.

613 **Helgason T, Daniell TJ, Husband R, Fitter AH, Young JPW. 1998.** Ploughing up the
614 wood-wide web? *Nature* **394:** 431.

615 **Herman DJ, Firestone MK, Nuccio E, Hodge A. 2012.** Interactions between an arbuscular
616 mycorrhizal fungus and a soil microbial community mediating litter decomposition. *FEMS*
617 *Microbiology Ecology* **80:** 236-247.

618 **Hino T, Matsumoto Y, Nagano S, Sugimoto H, Fukumori Y, Murata T, Iwata S, Shiro**
619 **Y. 2010.** Structural basis of biological N₂O generation by bacterial nitric oxide reductase.
620 *Science* **330:** 1666-1670.

621 **Hodge A. 2001.** Arbuscular mycorrhizal fungi influence decomposition of, but not plant
622 nutrient capture from, glycine patches in soil. *New Phytologist* **151:** 725-734.

623 **Hodge A. 2014.** Interactions between arbuscular mycorrhizal fungi and organic material
624 substrates. In: S. Sariaslani and G. M. Gadd, eds. *Advances in Applied Microbiology*, Vol. 89,
625 pp. 47-99.

626 **Hodge A, Campbell CD, Fitter AH. 2001.** An arbuscular mycorrhizal fungus accelerates
627 decomposition and acquires nitrogen directly from organic material. *Nature* **413:** 297-299.

628 **Hodge A, Fitter AH. 2010.** Substantial nitrogen acquisition by arbuscular mycorrhizal fungi
629 from organic material has implications for N cycling. *Proceedings of the National Academy of*
630 *Sciences* **107**: 13754-13759.

631 **Hodge A, Storer K. 2015.** Arbuscular mycorrhiza and nitrogen, implications for individual
632 plants through to ecosystems. *Plant and Soil* **386**: 1-19.

633 **Jiang Q-Q, Bakken LR. 1999.** Nitrous oxide production and methane oxidation by different
634 ammonia-oxidising bacteria. *Applied and Environmental Microbiology* **65**: 2879-2884.

635 **Jones CM, Graf DRH, Bru D, Philippot L, Hallin S. 2013.** The unaccounted yet abundant
636 nitrous oxide-reducing microbial community: a potential nitrous oxide sink. *ISME Journal* **7**:
637 417-426.

638 **Jones CM, Spor A, Brennan FP, Breuil M-C, Bru D, Lemanceau P, Griffiths B, Hallin S,**
639 **Philippot L. 2014.** Recently identified microbial guild mediates soil N₂O sink capacity.
640 *Nature Climate Change* **4**: 801-805.

641 **Jones DL, Hodge A, Kuzyakov Y. 2004.** Plant and mycorrhizal regulation of
642 rhizodeposition. *New Phytologist* **163**: 459-480.

643 **Jung M-Y, Well R, Min D, Giesemann A, Park S-J, Kim J-G, Rhee S-K. 2014.** Isotopic
644 signatures of N₂O produced by ammonia oxidising archaea from soils. *The ISME Journal* **8**:
645 1115-1125.

646 **Köhl L, van der Heijden MGA. 2016.** Arbuscular mycorrhizal fungal species differ in their
647 effect on nutrient leaching. *Soil Biology and Biochemistry* **94**: 191-199.

648 **Kool DM, Van Groenigen JW, Wrage N. 2011a.** Source determination of nitrous oxide
649 based on nitrogen and oxygen isotope tracing, dealing with oxygen exchange. *Methods in*
650 *Enzymology* **496**: 139-160.

651 **Kool DM, Dolfing J, Wrage N, van Groenigen JW. 2011b.** Nitrifier denitrification as a
652 distinct and significant source of nitrous oxide from soil. *Soil Biology and Biochemistry* **43**:
653 174-178.

654 **Lazcano C, Barrios-Masias FH, Jackson LE. 2014.** Arbuscular mycorrhizal effects on plant
655 water relations and soil greenhouse gas emissions under changing moisture regimes. *Soil*
656 *Biology and Biochemistry* **74**: 184-192.

657 **Leff B, Ramankutty N, Foley JA. 2004.** Geographic distribution of major crops across the
658 world. *Global Biogeochemical Cycles* **18**: 1-27.

659 **Leigh J, Hodge A, Fitter AH. 2009.** Arbuscular mycorrhizal fungi can transfer substantial
660 amounts of nitrogen to their host plant from organic material. *New Phytologist* **181**: 199-207.

661 **Li XL, George E, Marschner H. 1991.** Phosphorus depletion and pH decrease at the root-
662 soil and hyphae-soil interfaces of VA mycorrhizal white clover fertilized with ammonium.
663 *New Phytologist* **119**: 397-404.

664 **Miller RM, Jastrow JD. 1992.** Extraradical hyphal development of vesicular-arbuscular
665 mycorrhizal fungi in a chronosequence of prairie restoration. In: Read DJ, Lewis DH, Fitter
666 AH, Alexander IJ, eds. *Mycorrhizas in ecosystems*. Wallingford, UK: CAB International,
667 171-176.

668 **Mørkved PT, Dörsch P, Bakken LR. 2007.** The N₂O product ratio of nitrification and its
669 dependence on long-term changes in soil pH. *Soil Biology and Biochemistry* **39**: 2048-2057.

670 **Nuccio EE, Hodge A, Pett-Ridge J, Herman DJ, Weber PK, Firestone MK. 2013.** An
671 arbuscular mycorrhizal fungus significantly modifies the soil bacterial community and
672 nitrogen cycling during litter decomposition. *Environmental Microbiology* **15**: 1870-1881.

673 **Olsson PA, Thingstrup I, Jakobsen I, Bååth E. 1999.** Estimation of the biomass of
674 arbuscular mycorrhizal fungi in a linseed field. *Soil Biology and Biochemistry* **31**: 1879-1887.

675 **Ostrom NE, Ostrom PH. 2011.** The isotopomers of nitrous oxide, analytical considerations
676 and application to resolution of microbial production pathways. In: Baskaran M, ed.
677 *Handbook of environmental isotope geochemistry*, Berlin, Germany: Springer Heidelberg,
678 453-476.

679 **Park D, Kim H, Yoon S. 2017.** Nitrous oxide reduction by an obligate aerobic bacterium
680 *Gemmatimonas aurantiaca* T-27. *Applied and Environmental Microbiology* **83**: e00502-17.

681 **Park S, Croteau P, Boering KA, Etheridge DM, Ferretti D, Fraser PJ, Kim K-R,
682 Krummel PB, Langenfelds RL, van Ommen TD, Steele LP, Trudinger CM. 2012.** Trends
683 and seasonal cycles in the isotopic composition of nitrous oxide since 1940. *Nature*
684 *Geoscience* **5**: 261-265.

685 **Prosser JI. 2007.** The ecology of nitrifying bacteria. In: Bothe H, Ferguson S, Newton WE,
686 eds *Biology of the nitrogen cycle*. Oxford, UK: Elsevier Science, 223-243.

687 **Prosser JL, Nicol GW. 2012.** Archaeal and bacterial ammonia-oxidisers in soil: the quest for
688 niche specialisation and differentiation. *Trends in Microbiology* **11**: 523-531.

689 **Reay DS, Davidson EA., Smith KA, Smith P, Melillo JM, Dentener F, Crutzen PJ. 2012.**
690 Global agriculture and nitrous oxide emissions. *Nature Climate Change* **2**: 410-416.

691 **Sanford RA, Wagner DD, Wu Q, Chee-Sanford JC, Thomas SH, Cruz-García C,**
692 **Rodríguez G, Massol-Deyá A, Krishnani KK, Ritalahti KM, et al. 2012.** Unexpected
693 nondenitrifier nitrous oxide reductase gene diversity and abundance in soils. *Proceedings of*
694 *the National Academy of Sciences* **109**: 19709-19714.

695 **Šimek M, Cooper JE. 2002.** The influence of soil pH on denitrification: progress towards the
696 understanding of this interaction over the last 50 years. *European Journal of Soil Science* **53**:
697 345-354.

698 **Singh BK, Bardgett RD, Smith P, Reay DS. 2010.** Microorganisms and climate change:
699 terrestrial feedbacks and mitigation options. *Nature Reviews Microbiology* **8**: 779-790.

700 **Smith SE, Read DJ. 2008.** *Mycorrhizal Symbiosis*. Cambridge, UK: Academic Press.

701 **Storer KE. 2013.** *Interactions between arbuscular mycorrhizal fungi and soil greenhouse gas*
702 *fluxes*. PhD thesis, University of York, UK.

703 **Tanaka Y, Yano K. 2005.** Nitrogen delivery to maize via mycorrhizal hyphae depends on the
704 form of N supplied. *Plant Cell and Environment* **28**: 1247-1254.

705 **Thirkell TJ, Cameron DD, Hodge A. 2016.** Resolving the ‘nitrogen paradox’ of arbuscular
706 mycorrhizas: fertilization with organic matter brings considerable benefits for plant nutrition
707 and growth. *Plant, Cell & Environment* **39**: 1683-1690.

708 **Thomson AJ, Giannopoulos G, Pretty J, Baggs EM, Richardson DJ. 2012.** Biological
709 sources and sinks of nitrous oxide and strategies to mitigate emissions. *Philosophical*
710 *Transactions of the Royal Society B* **367**: 1157-1168.

711 **Toljander JF, Lindahl BD, Paul LR, Elfstrand M, Finlay RD. 2007.** Influence of
712 arbuscular mycorrhizal mycelial exudates on soil bacterial growth and community structure.
713 *FEMS Microbiology Ecology* **61**: 295-304.

714 **van Kessel MAHJ, Speth DR, Albertsen M, Nielsen PH, Op den Camp HJM, Kartal B,**
715 **Jetten MSM, Lückner S. 2015.** Complete nitrification by a single microorganism. *Nature*
716 **528:** 555-559.

717 **Veresoglou SD, Sen R, Mamolos AP, Veresoglou DS. 2011.** Plant species identity and
718 arbuscular mycorrhizal status modulate potential nitrification rates in nitrogen-limited
719 grassland soils. *Journal of Ecology* **99:** 1339-1349.

720 **Verhagen FJM, Laanbroek HJ, Woldendorp JW. 1995.** Competition for ammonium
721 between plant roots and nitrifying and heterotrophic bacteria and the effects of protozoan
722 grazing. *Plant and Soil* **170:** 241-250.

723 **Woldendorp JW, Laanbroek HJ. 1989.** Activity of nitrifiers in relation to nitrogen nutrition
724 of plants in natural ecosystems. *Plant and Soil* **115:** 217-228.

725 **Wrage N, Velthof GL, van Beusichem ML, Oenema O. 2001.** Role of nitrifier
726 denitrification in the production of nitrous oxide. *Soil Biology and Biochemistry* **33:** 1723-
727 1732.

728 **Wuebbles DJ, Hayhoe K. 2002.** Atmospheric methane and global change. *Earth-Science*
729 *Reviews* **57:** 177-210.

730 **Zhang X, Wang L, Ma F, Shan D. 2015.** Effects of arbuscular mycorrhizal fungi on N₂O
731 emissions from rice paddies. *Water Air and Soil Pollution* **226:** 1-10.

732 **Zhu X, Burger M, Doane TA, Horwath WR. 2013.** Ammonia oxidation pathways and
733 nitrifier denitrification are significant sources of N₂O and NO under low oxygen availability.
734 *Proceedings of the National Academy of Sciences* **110:** 6328-6333.

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

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

738

739

740 **Table 2.** Experiment 2 Friedman’s test statistics controlling for block comparing the post-N minus pre-N (61 days
 741 post-patch addition) patch N₂O concentrations (Δ N₂O concentrations) or compartment N₂O fluxes (Δ N₂O fluxes)
 742 among N addition treatments, for each of the gas sampling events.

	Time since N addition								
	48 h			96 h			192 h		
	<i>Q</i>	df	<i>P</i>	<i>Q</i>	df	<i>P</i>	<i>Q</i>	df	<i>P</i>
Patch Δ N ₂ O concentration	28.89	7	0.0002 ^{***}	14.35	7	0.045 [*]	3.79	7	0.804
Compartment Δ N ₂ O flux	44.85	7	<0.0001 ^{***}	25.63	7	0.0006 ^{***}	4.80	7	0.684

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

745

746

747 **Figure & Table Legends**

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

758

759 **Fig. 2** Mean N₂O concentration (ppm) in AMF access (AMF) and no AMF access (non-AMF)
760 organic matter patches (a) at 43 d post-patch addition in Experiment 1 and (b) at 58 d post-
761 patch addition in Experiment 2. Error bars represent ± standard error of the mean (a: *n* = 12, b:
762 *n* = 39). Different letters represent significant differences at *P* < 0.05 as determined using (a)
763 two-way ANOVAs and (b) by comparing the ΔAMF value to zero (Wilcoxon Signed Rank
764 test).

765

766 **Fig. 3** Mean patch N₂O concentration at 24, 48 and 96 h following inorganic N (NH₄NO₃:
767 filled symbols) or water (open symbols) addition for AMF access patches (AMF; solid lines)
768 and no AMF access patches (non-AMF; dashed lines) shown over time. Error bars represent
769 ± standard error of the mean (*n* = 6). Asterisks represent a significant difference among
770 treatments within each sample period (**P* < 0.05, ***P* < 0.01) as determined using a two-way
771 ANOVA. Different letters within each sample timing represent significant differences
772 between treatments for that sample timing (*P* < 0.05).

773

774 **Fig. 4** Mean difference between 48 h post-N addition (64 d post-patch addition) and pre-N
775 addition (61 days post-patch addition) N₂O flux (ΔN₂O flux) for AMF access (AMF; solid bars)
776 and no AMF access (non-AMF; open bars) treatments, split by N addition treatment. The N
777 addition treatments were: (NH₄)₂SO₄ (labelled as NH₄), KNO₃ (labelled as NO₃), K₂SO₄ or

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

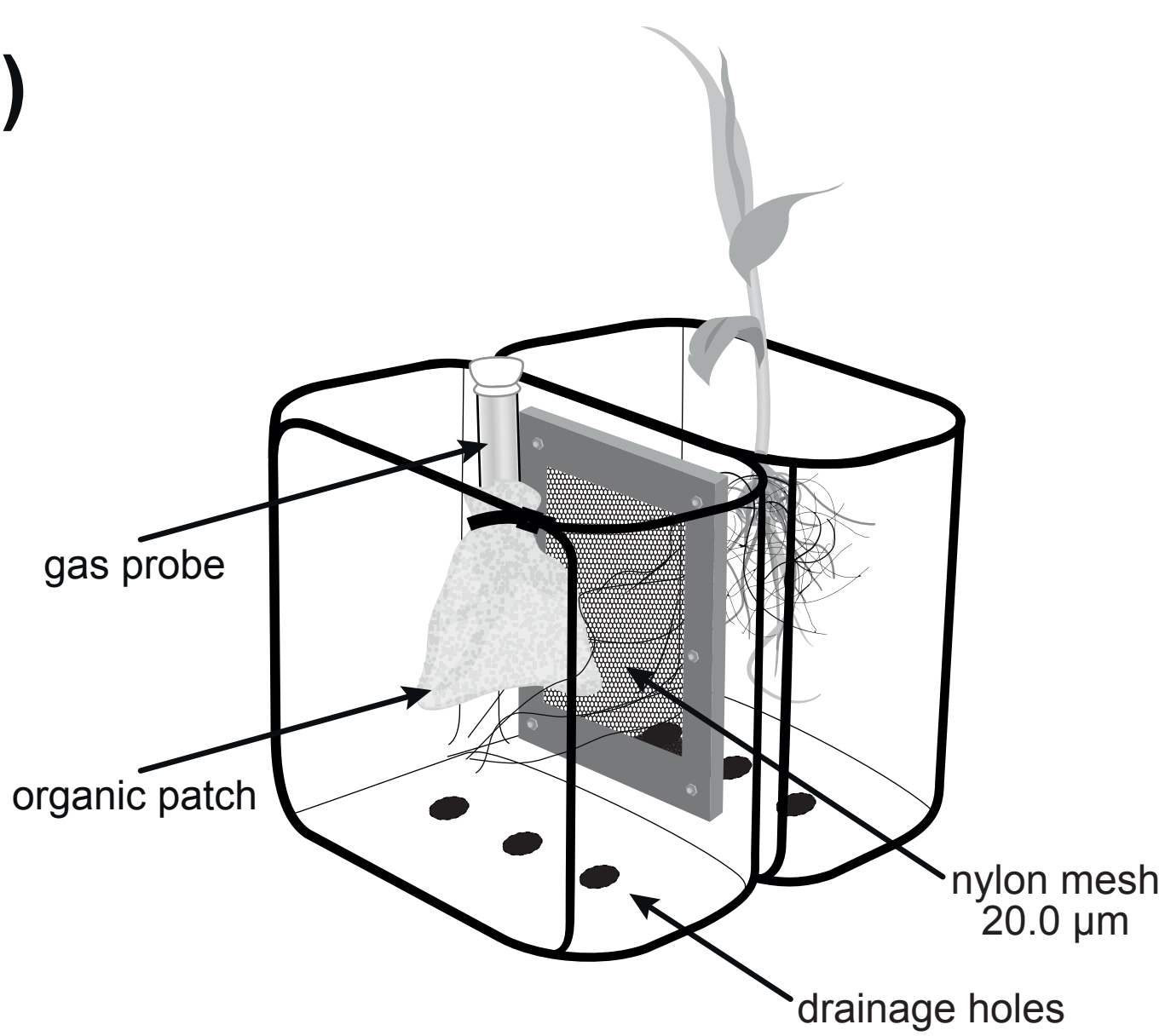
782

783 **Fig. 5** Summarized potential interactions between AMF hyphae and soil N₂O producing
784 processes as described in Baggs (2011) and Zhu *et al.* (2013). The solid and dashed bold lines
785 represent AMF effects that could result in an increase or decrease in N₂O production
786 respectively. AMF can affect the availability of nitrogen (N), phosphorus (P), copper (Cu)
787 and iron (Fe) in soils as well as potentially change soil pH. Nitrifier nitrification is generally
788 carried out by ammonia oxidising bacteria (AOB) and archaea (AOA). Dissimilatory
789 reduction of nitrate to ammonium (DNRA) may produce N₂O as a side product. DNRA is
790 also known as nitrate ammonification. There are various pathways and organisms capable of
791 carrying out these roles, but for simplicity, they are grouped by factors affecting the rate of
792 N₂O production (i.e. availability of O₂, or carbon (C)).

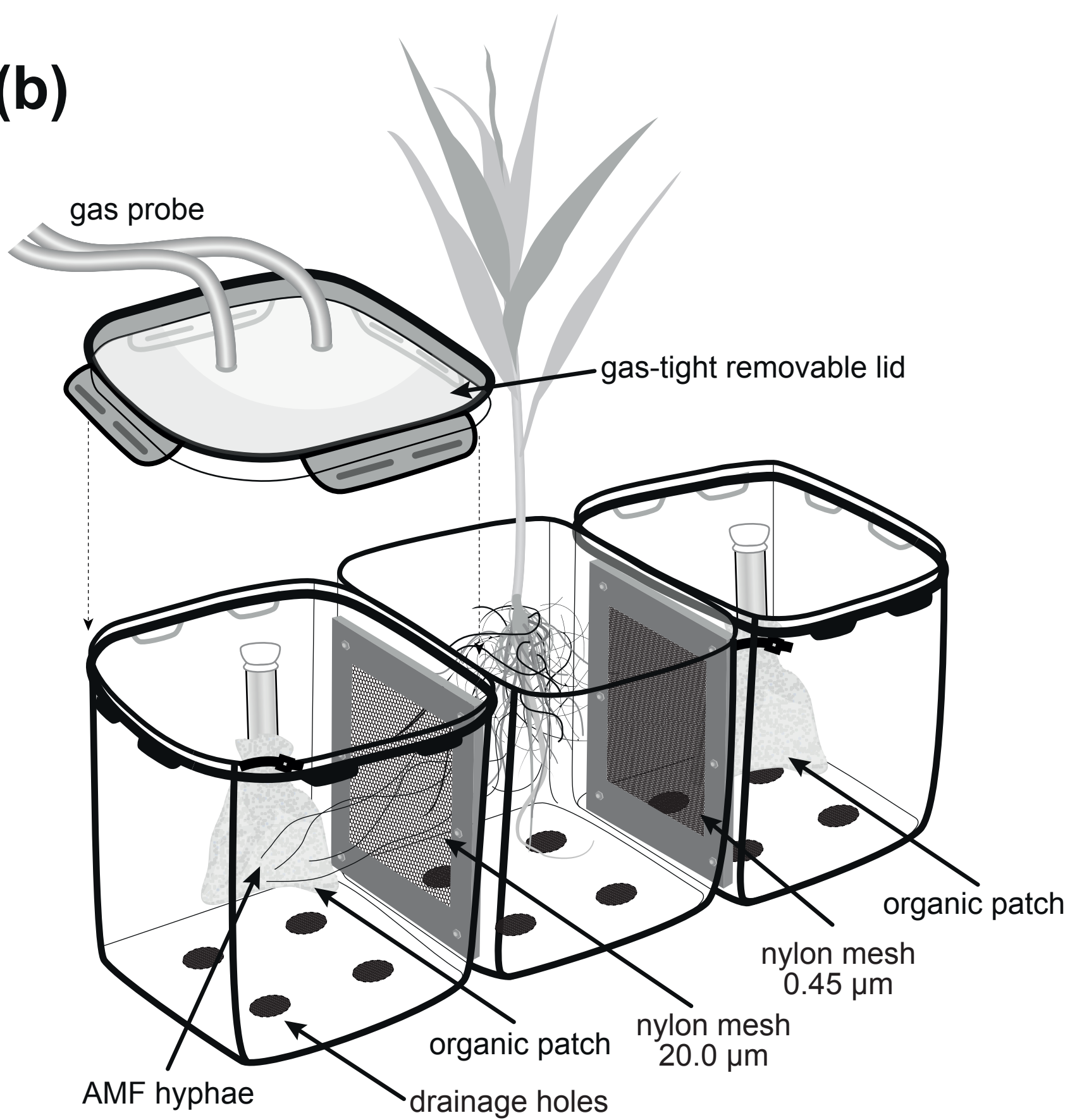
793

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

(a)



(b)



(c)

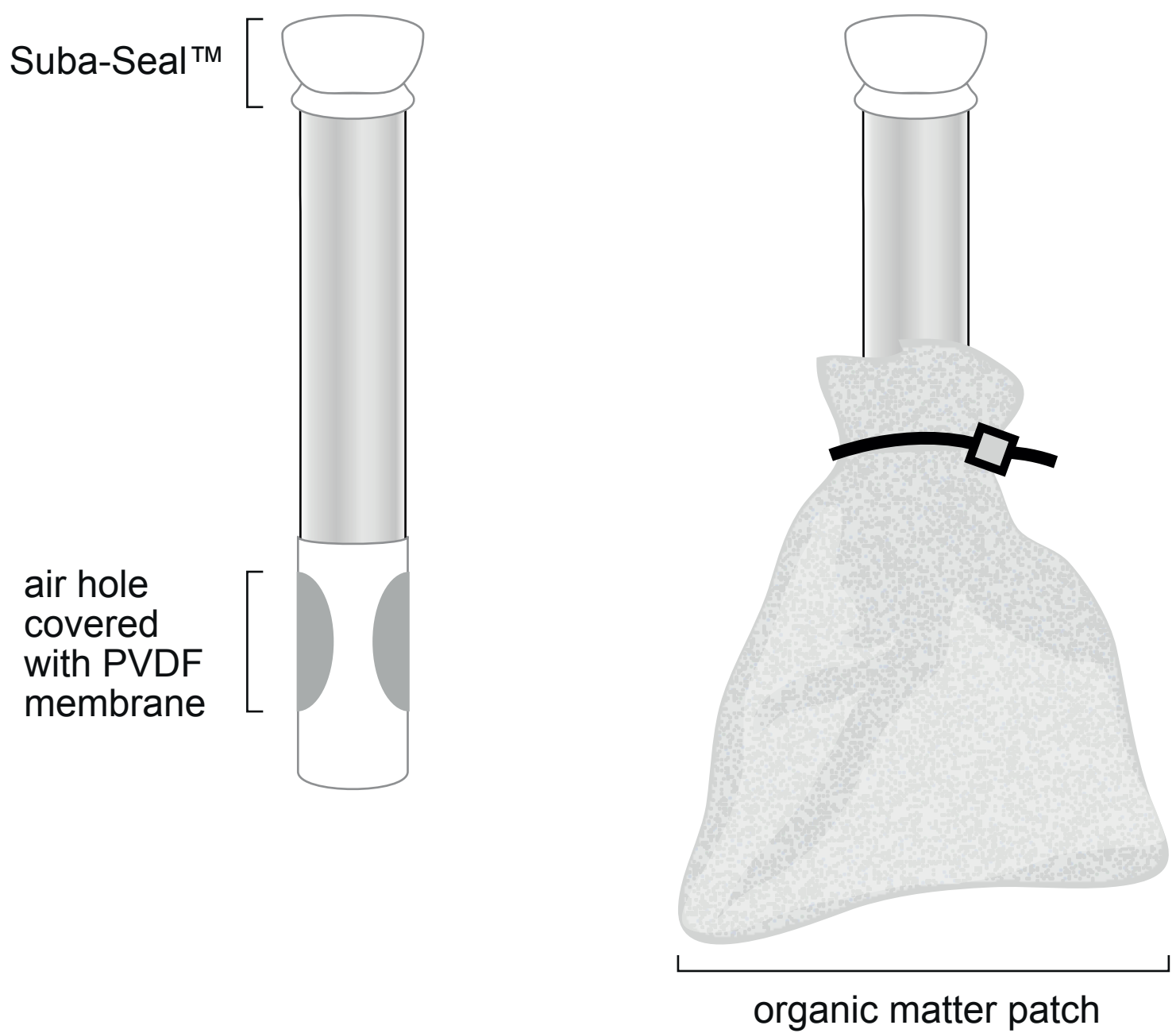


Figure 2

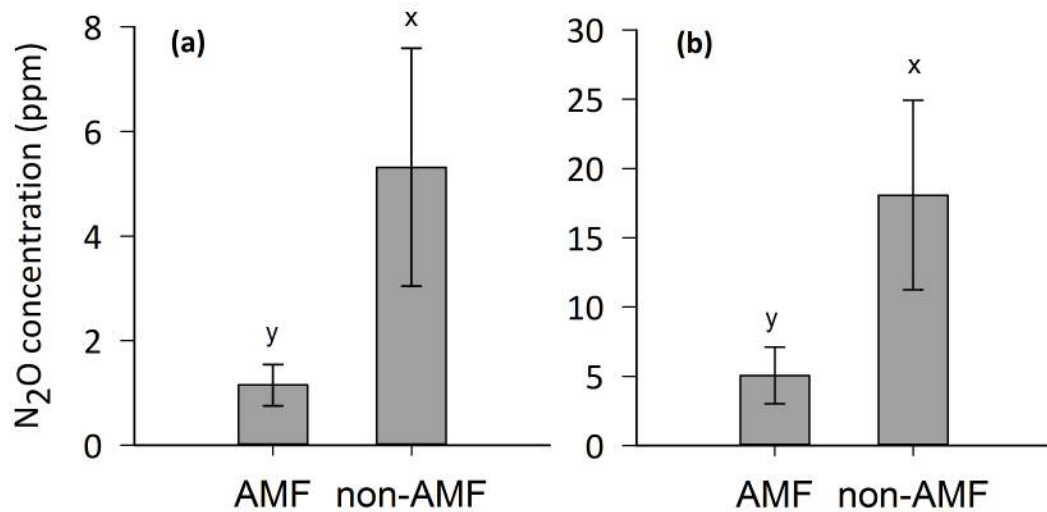


Figure 3.

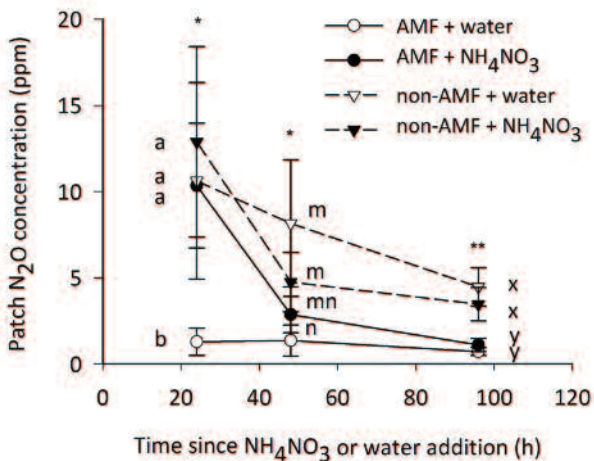


Figure 4.

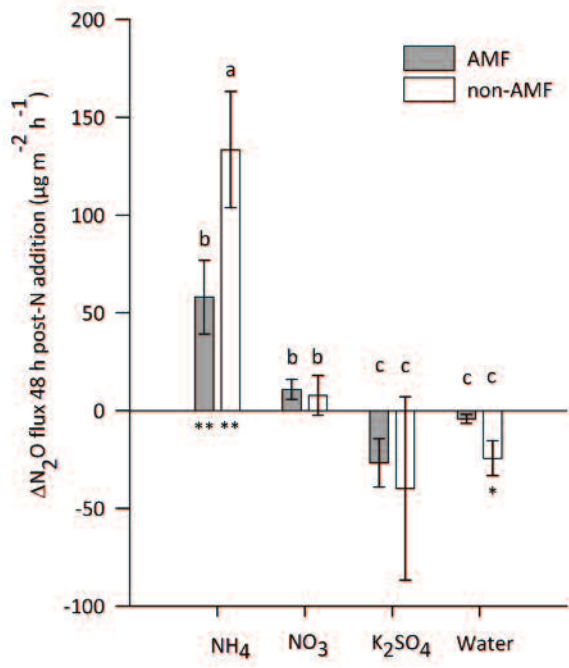
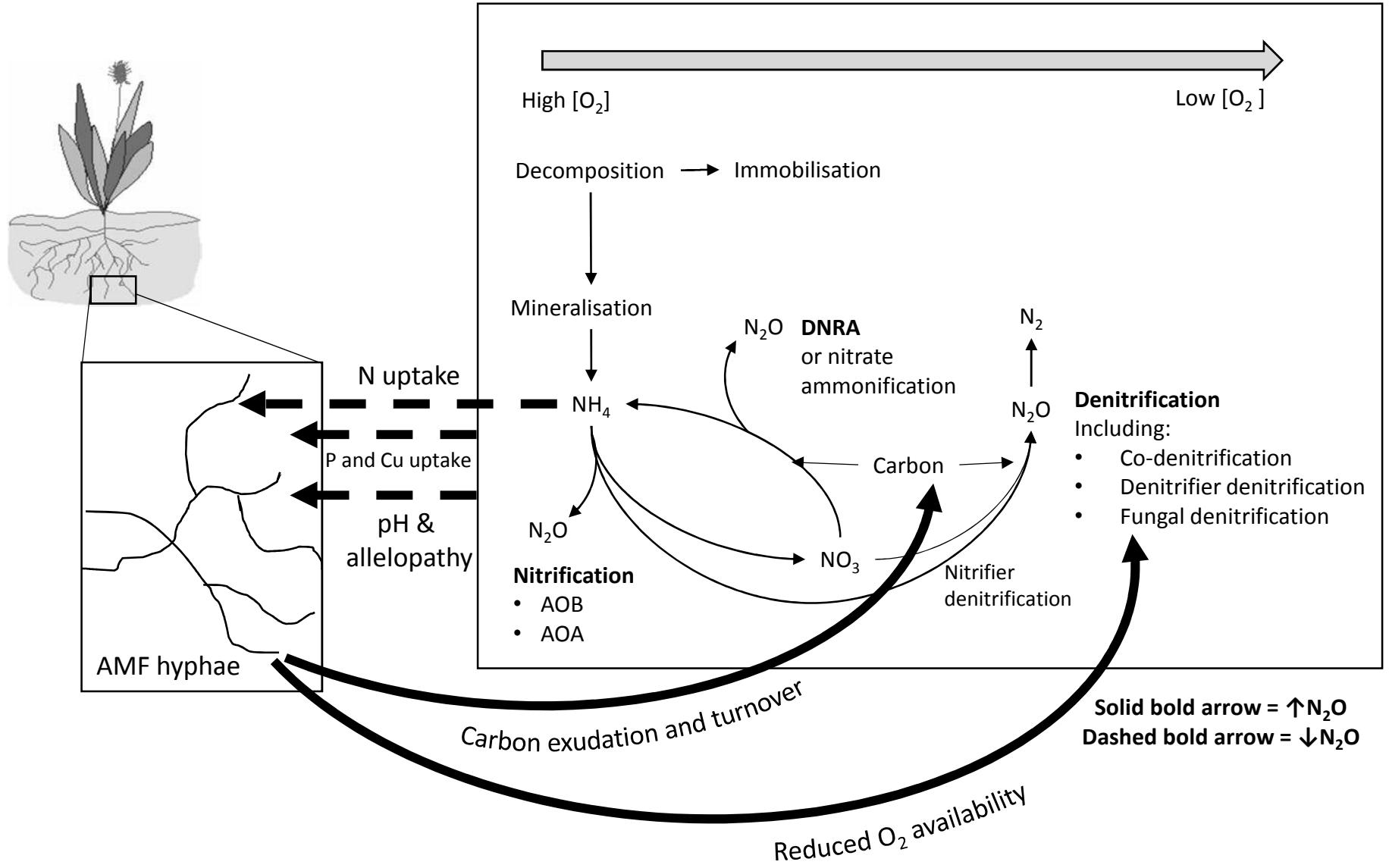


Figure 5



New Phytologist Supporting Information

Article title: **Arbuscular mycorrhizal fungi reduce nitrous oxide emissions via impacts on nitrification.**

Authors: Kate Storer, Aisha Coggan, Phil Ineson, Angela Hodge

The following Supporting Information is available for this article:

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

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