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Spore Density of Arbuscular Mycorrhizal Fungi is Fostered by Six Years of a No-Till System and is Correlated with Environmental Parameters in a Silty Loam Soil

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Abstract: Arbuscular mycorrhizal fungi (AMF) play major roles in nutrient acquisition by crops and are key actors of agroecosystems productivity. However, agricultural practices can have deleterious effects on plant–fungi symbiosis establishment in soils, thus inhibiting its potential benefits on plant growth and development. Therefore, we have studied the impact of different soil management techniques, including conventional moldboard ploughing and no-till under an optimal nitrogen (N) fertilization regime and in the absence of N fertilization, on AMF spore density and soil chemical, physical, and biological indicators in the top 20 cm of the soil horizon. A field experiment conducted over six years revealed that AMF spore density was significantly lower under conventional tillage (CT) combined with intensive synthetic N fertilization. Under no-till (NT) conditions, the density of AMF spore was at least two-fold higher, even under intensive N fertilization conditions. We also observed that there were positive correlations between spore density, soil dehydrogenase enzyme activity, and soil penetration resistance and negative correlations with soil phosphorus and mineral N contents. Therefore, soil dehydrogenase activity and soil penetration resistance can be considered as good indicators of soil quality in agrosystems. Furthermore, the high nitrate content of ploughed soils appears to be detrimental both for the dehydrogenase enzyme activity and the production of AMF spores. It can be concluded that no-till, by preventing soil from structural and chemical disturbances, is a farming system that preserves the entire fungal life cycle and as such the production of viable spores of AMF, even under intensive N fertilization.

Keywords: tillage; nitrogen fertilization; arbuscular mycorrhizal fungi; spore density; microbial activity

1. Introduction

Symbiosis between arbuscular mycorrhizal fungi (AMF) and plants arose on earth more than 400 million years ago [1]. The roots of the majority of land plant species are colonized by these fungi, extending the prospecting capacity of plants into the surrounding soil through mycorrhizosphere

(a network formed by root-like extensions of the fungi known as hyphae), where the fungal spores are also formed [2]. It is commonly accepted that AMF develop obligate symbioses with at least 65% of vascular plants [3], including a large number of cereals grain crops and legumes, thus increasing their ability to acquire nutrients. However, AMF are particularly sensitive to physical, chemical, and biological disturbances caused by human activities that limit their establishment in agrosystems. Although it has been shown that tillage (through aggregate disruption [4]) and N fertilization can reduce the colonization of crops by arbuscular mycorrhizal fungi [5–7], our knowledge of the factors that determine the successful establishment of an AMF symbiosis remains limited. Spore density of AMF together with the level of hyphal growth and branching are critical for successful root colonization [6,8]. The density of spores has been defined as an early and useful indicator of AMF colonization potential [9]. Spore density determines the capability of AMF to resist ecological and physical disturbances, such as periods during which suitable host plants are not present or following intensive tillage, as they both limit the ability of AMF to colonize the subsequent cultivated crops [10]. In addition, among AMF species, there are different levels of tolerance to the disruption of hyphae resulting from tillage [11]. Inoculation with AMF spores has recently been shown to increase plant growth [12] and the expression of nitrate and phosphate transporter genes in wheat roots [13]. Moreover, inoculation can be used to offset tillage effects on the number of indigenous spores. However, there is still a paucity of knowledge concerning the potential ecological impact resulting from propagule inoculation [6], as such methods could increase the competition between inoculated fungi and previously established indigenous AMF communities [14]. Therefore, the implementation of alternative farming practices that promote natural production of viable AMF spores, and thus the occurrence of an efficient symbiosis with crop plants, needs to be studied further. The aim of this study was to evaluate the impact of alternative farming practices on AMF spore density in order to propose if such practices could be a way to increase root mycorrhizal colonization and thus crop mineral nutrient use efficiency in the context of agricultural sustainability.

2. Results and Discussion

Monitoring the effects of contrasting agrosystems, both in terms of mineral fertilization and tillage practices on AMF development could lead to a better understanding of such an important biological process and improve soil fertility. In the present work, the effect of tillage under zero or optimal N fertilization on AMF spore density was investigated over a six-year period. Moreover, we have examined if there was any relationship between the AMF spore density and soil chemical as well as biological parameters.

When multiple comparisons were performed using the Conover post-hoc tests, soil DH activity was significantly higher (35%) in the absence of tillage and N fertilization (Table 1). In ploughed soils, the concentration of PO_4^{3-} was also slightly higher, but only in the absence of N fertilization. We also observed that both plant NO_3^- and soil soluble NO_3^- contents were approximately 3- and 2-fold higher, respectively, in ploughed soils as compared to those managed under no-till (NT) conditions, irrespective of the N fertilization regime. The higher NO_3^- content found in the water collected from lysimeters suggests that inorganic N availability for microorganisms was higher in ploughed soils during the inter-cropping period. The lower soil NO_3^- content in the NT plots could result from a greater uptake by soil microorganisms. Moreover, it has been shown that substantial amounts of soil inorganic N can be taken up by microorganisms [15] and that NT enhances microbial biomass as compared to conventional tillage (CT) [16]. Another hypothesis is that increased mineralization of soil organic matter pools, caused by the annual moldboard ploughing [17,18], leads to an accumulation of NO_3^- in CT plots. However, the DH enzyme activity, which reflects the metabolic state of microorganisms in soils [19], was higher in NT and N0 plots as compared to those that were ploughed or fertilized. The finding that NT-N0 soils, characterized by the lowest concentration of nutrients (i.e., available phosphorus and nitrate), are also the most microbiologically active is supported by the results obtained by Nivelle et al. [20], and is consistent with those obtained by Das et al. [21],

who also observed that the DH enzyme activity was approximately twice as high under NT than under CT conditions.

The highest value of penetration resistance (SPR) was obtained under NT conditions in N-fertilized soils (1.265 MPa), while the lowest value was found in ploughed soils without N fertilization (0.57 MPa) (Table 1). Such a result is not surprising as the upper soil layer under NT conditions is more compact [22], while annual moldboard ploughing destroys soil structure by mechanical aggregate disruption [4]. In contrast, annual moldboard ploughing could cause a strong subsoil compaction through the formation of a plough pan at a depth of 25–30 cm [23,24]. Such a soil compaction is a major agronomic concern that is detrimental for root system growth, soil aeration, and water infiltration. However, in our study, values for SPR at the 0–20 cm depth soil horizon under NT are not a problem from an agronomic point of view, as they do not reach levels that could limit crop production potential [25]. One can also hypothesize that the increased SPR induced by N fertilization in NT soils could be due either to chemically induced changes in soil physical properties or to changes in root development under low or high N fertilization inputs.

Table 1. Impact of tillage and nitrogen fertilization on the biological, chemical, and physical parameters of soil at a depth of 0–20 cm.

Soil Parameter	H (*p*)	NT-N0	NT-NX	CT-N0	CT-NX
DH (µg TPF g^{-1} 24 h^{-1})	7.35 (0.042)	12.58 ± 1.22 a	8.35 ± 1.6 b	8.22 ± 0.36 b	7.63 ± 0.55 b
NO_3^-s (mg kg^{-1})	12.93 (0.005)	2.91 ± 0.06 b	3.35 ± 0.18 b	11.25 ± 1.12 a	8.49 ± 1.21 a
NO_3^-w (mg L^{-1})	13.26 (0.004)	18.13 ± 2.04 b	27.62 ± 3.67 b	44.67 ± 2.65 a	61.76 ± 3.81 a
PO_4^{3-} (mg kg^{-1})	8.28 (0.040)	38.08 ± 2.21 b	41.58 ± 2.11 ab	51.37 ± 2.38 a	42.55 ± 3.45 ab
TN (g kg^{-1})	NS	1.18 ± 0.04	1.21 ± 0.06	1.18 ± 0.02	1.25 ± 0.07
TOC (g kg^{-1})	NS	11.54 ± 0.48	11.75 ± 0.51	12.04 ± 0.19	11.59 ± 0.89
C:N ratio	7.57 (0.048)	9.74 ± 0.12 b	9.74 ± 0.16 b	10.17 ± 0.08 a	9.29 ± 0.31 b
SPR (MPa)	12.11 (0.007)	0.88 ± 0.06 b	1.265 ± 0.08 a	0.57 ± 0.08 c	0.83 ± 0.04 bc

In H: Values of the Kruskal–Wallis test with its probability in brackets. Different letters a, b, and c indicate significant differences between treatments according to the Conover post-hoc test ($p < 0.05$), following a significant Kruskal–Wallis test. NS = not significant. CT: conventional tillage; NT: no-till; NX: with mineral N fertilization; N0: without N fertilization. NS: not significant. DH: dehydrogenase activity; NO_3^-s: nitrates extracted from soil; NO_3^-w: nitrates analyzed in soil solution; PO_4^{3-}: available phosphorus; TN: total nitrogen; TOC: total organic carbon; C:N ratio: carbon to nitrogen ratio; SPR: soil penetration resistance. Results are presented as mean values for soil parameters in the four treatments with standard errors.

Spore density was approximately two-fold higher ($p < 0.01$) after six years of experimentation under NT conditions irrespective of the N fertilization regime (Figure 1A). In contrast, under conventional ploughing less AMF spores were present in the soil, notably when mineral N fertilization occurred. It has already been shown that both spore density and AMF colonization are representative markers of the effect of tillage on AMF biological activity. For example, in comparison to CT, NT had a positive impact on both AMF colonization of wheat roots [7] and AMF spore density [26]. The lower density of AMF spores in CT treatments could be explained by hyphal networks disturbance resulting from ploughing [26]. However, Hu et al. [27] did not find any impact of tillage on AMF spore density in a sandy-loam soil in a temperate monsoon climate, which could be due to specific physical characteristics of such type of soil. In agreement with our results, Curaqueo et al. [28] showed that there was a larger density of AMF spores after six years of experimentation under NT conditions as compared to CT. However, after four additional years of NT, Curaqueo et al. [28] observed a reduction in the density of spores, likely due to instability of the favorable soil characteristics when NT was extended. Moreover, the results of Curaqueo et al. [28] were obtained under a continuous durum wheat–maize rotation, without any cover crop between the two main crops and with a cultivation system based on the use of intensive urea and triple super phosphate fertilization. It is therefore difficult to compare Curaqueo et al. results [28] with those obtained in the present investigation since we used cover crops as a healthier agronomic practice instead of fertilizer application. Indeed, it has been observed that the addition of mineral P fertilizers usually decreases the densities of AMF spores and hyphae under different soil and climatic conditions [29,30]. In the present study, cover

crops containing leguminous species were cultivated between the main crops during winter periods. Therefore, cultivating mixtures of cover crops including leguminous species, which are known to be effective AMF hosts within the tripartite symbiosis with rhizobia [31,32], appears to be beneficial for a more efficient fungal colonization.

We observed a significant decrease of spore density in CT under high N fertilization conditions. In the present study, N fertilization consisted in the application of urea, ammonium, and nitrate. Cornejo et al. [33] emphasized the importance of the type of applied fertilizer on spore density in an andosol, since the authors observed that, compared to an ammonium-based fertilization, there were more spores of *Glomus etunicatum* in soils fertilized with NO_3^-. In our study, we can only speculate that the frequent application of urea and ammonium in CT-NX plots may induce deleterious effects on the AMF life cycle, thus limiting the density of viable spores. Such a result is consistent with the findings of Bhadalung et al. [34], Egerton-Warburton and Allen [35], and Mbuthia et al. [36], who showed that the use of ammonium sulfate plus triple superphosphate or ammonium nitrate caused a decrease in the total density of AMF spores and in the level of expression of AMF biomarkers. This decrease is probably due to a lower root colonization of potential hosts throughout the crop rotation. However, in our study, the lack of difference between N0 and NX plots in terms of spore density under NT conditions (Figure 1A) indicates that the detrimental effect of N fertilization on AMF spore density is somehow buffered by the better soil quality over the six-year period.

Further analysis of the data showed that the AMF spore density was positively correlated with soil penetration resistance ($\rho = 0.69$) and DH activity ($\rho = 0.62$) and was negatively correlated with the nitrate content of the soil ($\rho = -0.62$) or the nitrate content present in the soil solution ($\rho = -0.76$) (Figure 1B).

The results of the correlation studies are further supported by the principal component analysis (PCA) on which the first axis (36.5% of explained variance) clearly separated ploughed soils from those managed under NT (Figure 1C). Interestingly, in NT-managed soils, there was a high DH, SPR, and spore density, irrespective of the N fertilization regime. In contrast, annually ploughed soils were characterized by a high phosphate and nitrate content (Figure 1D). Although a positive correlation between AMF spore density and alkaline phosphatase activity has already been reported in the literature [27], such a positive correlation has never been described for soil DH activity. Nevertheless, the finding that a decrease in AMF spore density is associated with soils exhibiting the lowest value of DH activity (i.e., CT-NX) is not surprising because both parameters are known to be disrupted by intensive tillage and high levels of inorganic N [17]. In contrast, the negative correlation found between spore density and both soil and water NO_3^- content has already been observed by Egerton-Warburton and Allen [35]. These authors observed that an anthropogenic N deposition gradient, characterized by increased concentrations of soil NO_3^-, led to a significant reduction in AMF spore density. Additionally, Egerton-Warburton et al. [37] reported that the decrease in AMF productivity in N-fertilized soils was associated with an enrichment in inorganic P and a lower inorganic N/P ratio. However, in the present study, performed over six years without any addition of inorganic P, we showed that neither nitrate concentration nor the inorganic N/P ratio were related to the fertilizer management regime (Table 1). We therefore suggest that, among the tested soil parameters, CT, by strongly and permanently modifying both the soil physicochemical properties and the microbial response, is the main driver causing the decrease in AMF spore density. Such a decrease is even more important under optimal N fertilizer input.

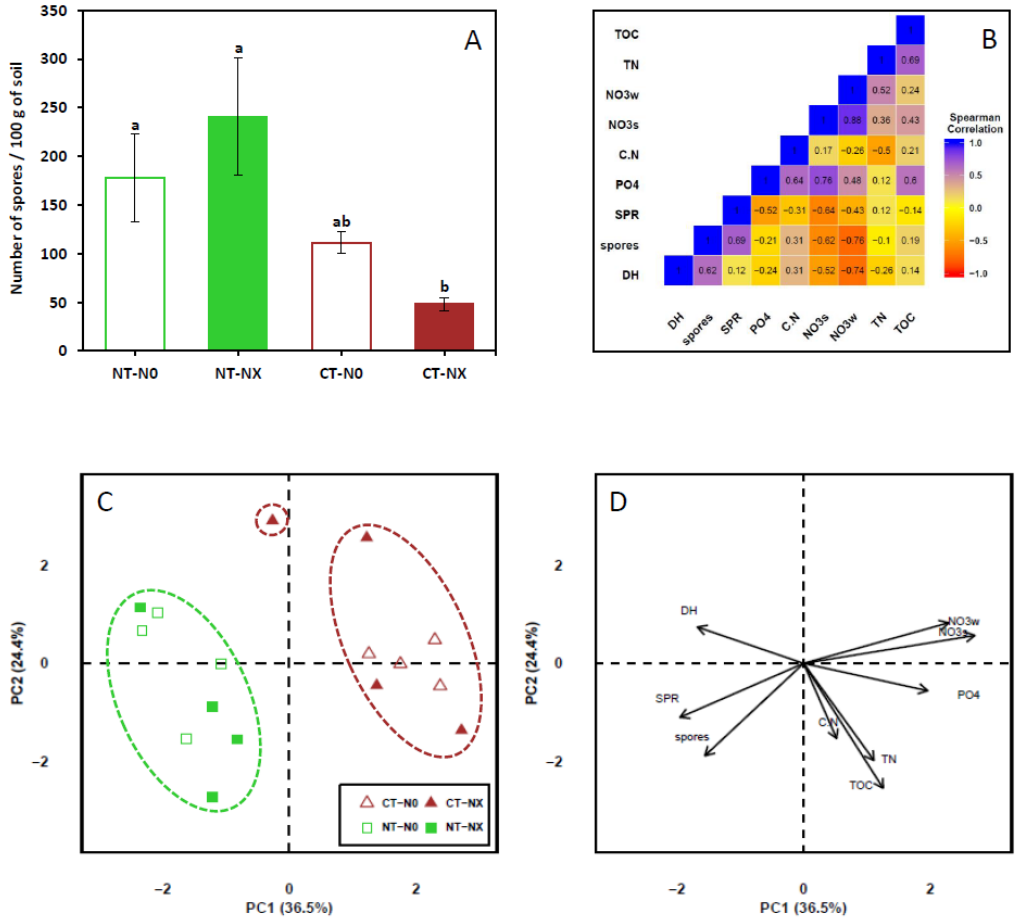

Figure 1. (**A**) Average number of spores per 100 g of soil ± standard error in each of the four treatments. Letters indicate differences among treatments according to a Conover post-hoc test ($p < 0.05$) following a significant Kruskal–Wallis test ($p < 0.01$). The filled bars correspond to the presence of nitrogen fertilization. The empty bars correspond to the absence of nitrogen fertilization. No-till is indicated in green while conventional tillage is indicated in brown. (**B**) Spearman correlations between measured parameters. Values of Spearman correlations are colored by blue as positive, red as negative, and yellow as neutral. Numbers in the squares are correlation coefficients. Factor map of the individuals (**C**) and variables (**D**) as obtained by principal component analysis based on spore density, dehydrogenase activity, soil penetration resistance and nutrient content. The green circle (- - - - -) groups samples collected in no-tilled soils while brown circles (- - - - -) group samples collected in ploughed soils. CT-N0: conventional tillage without nitrogen fertilization; CT-NX: conventional tillage with nitrogen fertilization; NT-N0: no-till without nitrogen fertilization; NT-NX: no-till with nitrogen fertilization; spores: spore density; DH: dehydrogenase activity; SPR: soil penetration resistance; NO_3w: NO_3^--N content measured from soil solution collected in lysimeters; NO_3s: NO_3^--N content extracted from soil samples; PO4: soil available phosphorus; TN: soil total nitrogen; TOC: soil total organic carbon; C:N: soil carbon/nitrogen ratio.

3. Materials and Methods

3.1. Site Description and Experimental Design

The field experiment was conducted at the *La Woestyne* experimental site, in Northern France (50°44′ N, 2°22′ E, 40 m above sea level). The average annual air temperature and total rainfall were 10.5 °C and 675 mm, respectively, with amounts of rainfall relatively homogeneous across the four seasons, these values being considered as normal for the region. The silty loam soil particle size

composition was as follows: 66.7% silt, 21.2% clay, and 12% sand. The soil pH of 6.7 was homogeneous in the entire field.

Prior to the establishment of the field experiment in 2010, the field was managed with chisel ploughing and a rotary power system. In order to study the effect of tillage and N fertilization on the density of arbuscular mycorrhizal fungi (AMF) spores in soils, the experimental field was split into four replicated plots placed randomly for each of the four treatments making 16 plots in total. The four different treatments consisted of conventional tillage (CT) with nitrogen (CT-NX) or without nitrogen (CT-N0) fertilization and no-till (NT) with nitrogen (NT-NX) or without nitrogen (NT-N0) fertilization. Each experiment plot measured 2 m × 2 m and was separated from other plots by 3-m-wide corridors. The crop rotation before the sampling date consisted of wheat (*Triticum aestivum* L.) in 2011, bean (*Phaseolus vulgaris* L.) in 2012, wheat in 2013, green peas (*Pisum sativum* L.) in 2014, and maize (*Zea mays* L.) in 2015 for the entire field. As maize was grown for silage, all the aboveground material was removed from the field. Bean and pea haulm as well as wheat straw, were mechanically ground and returned to the soil. In the whole field, winter cover crops were cultivated each year between the growing seasons of the major crops except in 2013 before wheat. The cover crop mixture was composed of 60 seeds m^{-2} of oats (*Avena sativa* L.), 200 seeds m^{-2} of phacelia (*Phacelia tanacetifolia* Benth.), 80 seeds m^{-2} of flax (*Linum usitatissimum* L.), 50 seeds m^{-2} of vetch (*Vicia sativa* L.), 30 seeds m^{-2} of faba bean (*Vicia faba* L.), and 400 seeds m^{-2} of Egyptian clover (*Trifolium alexandrinum* L.). All cover crop seeds were mixed and simultaneously sown in line using a conventional seeder. Each year, cover crops were sown immediately after the harvest of the previous crop and were terminated by grinding following a frost period. Before the main crops were sown, cover crop residues were buried by annual conventional moldboard ploughing to a depth of 30 cm in CT plots and left on the soil surface in NT plots. In the NX plots, wheat received 160 kg of N ha^{-1} in 2011 and 2013, bean received 80 kg of N ha^{-1} in 2012 and maize received 108 kg of N ha^{-1} in 2015 (50% urea, 25% ammonium, 25% nitrate). In 2014, green peas did not receive any N fertilization in NX plots in accordance with European policies. The amount of N fertilizer applied under NX conditions was determined according to the N budget method [38]. The N0 plots were not fertilized with N for the whole 6 years of the experiment. Apart from N, no additional fertilizer was added to the experimental fields.

3.2. Sample Collection and Analyzes

In April 2016 (6 years after the beginning of the different soil management practices) during the intercropping period (before sowing of the subsequent main crop), three 0–20-cm-deep soil cores were randomly collected using an auger 5 cm in diameter from each of the 16 plots. In each of the four replicated plots for each of the four treatments, the three soil cores were pooled to form a single sample. The pooled soil samples were then homogenized and sieved using a 2 mm mesh.

Plant-available nitrate (NO_3^-) was extracted from 20 g of fresh soil with 100 mL of 2 M KCl. After shaking for 1 h, the extracts were centrifuged for 10 min at 4000 rpm, and the supernatants were analyzed by using an Alpkem Flow Solution IV continuous flow analytical system (Alpkem, Wilsonville, VT, USA). One week before soil sampling, 3 lysimeters (Sdec France, Reignac-sur-Indre, France) were inserted at a 20 cm depth in each of the 16 plots to extract the soil solution. Samples used to measure NO_3^- content in the soil solution were collected simultaneously with the soil samples. The 3 samples of soil solution collected from the 3 lysimeters were pooled to form a single sample in each of the 16 plots. The NO_3^- content in the pooled soil samples solution was directly analyzed with the Alpkem Flow Solution IV continuous flow analytical system. Soil phosphorus (PO_4^{3-}) was extracted with 0.5 M $NaHCO_3$, pH 8.5, and quantified using the colorimetric method described by Olsen et al. [39]. Total soil carbon (TOC) and total soil N (TN) contents were determined using an elemental analyzer Flash EA 1112 series (Thermo Fisher Scientific, Waltham, MA, USA) after drying at 35 °C for 48 h and ball-milled using a grinder MM 400 (Retsch, Haan, Germany). The soil C:N ratio corresponds to the ratio TOC on TN.

Soil dehydrogenase activity (DH) was measured as described by Casida et al. [40]. Soil sub-samples were adjusted with $CaCO_3$ to a final mass ratio of 100:1 (soil:$CaCO_3$) using 5.94 g of fresh soil. One mL of 3% 2,3,5-triphenyltetrazolium chloride solution and 2.5 mL of ultrapure water were added to the soil sub-samples. After mixing, the tubes were incubated at 37 °C for 24 h. The resulting triphenylformazan (TPF) was extracted with 30 mL of pure methanol by stirring for 1 min. The solution was then filtered in a dark room and the intensity of TPF was measured at 485 nm using an Eon spectrophotometer (BioTek Instruments Inc., Winooski, VT, USA). The AMF spore density was determined using the sucrose extraction method described by McKenney and Lindsey [41]. Only bright, apparently viable spores were counted on a gridded Petri dish under a binocular stereomicroscope.

Near the soil sampling areas, penetration resistance (SPR) was measured with a penetrologger (Eijkelkamp, Giesbeek, The Netherlands) fitted with a 60 deg and 1 cm^2 base area cone. Measuring the resistance to penetration of the soil was executed according to the manufacturer's instructions by applying the electronic penetrometer together with a datalogger, allowing for immediate storage and processing of the data in the datalogger.

3.3. Statistical Analysis

All statistical analyzes were performed using R software, v. 3.1.2 (R Development Core Team, 2014 [42]). Mean values are given with their standard error. Because of the low number of replicates, spore density was compared among treatments by using a non-parametric Kruskal–Wallis one-way analysis of variance followed by a Conover post-hoc test whenever significant (*PMCMR* package, [43]). A principal component analysis (PCA) including spore density, DH, SPR, and soil chemical parameters was performed using the *vegan* package [44].

4. Conclusions

To summarize, this work demonstrates that NT under continuous cover cropping system is an agricultural practice that maintains a chemical and a biological soil environment that is favorable for AMF spore production. Such an environment appears to be a key factor for increasing the potential of root mycorrhizal colonization and thus for enhancing mineral nutrient use efficiency in crop plants.

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Conflicts of Interest: The authors declare no conflict of interest.

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