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Effect of different plant densities on the diversity of arbuscular mycorrhizal fungi community in a long-term maize monocrop system

Z. Sasvári and K. Posta*

Microbiology and Environmental Toxicology Group. Institute of Plant Protection. Szent István University. Páter K. str. 1. H-2103 Gödöllő. Hungary

Abstract

The association between terrestrial plants and arbuscular mycorrhizal (AM) fungi is one of the most widespread mutualistic plant-fungus interactions in natural and cropping systems. We studied the effect of different plant densities (70,000 and 100,000 plant ha^{-1}) on species diversity and community structure of AM fungi associated with maize (*Zea mays* L.) in a long-term crop production experiment established in Martonvásár, Hungary. Based on the differences in small subunit (SSU)/18S ribosomal genes nested-PCR procedure was used to identify groups of AM fungi that are active in the colonization of maize roots. Shannon-Wiener diversity index of AM fungi were 1.43 ± 0.37 and 1.31 ± 0.50 at 70,000 plant ha^{-1} and 100,000 plant ha^{-1} respectively. All of sequence types we found belonging to the *Glomus* clade. Besides *Glomus* A fungi, only the members of the *Glomus* B group occurred, however at significantly lower frequency. There were differences in the phylogenetic group composition of AM fungi demonstrating the effect of different plant densities on the diversity of arbuscular mycorrhizal fungi.

Additional key words: diversity, mycorrhiza, plant density, *Zea mays*.

Resumen

Efecto de diferentes densidades de plantación en la diversidad de la comunidad de hongos micorrizas arbusculares en un sistema de monocultivo de maíz a largo plazo

La asociación entre las plantas terrestres y los hongos micorrizas arbusculares (MA) es una de las simbiosis mutualistas más extendidas entre plantas y hongos en los sistemas naturales y de cultivo. Se ha estudiado el efecto de diferentes densidades de la plantación (70.000 y 100.000 plantas ha^{-1}) en diversas especies y la estructura de la comunidad de hongos MA asociados con el maíz (*Zea mays* L.), en un ensayo de producción a largo plazo en Martonvásár, Hungría. Utilizando las diferencias en la subunidad pequeña (SSU) /18S de los genes ribosomales, se usó un procedimiento de PCR anidada para identificar los grupos de hongos MA activos en las raíces de maíz colonizadas. El índice de diversidad de Shannon-Wiener respecto a los hongos MA fue 1,43 ± 0,37 y 1,31 ± 0,50 en los ensayos con densidades de 70.000 y 100.000 plantas ha^{-1}, respectivamente. Todos los tipos de secuencias encontradas pertenecieron al género *Glomus*. Además de hongos del grupo *Glomus* A, sólo se encontraron secuencias de especies del grupo *Glomus* B, pero con una frecuencia significativamente menor. Se encontraron diferencias en la composición de los grupos filogenéticos de hongos MA obtenidos, demostrando el efecto de las diferentes densidades de plantas en la diversidad de hongos micorrízicos arbusculares.

Palabras clave adicionales: densidad de plantas, diversidad, micorrizas, *Zea mays*.

Introduction

The association between terrestrial plants and arbuscular mycorrhizal (AM) fungi is one of the most widespread symbioses in natural and cropping systems. Different works have shown that besides natural ecosystem, arable crops can also be colonized extensively by arbuscular mycorrhizal fungi (AMF) however, the

* Corresponding author: posta.katalin@mkk.szie.hu
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Abbreviations used: AM (arbuscular mycorrhizal), AMF (arbuscular mycorrhizal fungi), H (diversity index), HD (high density), LD (low density), OTU (operational taxonomic unit), SSU (small subunit).

role of AMF in plant production often has been trivialized in high-input agriculture (Jansa *et al.*, 2002; Oehl *et al.*, 2004; Carrenho *et al.*, 2007). AM fungi are major contributors to plant nutrition, promoting mostly the uptake of phosphorus but also other ions such as zinc, cooper and nitrogen, protecting plants from infection by pathogenic fungi and nematodes, improving soil structure and conferring heavy metal resistance to plants (Leyval *et al.*, 1997; Gonzales-Chávez *et al.*, 2002; Smith *et al.*, 2003).

AM fungi comprise a group of more than 200 described species assigned to class *Glomeromycetes* within the new phylum *Glomeromycota* (http://www.lrz-muenchen.de/~schuessler/amphylo/). However only four orders, thirteen families and nineteen genera of AMF are recognized until now, molecular techniques showed that natural AM fungal populations exhibit unexpectedly high genetic diversity (Koch *et al*., 2004). Nevertheless, independently of the ecological zone, AMF species richness decreases by land use intensification (Tchabi *et al.*, 2008). This decrease in diversity is attributed to a complex selective pressure of agricultural practices, such as ploughing, fertilization and fungicide application, on the AMF communities (Douds and Millner, 1999; Roldan *et al*., 2007). Besides agroecosystems plant species, soil types, weeds and previous crop have an effect on AMF population as well (Jansa *et al.*, 2002; Mathimaran *et al*., 2005; Vestberg *et al*., 2005).

Van der Heijden *et al*. (1998) showed that both plant diversity and plant productivity rise with increasing diversity of mycorrhizal fungi. Nevertheless, pot experiments have shown that there is a large functional diversity among different AMF with respect to P uptake (Jakobsen *et al*., 1992; Munkvold *et al*., 2004; Smith *et al*., 2004).

There is only limited information on the relationships between AMF and intraspecific plant density (Schroeder and Janos, 2004, 2005). Some works showed that plant to plant allellopathyc interaction are more detrimental with mycorrhizal fungi than without them, implying that plant responsiveness to beneficial mycorrhizal fungi declines with increasing density (Koide, 1991; Hetrick *et al*., 1994; Maffia, 1997; Posta and Füleky, 1997; Facelli *et al*., 1999). Li *et al*. (2008) examined the effects of increasing plant density on negative growth responses of AM wheat inoculated with two different AM fungi and concluded that growth depressions are primarily due to the cost of C drain towards the fungus, exceeding the benefits resulting from P uptake via the AM fungal pathway.

However, some studies have investigated how phosphorus availability, intraspecific plant density and their interactions affect plant responses to arbuscular mycorrhizas, but there are no data on how plant density influences species diversity and community structure of AM fungi.

The aim of this work was to estimate the effect of different plant densities on the diversity of AMF community in maize monoculture. In order to understand the role of mycorrhizal diversity in plant populations and communities, we need a better understanding of the impact of the mycorrhizal fungus community structure on plant-plant interactions. The experimental results presented here represent one step towards such an understanding.

Material and methods

Study site and sampling

Root samples were collected from a long-term experiment, which was set up in Martonvásár (47°21' N, 18°49' E), Hungary in 1961, with the same treatments applied to the same plots year after year. The climate is temperate, with 514 mm of yearly precipitation (292 mm between 1st of April and 30th of September) and 10.6°C of annual temperature.

The soil is a humous loam of the chernozem type with forest residues, slightly acidic in the ploughed layer, with poor supplies of available phosphorus. The main characteristics in the 0-20 cm soil layer are the following: pH (KCl): 6.9; humus content: 1.85%; AL-P_2O_5: 29.95 ppm; AL-K_2O: 320.15 ppm. The 8×7 m plots had been arranged in a completely randomized block design and had two different plant densities (LD) 70,000 plant ha^{-1} and (HD) 100,000 plant ha^{-1} with four replications per each treatment/density. For each plot, a mouldboard plough to 30 cm depth was used for soil tillage after harvesting time. Annually, the fertilizers were homogenously spread out on the soil surface in September and incorporated with a disc harrow to a depth of 12-15 cm. Field cultivation to a depth of 20-25 cm was made to prepare a smooth seedbed before sowing. During the past 45 years, maize has been sown each April and harvested in August.

Maize plants were removed with 25-25 cm-deep soil cores in July. Roots were washed from soil with tap water, cut into segments approximately 1 cm long. Roots were divided into two subsamples and used for DNA extraction and for measurement of mycorrhizal

colonization. The percentage of root length infected was evaluated by the grid line intersect method (Giovanetti and Mosse, 1980) after staining with Trypan blue, a phenol-free modification of Phillips and Hayman (1970) method.

DNA extraction and nested-PCR

DNA was extracted from lateral roots of a root subsample using a boiling procedure (Di Bonito *et al.*, 1995; Saito *et al.*, 2001). DNA extracted from each of five roots cores per plant was pooled and stored at –20°C until subsequent PCR amplification.

Amplification of partial 18S rRNA gene sequence by nested-PCR was performed on a T3 Thermoblock (Biometra) in a total volume of 25 µL containing 15 mM Tris-HCl (pH 8.0), 50 mM KCl, 1.5 mM $MgCl_2$, 200 µM of each dNTP, 0.025 U μL^{-1} AmpliTaq Gold (Applied Biosystems), 0.5 µM of each primer, and 2.5 µL template DNA. The two nesterd-PCR primer-pair were AMV4.5F-AMV4.5R and AMV4.5NF-AMV4.5NR (Saito *et al.,* 2004). The PCR program comprised 20 (first round of the nested-PCR) or 40 (second round of the amplification) cycles (94°C for 30 s, 60°C for 30 s, 72°C for 1 min) after denaturation at 95°C for 10 min. A final elongation of 10 min at 72°C followed the last cycle. Amplified products (~650 bp) were separated by electrophoresis on 1.5% agarose gel and visualized with on UV transilluminator after staining with ethidium bromide.

Cloning and sequencing

PCR products were purified from agarose gels with GFX PCR DNA and Gel Band Purification Kit (GE Healthcare, Amersham Biosciences), inserted into pGEM®-T vector (3,000 bp) using pGEM®-T Easy Vector System Kit (Promega) and transformed into *E. coli* DH5α bacteria. Putitative positives were sequenced with ABI PRISM 3100 (Hitachi) using the AMV4.5NF primer as sequencing primer.

Sequence analysis

Sequences were identified with the BLAST tool (Altschul *et al*., 1997) provided in GenBank (http://www.ncbi.nlm.nih.gov/), and were submitted to GenBank under accession numbers FJ794852-FJ794820.

The program CHIMERA_CHECK 2.7 (Ribosomal Database Project II; http://rdp.cme.msu.edu) was used to check for chimeric artifacts among the 18S rDNA sequences. Alignments of sequences were performed using a ClustalW algorithm, version 1.6 (http://www.ebi.ac.uk/clustalw/), and were converted into a distance matrix using DNADIST from PHYLIP software (Felsenstein, 2005). OTU (operational taxonomic unit), species rarefaction curves, Shannon-Wiener diversity index (H) and diversity estimators (ACE, Chao) were calculated on 97% of similarity level using the DOTUR program (Schloss and Handelsman, 2005). Phylogenetic analyses were performed by the neighbour-joining algorithm (Saitou and Nei, 1987) with Kimura 2 parameter distance (Kimura, 1980) and bootstrapping of 1,000 replicates in Molecular Evolutionary Genetics Analysis MEGA 4.0 software (Tamura *et al.*, 2007). *Endogone pisiformis*, which is a sister group of *Glomeromycota*, was used as the outgroup.

Results and discussion

Surprisingly, there were no significant differences in the root colonization attributable to different plant densities however, a shift in spore numbers could be detected (Table 1).

The effect of plant density on AMF root colonization is well studied and most of the works have shown that as density increases, the degree to which plants respond to arbuscular mycorrhizal colonization decreases (Bååth and Hayman, 1984). The lack of a decrease in root colonization on higher plant density treatments may be due to the long-term, repeated experiment (45 years), which may cause a selection of AMF with different functional roles. The colonization was measured only once in 12 weeks, just before the flowering, however,

Table 1. Root colonization and spore number of AM fungi at different plant densities

Plant density	Colonization (%)	Spore number (number g^{-1} soil)
LD: 70,000 plant ha^{-1}	36.5 ± 5.5	15 ± 1
HD: 100,000 plant ha^{-1}	37.5 ±7.5	11 ± 1

the seasonal change in the root colonization of AMF has been documented elsewhere (Oehl *et al.*, 2005).

Three mechanisms have been proposed to account for plant density-dependent regulation of mycorrhizal colonization (Koide and Dickie, 2002). Higher plant together with higher root density cause (i) an increase in overlap of phosphorus depletion zones, (ii) a competition for light results in less carbohydrate available for mycorrhizal fungi, (iii) an increase in the cost:benefit ratio of mycorrhizal colonization. As plant density increases, competition for light increases and photosynthesis per plant declines and plants become limited more by carbon than by P reducing the root colonization. However, in some cases, increasing plant density has been associated with increasing mycorrhizal colonization (Eissenstat and Newman, 1990; Allsopp and Stock, 1992).

Higher plant density decreased the spore production (Table 1) which is connected to the life cycle of arbuscular mycorrhizal fungi. AM fungi are obligate symbionts relying on carbon provided by the host plants, the increase in plant density might decrease the carbohydrates available to the fungus and subsequently lower spore numbers might be produced and detected. However, the change in spore numbers gives no information about changes in AMF diversity due to different plant densities.

Maize monoculture has been estimated to contain up to 5-18 AMF species related with different agricultural practices (Oehl *et al.*, 2004; Bhadalung *et al.*, 2005; Jefwa *et al.*, 2006). Hence, it is difficult to compare results using different methods for estimating AMF diversity. Most of the studies have relied on the spore morphology for identification of AMF, however, the spore production does not always reflect the abundance of the mycorrhizal species in roots (Clapp *et al*., 1995). Sporulation of many *Glomus* species is not necessary for the colonisation of new roots (Klironomos and Hart, 2002) but some AMF, especially members of *Gigaspora* and *Scutellospora* genera, need to be preceded by a high colonization (Dodd *et al*., 2000). The estimation of the AM fungi actively colonizing the roots, using molecular tools, can give real information about the mycorrhizal population. For molecular identification of AMF the most widely used primer pair is NS31 (Simon *et al.*, 1992) coupled with AM1 (Helgason *et al.*, 1998) targeting the 18S rDNA region, although AM1 presents some mismatches, and it can exclude some members of *Archaeosporaceae* and *Paraglomaceae*. Using the primer pairs designed by Saito *et al*. (2004) most of the sequences clustered into the *Glomeromycota* could be detected in the roots giving more information than with other AMF specific primer pairs.

Most of the sequences obtained with AMF specific AMV4.5(N)F-AMV4.5(N)R primers represent members of the *Glomeromycota* but several other fungal and unknown sequences were also observed (data not shown). Showing the reality of AMF species in the rhizosphere of maize roots at different plant densities, OTUs were determined by DOTUR program (Schloss and Handelsman, 2005). The rarefaction curve of our data showed saturation, and the diversity estimators had the same richness as the refraction curve suggesting that the number of clone sequences was sufficient to estimate the diversity of AMF colonizing the maize roots at different plant densities (data not shown). A similarity level of 97% has been used as the lower boundary to define OTUs and the phylogenetic tree was constructed using neighbour-joining algorithm (Fig. 1). Taking all the AMF species identified directly in the root samples into account, only a lower number of AMF species were detected which all belonged to *Glomus* species. This result is in accordance with the value of Shannon-Wiener diversity (H) showing no significant differences between different plant densities ($H_{70,000} = 1.43 \pm 0.37$ and $H_{100,000} = 1.31 \pm 0.50$). The Shannon-Wiener diversity index showed no significant differences in the AMF communities with the two plant densities, however a change in the phylogenetic composition of AMF could be well documented (Fig. 2). A total of 7 OTUs were found, five at higher and six at lower (normal) plant densities containing only *Glomus* species. *Glomus* is a diverse genus (Simon *et al.*, 1993) involving three distinct clades: *Glomus* A, B and C (Schüßler *et al.*, 2001) furthermore, *Glomus* A is composed of at least five subclades: *Glomus* Aa, *Glomus* Ab, *Glomus* Ac, *Glomus* Ad and *Glomus* Ae (Schwarzott *et al.*, 2001; Saito *et al.*, 2004).

Among different *Glomus* lineages *Glomus* A clade could be found dominantly and *Glomus* B could be detected at lower intensity (Figure 2). The dominance of *Glomus* A phylotypes in AMF communities is in agreement with previous reports for agricultural lands (Daniell *et al.*, 2001; Galván *et al*., 2009). Other AMF phylotypes and species are absent which is similar to the findings of other authors (Oehl *et al.*, 2004; Hijri *et al.*, 2006).

The most abundant OTU-s was OTU 4 and OTU 7 at normal and higher plant densities, respectively. OTU 4

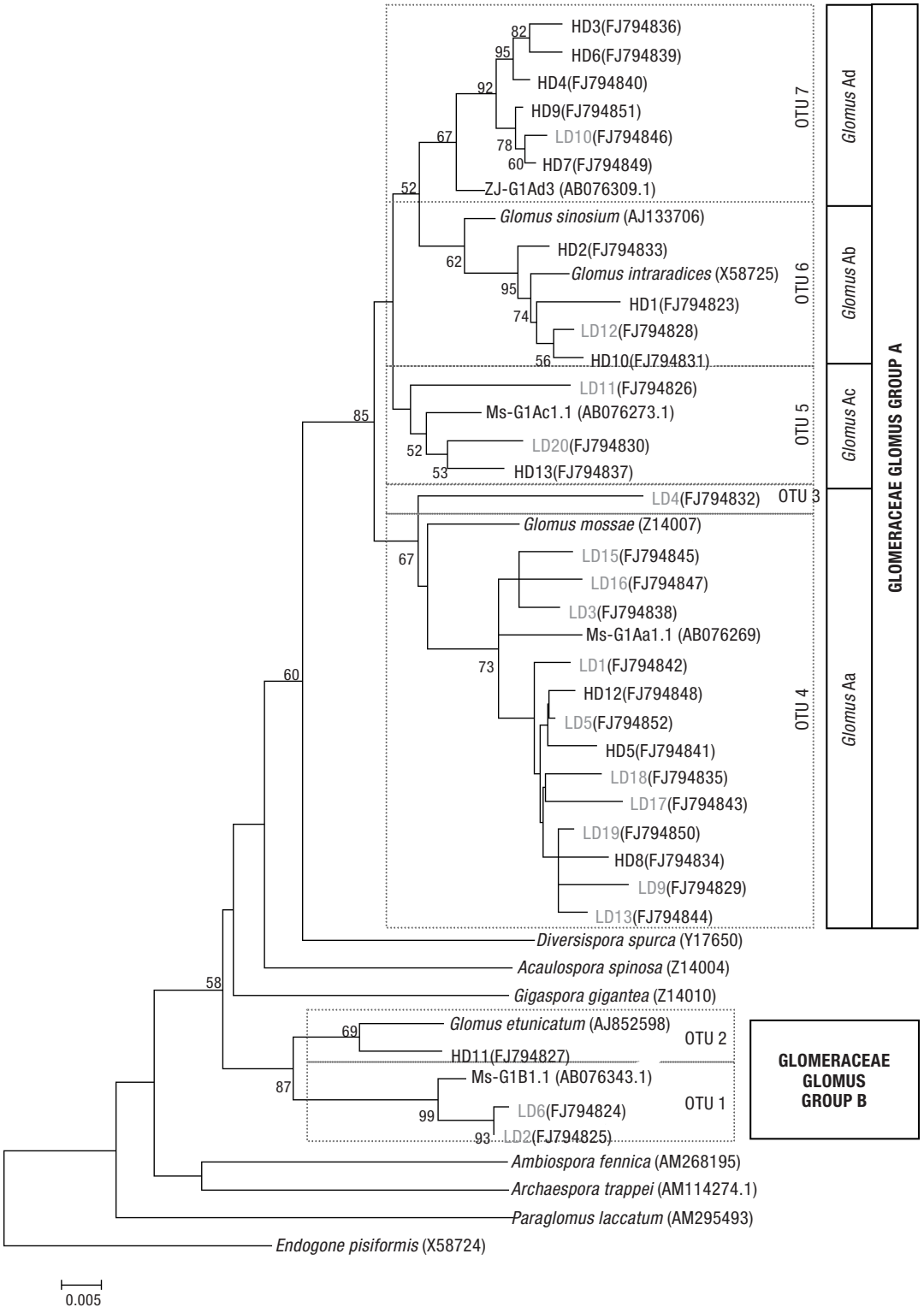

Figure 1. Neighbor-joining tree (Kimura-2-parameter distance) based on partial 18S rDNA sequences obtained from *Zea mays* roots using the primer AMV4.5NF-AMV4.5NR, aligned with 15 other fungal sequences from the database; 416 positions certain to be alignment were used for tree construction. *Endogone pisiformis* was used as an outgroup taxon. Bootstrap values > 50% (1,000 replicates) are shown. Sequence identifiers from this study are in bold type, indicate the plant densities (LD with grey, 70,000 plant ha^{-1}; HD with black, 100,000 plant ha^{-1}).

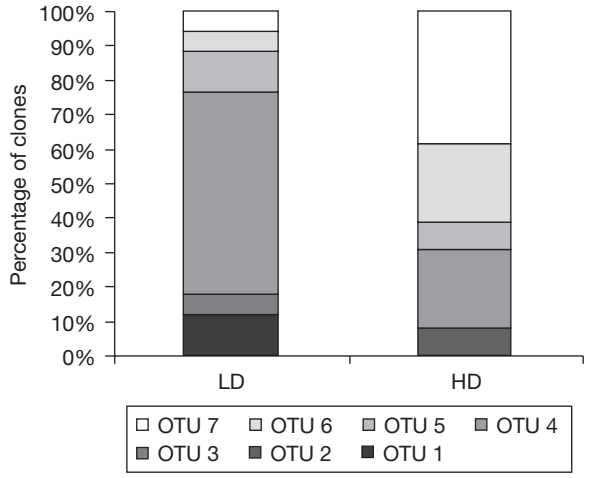

Figure 2. Proportional distribution of AMF sequence types in the roots of maize at different plant densities (LD: 70,000 plant ha^{-1}; HD: 100,000 plant ha^{-1}). Sequences in OUT 1 are closely related to the *Glomus* group B, phylotype Bb1.1; in OTU 2 *Glomus* group B, phylotype *G. etunicatum;* in OTU 3 *Glomus* group A, phylotype Aa2; in OTU 4 *Glomus* group A, phylotype Aa1; in OTU 5 *Glomus* group A, phylotype Ac; in OTU 6 *Glomus* group A, phylotype *G. intraradices* Ab; in OUT 7 *Glomus* group A, phylotype Ad3.1.

presented about 60% of all AMF sequences and closely related to *Glomus* Aa1. This phylotype was the second most abundant one at higher plant densities. Another phylotype, named *Glomus* Aa2 could be detected at lower frequency only at 70,000 plant ha^{-1} density. Both phylotypes belong to *Glomus* Aa group which is closely related to *G. mosseae* and *G. geosporum.*

Saito *et al.* (2004) mentioned that *Glomus* Ac and *Glomus* Ad groups consist of fungi which demand large amounts of carbohydrates from seminatural grassland while *Glomus* Ab group is independent of CH levels. These results are seemingly in contrast with dominant occurrence of *Glomus* Ad group represented as OTU 7 at high plant density, suggesting the influence of not only the quantity but also the quality of carbohydrates. The high occurrence of *Glomus* Ad phylotype in seminatural grassland and woodlands (Helgason *et al.*, 1998, 1999; Saito *et al.*, 2004) together with our results indicate that this fungal group might prefer less disturbed environments provided by higher plant density here.

In conclusion, our study confirms the low diversity of AMF in maize monoculture found in other works; nevertheless demonstrate first the influence of different plant densities on the diversity of arbuscular mycorrhizal fungi. Future work should be required to understand the reasons of changes in the phylogenetic group composition of AMF caused by intraspecific density.

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