



RESEARCH PAPER

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## Varietal differences in the growth responses of rice to an arbuscular mycorrhizal fungus under natural upland conditions

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### ABSTRACT

Seedlings of three rice (*Oryza sativa* L.) varieties (one indica, ARC5955; and two japonica, Nipponbare and Koshihikari) with or without pre-colonization by the arbuscular mycorrhizal fungus *Funneliformis mosseae* were transplanted into an upland field and grown to maturity. Pre-colonization had no effect on the yield of Nipponbare or Koshihikari. However, pre-colonized ARC5955 exhibited a strong tendency toward increased yield, which was accompanied by increases in the percentage of ripened grain and the 1000-grain weight. The rice roots were also colonized by indigenous arbuscular mycorrhizal fungi in the field, but these had only limited effects on shoot biomass and grain yields. We speculate that *F. mosseae* may have exhibited priority effects, allowing it to dominate the rice roots. There was no significant difference in the contents of most mineral elements in the shoots of pre-colonized ARC5955 at harvest, indicating that some other factor is responsible for the observed yield increase.

**Abbreviations:** AM, arbuscular mycorrhiza or arbuscular mycorrhizal; MGR, mycorrhizal growth response

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## Introduction

There is currently an interest in not only elucidating the molecular mechanisms of symbiosis between rice (*Oryza sativa* L.) and arbuscular mycorrhizal (AM) fungi through laboratory experiments<sup>1,2</sup> but also promoting the growth and increasing the yield of rice in the field through colonization with AM fungi. Although AM fungi are present in paddy wetlands,<sup>3,4</sup> their colonization rate decreases with increased development of aerenchyma in the roots.<sup>5</sup> Therefore, here we cultivated rice plants in an upland region to assess the effects of AM fungi on rice growth, like field experiments using other crops.<sup>6-8</sup>

As a first step toward identifying quantitative trait loci (QTLs), which are important for producing a positive growth response and yield increase in rice, we grew seedlings that had or had not previously been inoculated with the AM fungus *Funneliformis mosseae* (formerly *Glomus mosseae*)<sup>9,10</sup> for 4 weeks in a glasshouse, and looked for a combination of rice varieties that showed high and low growth responses to the fungus.<sup>11</sup> Among the NIAS Global Rice Core Collection,<sup>12</sup> the indica variety ARC5955 showed the strongest positive growth response to colonization by *F. mosseae*, whereas the japonica varieties Nipponbare and Koshihikari exhibited only low response.<sup>11</sup> Furthermore, although seedlings of ARC5955 and Nipponbare exhibited similar changes in their mineral contents following colonization, only ARC5955 experienced growth stimulation, with a particularly large increase in P content.<sup>11</sup>

Therefore, in the present study, we used pre-colonized seedlings of these three rice varieties that were grown to maturity. For field experiments, large amounts of inoculum are often needed.<sup>6,7</sup> However, most of them can be saved through pre-colonization and transplantation of the seedlings. To our knowledge, this is the first trial to evaluate the effects of pre-colonization of AM fungus on the growth and yield of rice under natural upland conditions.

## Results and discussion

In each year of the experiment, we applied less basal dressing than is standard (N, P, and K were c. 40%, 20%, and 20% of the conventional amounts, respectively) and no top dressing to the field to allow the effects of AM colonization to be clearly observed. Four 4-week-old seedlings that were at the 4–5 leaf stages and had not yet started to tiller were transplanted into each plot. The colonization percentages were between 3 and 12% for the pre-inoculated seedlings, whereas no fungal structures were observed in the control seedlings. At this point, the shoot biomass (mg) per plant of non-inoculated controls and that of pre-colonized seedlings were  $110.4 \pm 10.2$  and  $127.0 \pm 6.7$ , respectively, for Nipponbare,  $86.0 \pm 4.1$  and  $106.7 \pm 5.8$ , respectively, for Koshihikari, and  $89.8 \pm 21.1$  and  $141.3 \pm 34.0$ , respectively, for ARC5955. We defined mycorrhizal growth

response (MGR) by the following equation:

$$\text{MGR (\%)} = 100 \times (\text{DW}_{\text{AM}} - \text{DW}_{\text{NM}}) / \text{DW}_{\text{NM}}$$

where  $\text{DW}_{\text{AM}}$  and  $\text{DW}_{\text{NM}}$  were shoot dry weights of AM and non-mycorrhizal seedlings, respectively. The MGR values (%) for Nipponbare, Koshihikari, and ARC5955 were 15.0, 24.1, and 57.3, respectively. The hill spacing was c. 50 cm × 50 cm, to minimize the cross-infection of AM fungi between the roots of different plants, and pre-colonized seedlings were transplanted into a separate bed from non-inoculated control seedlings to prevent the migration of AM fungi (Fig. 1). The mean ambient temperatures during the field experiments are shown in Fig. 2. In 2011, it became hot earlier than average, resulting in a large temperature difference between 2011 and 2012. However, according to the Ministry of Agriculture, Forestry and Fisheries of Japan ([http://www.maff.go.jp/j/study/suito\\_sakugara/h2403/pdf/ref\\_data2-3.pdf](http://www.maff.go.jp/j/study/suito_sakugara/h2403/pdf/ref_data2-3.pdf)), the rice yield indexes in Aichi Prefecture were the same for both years (101).

Nipponbare and Koshihikari form nonglutinous short grains, while ARC5955 forms nonglutinous long grains. Although all of these are intrinsically paddy rice varieties, we grew them in upland fields in the present experiment to remove the effect of waterlogging from the activities of the fungi. Table 1 shows the yield and yield components of the three varieties in 2011 and 2012. For Nipponbare, the pre-colonization of *F. mosseae* had no significant effect on any of the yield and yield components, and there was no interaction between pre-colonization and year; pre-colonization also had a variable effect on grain yield, decreasing it in 2011 and increasing it in 2012. For Koshihikari, the growth was unstable, with all of the yield and yield components showing significant year effects, possibly indicating that the upland and nutrient-poor conditions were particularly unsuitable for this variety; however, it is noteworthy that the pre-colonization of *F. mosseae* also had no significant effects on this variety, with a variable effect on grain

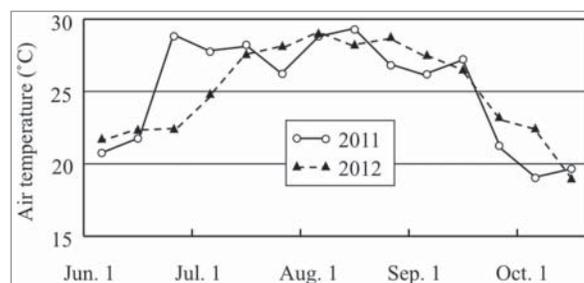


Figure 2. Mean ambient temperatures measured every 10 days at the experimental site at Nagoya during the cultivation periods. Solid and dashed lines represent 2011 and 2012, respectively. Source: Local Meteorological Observatory of Nagoya.

yield again being observed. By contrast, ARC5955 that had been pre-colonized by the AM fungus showed a strong tendency toward increased yield in both 2011 and 2012, although this effect was not statistically significant ( $p = 0.070$ ). Furthermore, the 1000-grain weight of pre-colonized ARC5955 was significantly higher than the control. It is also noteworthy that the percentage of ripened grain was also higher in pre-colonized ARC5955, and this difference was marginally significant ( $p = 0.054$ ). Therefore, the yield increase that was observed in pre-colonized ARC5955 was likely due to combined increases in the percentage of ripened grain and the 1000-grain weight.

During the cultivation period, the rice roots were also colonized by indigenous AM fungi in the field, resulting in the percentage of the root length colonized in non-inoculated controls being quite similar to that of pre-colonized plants (Table 2). Infection by indigenous fungi was also observed in 2013, although to a much lower extent than in 2011. The percentages of the root length colonized in non-inoculated controls and that of pre-colonized plants were  $5.8 \pm 1.2$  and  $16.8 \pm 1.8$ , respectively, for Koshihikari, and  $9.3 \pm 1.2$  and  $23.5 \pm 3.5$ , respectively, for ARC5955. Infection by indigenous AM fungi

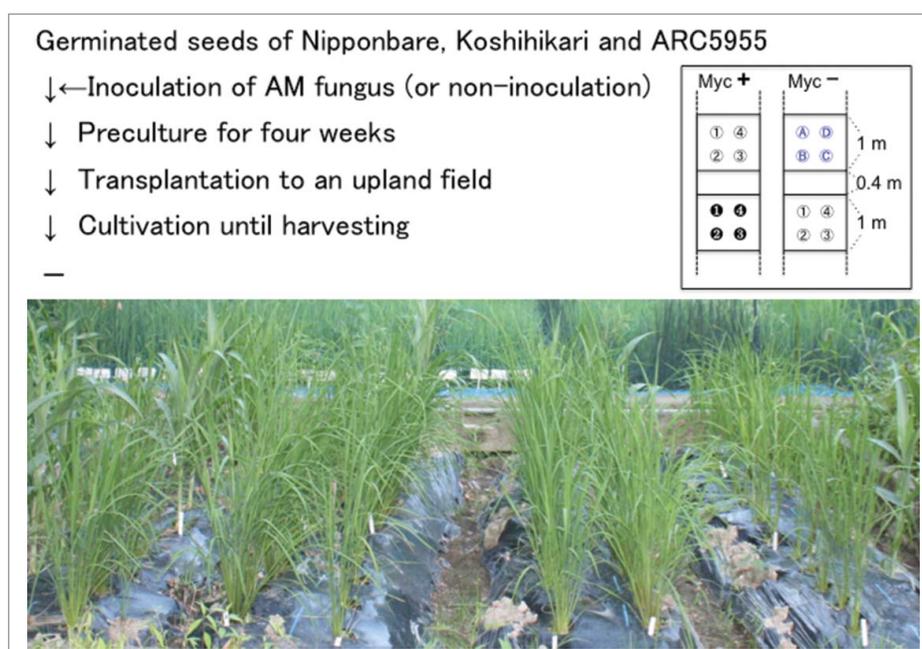


Figure 1. Design of the field experiment conducted in 2011.

**Table 1.** Yield and yield components of the three rice varieties with and without pre-inoculation by *Funneliformis mosseae*.

Variety	Year	<i>F. mosseae</i>	Panicle number hill <sup>-1</sup>	Spiklet number panicle <sup>-1</sup>	Total spikelet number hill <sup>-1</sup>	Percentage of ripened grain (%)	1000-grain weight (g)	Grain yield (g hill <sup>-1</sup> )
Nipponbare	2011	–	14.0 ± 5.3	85.2 ± 12.7	1194.0 ± 270.0	84.7 ± 3.1	23.8 ± 0.5	23.7 ± 4.4
		+	11.9 ± 4.4	94.9 ± 5.3	1096.3 ± 372.4	85.7 ± 1.0	24.0 ± 0.7	22.5 ± 7.8
	2012	–	9.4 ± 0.9	80.8 ± 4.9	751.3 ± 78.7	72.2 ± 10.1	21.8 ± 0.5	12.1 ± 2.1
		+	11.6 ± 4.7	88.0 ± 10.1	1010.0 ± 360.2	84.0 ± 1.9	22.4 ± 0.3	19.0 ± 6.7
ANOVA								
Year			ns	ns	ns	ns (p = 0.051)	***	ns (p = 0.051)
<i>F. mosseae</i>			ns	ns	ns	ns (p = 0.073)	ns	ns
Year x <i>F. mosseae</i>			ns	ns	ns	ns	ns	ns
Koshihikari	2011	–	11.6 ± 2.2	84.3 ± 8.2	976.0 ± 120.9	84.4 ± 0.6	23.5 ± 0.5	19.4 ± 2.8
		+	8.1 ± 1.9	83.7 ± 2.4	670.0 ± 138.1	84.1 ± 1.9	23.3 ± 0.6	13.1 ± 2.6
	2012	–	7.0 ± 1.4	62.0 ± 3.5	369.0 ± 206.5	62.0 ± 3.5	22.0 ± 0.6	7.5 ± 2.2
		+	6.7 ± 0.7	64.8 ± 1.6	430.7 ± 55.3	64.8 ± 1.6	22.3 ± 1.1	8.2 ± 0.9
Year			*	***	***	***	*	***
<i>F. mosseae</i>			ns	ns	ns	ns	ns	ns
Year x <i>F. mosseae</i>			ns	ns	ns	ns	ns	*
ARC5955	2011	–	24.4 ± 2.5	177.2 ± 23.0	4390.7 ± 942.1	89.0 ± 1.2	15.7 ± 0.3	62.3 ± 14.0
		+	26.8 ± 4.7	188.0 ± 18.3	5016.7 ± 1236.3	93.2 ± 1.2	15.9 ± 0.4	73.7 ± 18.8
	2012	–	26.8 ± 5.2	141.2 ± 12.6	3826.0 ± 814.7	77.2 ± 10.5	13.7 ± 1.1	41.1 ± 15.5
		+	37.1 ± 8.0	139.2 ± 12.5	5271.0 ± 1194.7	87.3 ± 2.8	15.4 ± 0.2	70.7 ± 18.9
Year			ns (p = 0.079)	**	ns	*	**	ns
<i>F. mosseae</i>			ns	ns	ns	ns (p = 0.054)	*	ns (p = 0.070)
Year x <i>F. mosseae</i>			ns	ns	ns	ns	ns (p = 0.078)	ns

Values are means ± SD (n = 3). \*p < 0.05; \*\*p < 0.01; \*\*\*p < 0.001; ns, not significant.

and yield increase by beneficial inoculum are in accord with the results of field experiments using alfalfa,<sup>6</sup> maize,<sup>7</sup> soybean and cotton.<sup>8</sup> Jeong et al. previously performed pot experiments under upland conditions, which demonstrated that indigenous AM fungi colonize rice plants, resulting in the upregulation of AM marker genes and AM-inducible phosphate transporter genes.<sup>13</sup> Unfortunately, however, we were unable to discriminate between pre-colonized and indigenous AM fungi under the microscope when we conducted the experiments. The differences between our results of 2011 and 2013 may have been due to the use of fallow upland that was full of weeds in 2011, as fallowed land often results in higher root colonization by AM fungi.<sup>13,14</sup> Alternatively, it could have resulted from the use of an upland field that had freshly been converted from a paddy field in 2013. However, considering these results, we suggest that the roots of control plants in 2012 will also have been colonized by indigenous AM fungi.

Indigenous AM fungi appeared to have only a limited effect on grain yield, as the yield of ARC5955 was repeatedly found

to increase with pre-colonization by *F. mosseae*. This may be because the indigenous AM fungi were not effective in improving the growth of rice plants as it is well known that the plant growth response to AM fungi depends on a combination of the plants and the fungi,<sup>15</sup> and that there are competitive interactions between different AM fungi during root colonization.<sup>16–18</sup> Another possibility is that an effect of each AM fungus on the grain yield might have been masked by effects of other fungi, because indigenous AM fungi consist of many species. We also speculate that *F. mosseae* may have exhibited priority effects, in which the AM fungus that initially colonizes the roots determines the eventual microbial population in them.<sup>19,20</sup> Such an effect was recently demonstrated by Werner and Kiers in a glasshouse experiment with special pots, which showed that the initially inoculated AM fungus (the resident) dominates the plant roots, while another later inoculated AM fungus (the invader) fails to colonize them, likely due to host downregulation of the second fungal infection.<sup>20</sup> Those effects can occur not only for growth-promoting fungi but also for growth-depressing fungi.<sup>21</sup> Thus, in the present study, rice plants pre-colonized by *F. mosseae*, which is a beneficial fungus for ARC5955, may have blocked infection of indigenous fungi in the field. Newly established methods such as quantitative PCR targeting fungal mitochondrial genome<sup>22</sup> and high-resolution discrimination of fungal ribosomal DNA<sup>23</sup> may be useful to confirm the occurrence of such priority effects. Alternatively, *F. mosseae* will need to be tagged with a unique molecular marker that is not found in any indigenous fungi.

In 2012, we also examined the shoot biomass of the three varieties at harvest. The shoot biomass (g) per plant of non-inoculated controls and that of pre-colonized plants were 49.4 ± 18.6 and 76.0 ± 33.5, respectively, for Nipponbare,

**Table 2.** Colonization of rice roots by arbuscular mycorrhizal (AM) fungi in 2011 at harvest. – and + indicate plants that were not inoculated and pre-inoculated with *Funneliformis mosseae*, respectively. Values are means ± SD (n = 3).

Variety	<i>F. mosseae</i>	Total colonization (%)	Specific internal colonization		
			Intraradical hyphae (%)	Arbuscule (%)	Vesicle (%)
Nipponbare	–	46.8 ± 7.9	43.1 ± 8.5	3.4 ± 3.1	0.7 ± 1.2
	+	64.6 ± 0.6	58.0 ± 5.3	1.3 ± 1.1	6.0 ± 5.3
Koshihikari	–	51.3 ± 3.0	48.0 ± 2.0	2.7 ± 1.1	0.7 ± 1.1
	+	63.9 ± 1.2	59.3 ± 3.1	2.0 ± 0.0	3.3 ± 2.3
ARC5955	–	73.3 ± 2.1	66.7 ± 6.1	4.7 ± 4.6	2.7 ± 1.1
	+	78.0 ± 6.0	76.7 ± 7.0	1.3 ± 1.1	0.0 ± 0.0

**Table 3.** Mineral contents ( $\mu\text{g/g DW}$ ) of the aboveground parts of rice at harvest. – and + indicate plants that were not inoculated and pre-inoculated with *Funneliformis mosseae*, respectively. Values are means  $\pm$  SD ( $n = 3$ ).

Variety	<i>F. mosseae</i>	N	P	K	Ca	Fe	S
Nipponbare	–	25567 $\pm$ 7315	1082 $\pm$ 135	17502 $\pm$ 2814	1894 $\pm$ 400	199 $\pm$ 26	1163 $\pm$ 250
	+	25567 $\pm$ 8541	1038 $\pm$ 180	20609 $\pm$ 4043	1827 $\pm$ 261	192 $\pm$ 86	1141 $\pm$ 192
Koshihikari	–	26933 $\pm$ 2511	1153 $\pm$ 249	15930 $\pm$ 3509	2003 $\pm$ 238	168 $\pm$ 30	1435 $\pm$ 438
	+	24400 $\pm$ 10739	1130 $\pm$ 491	13487 $\pm$ 5042	1669 $\pm$ 477	194 $\pm$ 118	1424 $\pm$ 180
ARC5955	–	38800 $\pm$ 9111	1422 $\pm$ 334	15036 $\pm$ 2859	1697 $\pm$ 81	250 $\pm$ 70	1258 $\pm$ 185
	+	29700 $\pm$ 3904	1234 $\pm$ 182	16494 $\pm$ 1422	2034 $\pm$ 257	301 $\pm$ 132	964 $\pm$ 151

Variety	<i>F. mosseae</i>	Mg	Mn	Cu	Zn	Al
Nipponbare	–	1620 $\pm$ 260	95 $\pm$ 8	10.2 $\pm$ 2.3	59.1 $\pm$ 19.0	1763 $\pm$ 99
	+	1641 $\pm$ 52	121 $\pm$ 37	15.1 $\pm$ 6.9	36.4 $\pm$ 10.7	1477 $\pm$ 596
Koshihikari	–	1673 $\pm$ 165	140 $\pm$ 14	11.9 $\pm$ 7.8	59.8 $\pm$ 40.9	943 $\pm$ 177
	+	1549 $\pm$ 475	97 $\pm$ 28	6.2 $\pm$ 1.6	33.7 $\pm$ 7.3	1689 $\pm$ 917
ARC5955	–	1846 $\pm$ 215	86 $\pm$ 21	21.0 $\pm$ 14.6	47.6 $\pm$ 4.1	1416 $\pm$ 323
	+	1855 $\pm$ 174	101 $\pm$ 10	9.4 $\pm$ 2.3	35.3 $\pm$ 1.1**	1701 $\pm$ 650

\*\*significance at  $p < 0.01$ . A one-way ANOVA was used to analyze effects of pre-colonization.

19.3  $\pm$  8.7 and 22.8  $\pm$  2.0, respectively, for Koshihikari, and 126.9  $\pm$  25.8 and 182.2  $\pm$  34.0, respectively, for ARC5955. Interestingly, we found that pre-colonization by *F. mosseae* resulted in similar changes in grain yield and shoot biomass. Thus, the effects of the indigenous AM fungi again appeared to be limited, even if they infected rice roots during the cultivation period.

To understand why the shoot biomass and grain yield of pre-colonized ARC5955 increased, we measured the shoot mineral contents using ICP-AES. This showed that the Zn content was significantly lower in pre-colonized plants, while the other 10 elements remained at similar levels (Table 3). These results are in contrast with increased contents of P and N by inoculation of other crops with AM fungi.<sup>6,8</sup> It is currently difficult to explain why a reduced Zn content would increase the grain yield, and so we cannot exclude the possibility that the different growth responses of plants may have been caused by some other factor, such as different plant hormone levels. Alternatively, the yield increase may have simply been a secondary effect of the excellent growth of ARC5955 seedlings due to *F. mosseae* colonization<sup>11</sup> – indeed, there is an old saying in Japan, “Nae Hansaku,” which means “If healthy seedlings are prepared, good yield of rice grains is guaranteed by half.” Therefore, a QTL analysis between ARC5955 and a japonica variety may help to clarify the mechanism of yield increase by *F. mosseae*, which, in turn, may be effective for improving the yield of good tasting japonica rice through the use of AM fungi in sustainable agricultural systems.

## Materials and methods

Seeds of the indica rice variety ARC5955 and the japonica rice varieties Nipponbare and Koshihikari were germinated and then transplanted individually into 40-mL cells in a plastic tray containing Kanuma soil. A soil inoculant of *F. mosseae* was a kind gift from Idemitsu Kosan (Tokyo, Japan). We initially found that the contaminating microorganisms in the filtrate of a soil inoculant of *F. mosseae* that had been passed through 38- $\mu\text{m}$  mesh<sup>10</sup> had no significant effect on plant growth. Therefore, we directly added the entire inoculant to the soil by

shaking 1 L of Kanuma soil (c. 420 g) and 45 mL (30 g) of the *F. mosseae* inoculant in a plastic bag and then adding 40 mL of the mixture to each cell for pre-colonization. For the control, 40 mL of pure Kanuma soil was added to the cell. The seedlings were grown in a cultivation room that was maintained at 28°C under a 16 h day / 8 h night cycle at a light intensity of 204  $\mu\text{mol m}^{-2} \text{s}^{-1}$  for 4 weeks. Watering was carried out as required and the soil in each cell was supplemented twice during the cultivation period with 20 mL of 0.5 $\times$  modified Hoagland solution, which contained 100  $\mu\text{M}$  phosphate.

The field experiments were carried out at the Higashiyama Campus of Nagoya University, Aichi Prefecture, Japan (35° 1' N, 137° 0' E, 61 m a.s.l.) from 2011 to 2013. In 2011, we performed the experiments in a fallow upland area that had been covered with weeds in the previous year, whereas in 2012 and 2013, we used an upland area that had been converted from flooded paddy fields. The land was plowed twice with a small tilling machine, and basal dressing (N, P, K = 2.2, 1.7, 1.5 g  $\text{m}^{-2}$ ) was applied as a 1:1 mixture (w/w) of a quick-acting compound fertilizer (Toho Co., Osaka, Japan) and the coated fertilizer Hitomaki-kun (JA Aichi, Nagoya, Japan). We then constructed beds that were 40 cm high  $\times$  1 m wide with 60-cm ditches between them, and covered these with thin black polyethylene sheets as mulch (Fig. 1). At the beginning of June, we transplanted the pre-inoculated and non-inoculated (control) seedlings into different beds. Each field was laid out in a split-plot design with three replications, whereby each plot (1 m long  $\times$  1 m wide) consisted of four plants (Fig. 1). The plants were grown until harvest at the end of October. Watering was carried out as required and no top dressing was applied during the cultivation period.

In 2011 and 2012, we harvested three and four hills, respectively, in each plot. All panicles were counted and threshed by hand, and filled and unfilled spikelets were separated and counted. After oven-drying, we determined the 1000-grain weight and calculated the grain yield for the filled unhulled grains. Unfortunately, no yield measurements could be made in 2013 because two typhoons occurred in September, severely damaging the rice plants.

In 2012, all of the stems and leaves on each of the sampled hills were also oven-dried and combined with the unhulled

grains to determine shoot biomass. The combined materials were then pulverized with a small electric crusher and ground with a mortar and pestle. A portion of each ground sample was digested by heating it with HNO<sub>3</sub>, allowing the inorganic element contents to be determined using inductively coupled plasma atomic emission spectroscopy (ICP-AES; IRIS ICAP, Nippon Jarrell Ash, Tokyo, Japan). Another portion of the ground sample was digested by heating it with NaOH and K<sub>2</sub>S<sub>2</sub>O<sub>5</sub> and then neutralized with HCl, allowing the nitrate content to be determined spectrophotometrically, from which we calculated the nitrogen content.

In 2011 and 2013, portions of the roots were also collected at harvest, cleared with KOH, and stained with trypan blue, following which we determined the root length colonization by AM fungi (%), as described by McGonigle et al.<sup>24</sup>

We analyzed the data statistically using analysis of variance (ANOVA).

### Disclosure of potential conflict of interest

No potential conflicts of interest were disclosed.

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