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Application of nonspecific commercial AMF inocula results in poor mycorrhization in *Camellia japonica* L.

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Abstract

Camellia japonica L. (*Theaceae, Theales*) is an acidophilic evergreen flowering shrub and is traded worldwide. Symbiotic associations between the roots of this plant species and arbuscular mycorrhizal fungi (AMF), commonly recognized as natural biofertilizers and biocontrol agents, have been poorly studied so far. The aim of our study was to verify whether the application of nonspecific commercial AMF-based inocula could succeed and improve the growth of *C. japonica*. An experiment was conducted concerning the application of commercial inocula constituted by a specific AMF isolate (*Funneliformis mosseae*) or a consortium of different fungi and bacteria as alternatives to fertilization in pot cultivated *C. japonica* 'Dr. Burnside'. Several growth parameters, plant nutrition and mycorrhization levels were monitored at the end of plant cultivation. Generally, increases in some macroelements (Ca, Mg, K) and microelements (Cu, Mn, Fe, Zn) were detected in the root system of inoculated camellias, while only Cu and Mn accumulation was increased in the leaves. Plants inoculated with the consortium inoculum exhibited increased chlorophyll content and foliage diameter while plants inoculated with *F. mosseae* exhibited increased the number of flowers. Based on the polymorphism of an 18S rDNA region, we assessed the inoculated AMF that colonized *C. japonica* roots. The experiments showed that the applied AMF poorly colonized the root system of *C. japonica*. We suggest that commercial AMF formulations should be more targeted and host-specific in order to successfully colonize the host root and, potentially, fully express their benefits.

Keywords*: arbuscular mycorrhizal fungi, biofertilizer inoculation, microbial consortium, 18S rDNA, sustainable floriculture*

1. Introduction

Camellia japonica L. (*Theaceae, Theales*) is an evergreen ornamental shrub. In floriculture, *C. japonica* importance ranks the highest due its ornamental characteristics and it is traded worldwide as potted plant (Mondal 2011). Nevertheless, limited research information is available on the species. Camellia is an acidophilic plant and is characterized by a specific rhizospheric chemistry because of the phenolic-rich root exudates and low pH characteristics that make this species a difficult plant material to work with.

The cultivation protocol adopted for this plant includes the use of high quantities of fertilizers and pesticides that have an impact on production costs (Larcher et al. 2011; Berruti and Scariot 2013). Moreover, the heightened social sensitivity and concern for the environmental problems require growers to reconsider the conventional fertilization strategies (Gomiero et al. 2011; Marucci et al. 2011). To overcome this problem, recent trends point at the use of arbuscular mycorrhizal fungi as natural biofertilizers. The symbiotic fungi that form arbuscular mycorrhizae (AM; belonging to the phylum Glomeromycota) are among the most important soil organisms. AM fungi (AMF) colonize the roots of most land plants, where they facilitate mineral nutrient uptake from the soil in exchange of plant-assimilated carbon (Smith and Read 2008). Thus, the early inoculation of potted plants with selected AMF based inocula could help improve plant survival and performance and lead to the need for lower chemical inputs, e.g., during fertilization and pathogen control (Azcón-Aguilar and Barea 1996; Neumann and George 2010).

The significance of AMF has not been ignored by the commercial sector and many AMF inocula are nowadays available for sale (Gianinazzi and Vosátka 2004). Most manufacturers advertise their products by supporting their suitability for a wide range of plants, although the success of root colonization is not predictable, as seen during several evaluations of the performance of commercial inocula in nurseries (Corkidi et al. 2004; Tarbell and Koske 2007). There is some evidence that experimental (van der Heijden et al. 1998) and natural (Moora et al. 2004) AM fungal communities, with different taxon compositions, may induce a different growth response in plants. Although large numbers of plant–fungus partnerships occur naturally, not all of them behave the same, implying that the cost-benefit ratio differs for different plant-fungus combinations (Helgason and Fitter 2009). As with natural host plants, many important ornamental and indoor plants (*Chrysanthemum*, *Impatiens*, *Rhododendron, Petunia, Callistephus*) are able to establish symbiosis with mycorrhizal fungi (Gaur et al. 2000; Jansa and Vosatka 2000; Sohn et al. 2003). However,

there is still little information in floriculture about the application of AMF inoculation to commercial productions (Blal 1999; Scagel et al. 2003; Koltai 2010). Encouraging results have been obtained on roses, where rooting was shown to improve (Scagel 2001). Although some data regarding yield and growth increase in inoculated ornamental plants are in fact available, only limited research has been conducted on investigating and optimizing the AMF species composition of the inocula in use. Generally, the technology used to propagate ornamental plants in nurseries does not take into consideration the existence of this mutualistic symbiosis and other associative plant growth-promoting microorganisms. Peat, the most used substrate, is usually devoid of AMF and, therefore, plants obtained from non-inoculated pots are poorly colonized or not mycorrhizal at all (Porras-Soriano et al. 2009; Martinez-Medina et al. 2011). Such plants eventually become mycorrhizal when they are planted in field soil (Mosse and Hayman 1971).

In this study, we raise the question whether or not the application of two commercial AMF based inocula is an effective alternative to high fertilization levels in pot cultivated *C. japonica* 'Dr. Burnside', one of the most commercialized camellia cultivars.

2. Materials and Methods

2.1 Plant material and growth conditions

C. japonica 'Dr. Burnside' was selected as test plant owing to its commercial and ornamental value. The cultivation cycle lasted two years (2008-2010) and was carried out in a commercial nursery devoted to the production of acidophilic ornamental plants (Tecnoverde s.p.a., Verbania, Piedmont - Italy). A peat mixture (89% commercial *Sphagnum* peat and 11% agriperlite) was used as the growing medium. This substrate had a pH of 5.0 (substrate/water ratio: 1/5 v/v), an electrical conductivity (EC) of 44.3 μS/cm and a C/N ratio of 63.3. The contents of K, Ca and Mg were of 0.05%, 0.21% and 0.09%, respectively, while no detectable concentrations of P were present. Neither a spore count nor a PCR-based approach with specific primers (see the Materials and Methods section below) showed any presence of AMF propagules in the peat mixture. A fertilizer dosage of 2.25 kg m^{-3} $CaCO_3$, 1.12 kg m^{-3} Osmocote Exact® (15N-3.9P-7.5K + trace micronutrients, 8-9 months, Scotts, Merysville, Ohio) and 0.5 kg m^{-3} Scorie Thomas (6.9P), a phosphatic inorganic fertilizer (Timac Italia, Ripalta Arpina, Italy), was added to half of the peat mixture. The two resulting mixtures, fertilized and non-fertilized were each divided into three parts. These were respectively added with a mixed beneficial microorganism inoculum (BM), a single AMF isolate inoculum (SI) or not inoculated (NI). The BM inoculum is available on the market under the name 'Micosat F® for acidophilic plants' (C.C.S. Aosta Srl, Aosta,

Italy) and, according to its label, is composed by 20% propagules of three morphologically characterized AM isolates (*Glomus intraradices* GB67, *G. viscosum* GC41, and *G. viscosum* GA74, of crude inoculum), and $5x10^8$ CFU/g *Trichoderma viridae* and several plant growth promoting rhizobacteria (PGPR – *Pseudomonas* spp., *Streptomyces* spp, *Bacillus subtilis*). The other inoculum, SI, carries propagules (920 propagules/cm^3) of a single molecularly identified isolate, i.e. *Funneliformis mosseae* BEG12 (Agroauxine, Saint Evarzec, France). The inert material used as carrier in the BM inoculum is micronized zeolite while the one used in the SI inoculum is quartz sand. These two inocula were chosen on the basis of their high commercial diffusion and successful application on several plant species, including ranunculus, tomato, kiwifruit and grapevine. Thus, a completely randomized design was created (2 fertilization regimes X 3 types of inoculation) for a total of 6 combinations, each represented by 50 replicates (camellia pots). In the first week of February 2008, three camellia rooted cuttings per pot (9 cm in diameter) were planted for a total of 300 pots (900 rooted cuttings). These were randomly distributed on four benches in a frost-free greenhouse (2.5°C minimum, 16°C average). The shading was set at constant 30%. Additional 40% shading was provided when illumination exceeded 60 Klux. No supplementary lighting was ever applied. All the camellias were fertigated with 20N-8.7P-16.6K Peter Professional hydrosoluble fertilizer (Scotts, Merysville, Ohio) at 0.8-0.9 g l^{-1} about once every two weeks from March to October, according to the weather conditions. After 9 months of cultivation, the plants were transplanted into 15 cm diameter pots and pruned. The cultivation ended on April 20th 2010, when flowering was completed.

2.2 Morphological parameters and nutrient uptake

Plant growth and ornamental characteristics were monitored at the end of the cultivation cycle (April 20th 2010). Plant height and diameter, which was measured across the widest side of the plant, were recorded for each pot. A Chlorophyll Meter SPAD-502 Konica Minolta (Nieuwegein, Netherlands), characterized by a measuring accuracy of ±1.0 SPAD unit, determined on rice-plant leaves, was used to indirectly measure leaf chlorophyll content and to assess plant nitrogen status (Gianquinto et al. 2004, Smith et al. 2004). Every SPAD value was the mean of three measurements performed on three mature leaves randomly chosen on each replicate. After flowering was reached, the aerial part of 20 randomly selected plants per treatment was oven-dried at 90°C for two days, and the dry weights were determined for the total leaves and branches. During the whole flowering period (from late December until late April), the total number of flowers produced was calculated and the size of 10 flowers for each treatment was measured. A quantification of the macronutrients (P, K, Ca, Mg) and micronutrients (Fe, Zn, Cu, Mn) was carried out in triplicate in roots and leaves with ultraviolet-VIS and atomic emission spectrophotometry, according to European Standard methods (EN) with minor modifications to assess the scale of the response to inoculation of plant nutrition. The results were

subjected to a two-way analysis of variance (ANOVA) to analyze the camellia growth, health and ornamental related data and to assess the interactions between the two fixed factors (inoculum + fertilization regime). All the data were post-hoc tested (alpha = 0.05) using the Ryan-Einot-Gabriel-Welsch-F test (REGW-F), by means of the SPSS statistical package (version 16.0; SPSS Inc., Chicago). A secondary one-way ANOVA and a following REGW-F post-hoc test (alpha = 0.05) were performed for variables characterized by a significant interaction between the two fixed factors.

2.3 DNA extraction and amplification from BM inoculum

DNA extraction from the BM inoculum was performed on a 0.5 g sample using a FastDNA Spin Kit for Soil (MP BIOMEDICALS), according to the manufacturer's recommendations. An additional step, consisting of 6 washes with guanidine thiocyanate (6M), was introduced after the addition of the binding matrix suspension, due to possible high levels of PCR inhibitors in the inoculum sample (mostly humic acids).

Three sets of primers were used to amplify two different regions of the Glomeromycota ribosomal DNA. The universal eukaryotic primer NS31 (Simon et al. 1992) was used as the forward primer for the first primer set, combined with AM1 (Helgason et al. 1998) as the reverse primer, which was designed to amplify ~550bp fragments of SSU rDNA. In addition, a nested PCR approach was applied (Millner et al. 2001), with the first primer set LSUGlom1/SSUGlom1 and the second PCR round with the Paraglomeraceae family-specific primer pair GOCC56/GOCC427 designed to amplify the ITS region. The annealing temperature was raised to 58°C when these primers were used to increase specificity, as suggested by Hempel et al. (2007). PCR reactions were performed using 0.1 μg μl^{-1} BSA (Bovine Serum Albumine), 0.2 mM dNTPs, 3.5 mM $MgCl_2$, 0.5 μM of each primer and the supplied reaction buffer, with 2 units of High Fidelity Taq (ROCHE) to obtain a final volume of 20μl. Amplifications were carried out in 0.2 ml PCR tubes using a Biometra T Gradient thermocycler according to the following steps: 5 min initial denaturation at 94°C; 35 cycles of 1 min at 94°C, 1 min at 55°C, 1 min at 72°C; and a final elongation of 7 min at 72°C. A negative control was included in the PCR to check for contamination.

2.4 Cloning, RFLP and sequencing

The PCR products were purified using QIAquick (Qiagen, Hilden, Germany), cloned in a pGEM-T Easy vector (Promega, Madison, Wisconsin, USA) and transformed into *Escherichia coli* competent cells (Xl1 blue). After colony PCR, inserts of the correct size (~550bp) were submitted to restriction fragment length polymorphism (RFLP) analysis using two endonucleases, Hsp92II (|CATG|) and HinfI (G|ANT|C), in order to reduce the number of clones to

be sequenced by comparing the RFLP-types found with a CNR-IPP (Turin, Italy) internal database based on the same enzyme couple used in the present work. Subsequently, the selected clones were sequenced using T7 vector primers by Dynamicode sequencing services (Turin, Italy). The sequences generated in this study were submitted to GeneBank and recorded under the following string of accession numbers: JF951398-JF951426.

2.5 Sequence analyses and phylogenetic inference

The sequences were viewed and edited using Chromas Lite 2.01 (Technelysium Pty Ltd). Possible chimera sequences were identified using the Mallard Chimera Detection programme (Kevin Ashelford, Cardiff University). All the sequences were aligned using the multiple sequence comparison alignment tool by MAFFT which is available online (Katoh et al. 2002). Distance matrices were constructed using DNAdist from the PHYLIP suite of programmes, version 3.6, with default parameters (Felsenstein 2005). These pairwise distances were used as input for MOTHUR (Schloss et al. 2009) in order to cluster the sequences into Operational Taxonomic Units (OTUs) of a defined sequence identity. A threshold 97% identity value, corresponding to 0.03 dissimilarity ($OTU_{0.03}$), was used to define OTUs. Although this distance cut-off is arbitrary and can be considered controversial, it was chosen on the basis of previous studies on AMF biodiversity (Lumini et al. 2010). A query for sequences characterization was conducted with the BLASTn similarity tool using a megablast algorithm (Zhang et al. 2000), provided by GenBank. The alignment built in a recent study by Krüger et al. (2011) was retrieved online (www.amf-phylogeny.com) and used as a reference for 1-3 representative sequences from each OTU. A new alignment was run, manually edited, and a Neighbour-joining (NJ) phylogenetic analysis (1000 bootstrap replicates) was performed with Mega Version 5 (Tamura et al. 2007) using default parameters. A Maximum Likelihood phylogenetic analysis (1000 bootstrap replicates) was also done but gave comparable results. The recent systematic reorganization of the Glomeromycota phylum published by Krüger et al (2012) was adopted.

2.6 Morphological and molecular assessment of root colonization by AM fungi

Three randomly chosen camellia plants were collected from each treatment at two experimental points, after potting (July 17th 2008) and after branching (January 14th 2009), and taken to the laboratory (a total of 18 plants per sampling). A small portion of the root systems from the second experiment point was cleaned and stored at -80°C for further molecular analyses. The resting parts of each plant's root system were washed free of peat and immediately used for morphological analyses. The roots were cleared with 10% KOH overnight, stained with 0.1 % cotton blue in lactic

acid overnight and then de-stained 6-8 times with lactic acid. The roots were cut into small fragments (about 1 cm each) and mounted onto microscope slides with some drops of lactic acid. About 60-90 fragments were observed for each treatment per sampling. AMF colonisation intensity in the root cortex and arbuscule presence was determined according to Trouvelot et al. (1986). Mycorrhization frequency (the percentage of root fragments showing fungal colonization), AMF colonization intensity (the percentage of the root system area showing fungal colonization) and the presence of arbuscules (the percentage of the root system area showing presence of arbuscules) percentages were calculated for each plant according to the freeware Mycocalc, which is available online (http://www2.dijon.inra.fr/mychintec/Mycocalc-prg/download.html). The mean values of the two experimental points were considered for the statistical elaboration. All the percentages calculated were arccosine transformed and subjected to a two-way analysis of variance (ANOVA) to assess the interactions between the two fixed factors (inoculum + fertilization regime). The data were post-hoc tested (alpha = 0.05) using the Ryan-Einot-Gabriel-Welsch-F test (REGW-F), by means of the SPSS statistical package (version 16.0; SPSS Inc., Chicago).

DNA extraction was performed on frozen samples 0.1 g of finely ground root samples with the DNeasy Plant Mini Kit (Qiagen, Crawley, UK), according to the protocol for frozen samples. The PCR-RFLP protocol was carried out as described in the previous paragraphs 2.3 and 2.4, with the only modification consisting in the use of PCR-primer pair NS31/AM1 only. The PCR products obtained from the same treatment were pooled together before the cloning step. Two approaches were adopted. The RFLP types found were compared with those identified from the BM inoculum and with the ones provided by the CNR-IPP lab internal database.

3. Results

3.1 Effects of inoculation on growth and nutrition

The mean values that describe the effect of the treatments at the end of cultivation are reported in table 1. All measured parameters, apart from the flower number, were significantly affected by the application of AMF inocula. Foliage diameter, chlorophyll content and flower depth significantly increased after inoculation with the BM inoculum. Plant height and leaf and branch dry weight were significantly reduced by inoculation with SI. Both BM and SI inocula gave height/diameter ratios statistically lower than the non-inoculated control. Flowering was also enhanced in plants inoculated with AMF but no statistical relevance was assumed. No effect on flowering earliness or uniformity was noted (data not shown).

High fertilization levels were fundamental for good plant health and development. All growth related parameters (height, foliage diameter, dry weights and chlorophyll content) were largely affected by high fertilization levels (p-value<0.001). Moreover, no flowering was registered when the low fertilization regime was applied.

Significant interactions between inoculation and fertilization occurred for foliage diameter, height/diameter ratio and branch dry weight. These interactions are explained in figure 1. The foliage diameter seems to be particularly boosted after inoculation with the BM inoculum in the presence of high fertilization. In the same situation, the height/diameter ratio remained low, differently from the other inoculation conditions. In general, for all three parameters, low fertilization levels repressed the differences produced by the three inoculation types in high fertilization conditions.

To characterize plant nutritional status as affected by both inoculation and fertilization, the radical and foliar accumulation of a wide range of pivotal macro and micronutrient elements was measured. Inoculation and fertilization practices affected the uptake of most of these elements (Table 2). The application of both inocula significantly improved Ca, Mg, K, Mn and Fe uptake in the roots, while Cu and Zn absorption was promoted by BM inoculation and reduced by SI inoculation, with respect to the non-inoculated control. Root P was not affected by BM, while the SI inoculum significantly decreased the accumulation of this macroelement. High fertilization provided significantly higher levels of P, Cu and Zn, while the accumulation of Ca, Mg, Mn and Fe was significantly higher in roots of low-fertilized plants. Fertilization did not statistically affect K uptake.

In leaves, inoculation with AMF gave a significant reduction of P, Ca and Zn. Conversely, AMF inoculation significantly enhanced the accumulation of Cu and Mn, particularly when inoculating with SI. This latter inoculum also improved K accumulation. No statistically significant changes were recorded in the Mg and Fe concentrations. In leaves, high fertilization promoted significantly higher levels of P, Ca and Mg, but lower levels of Cu and Zn. No influence of fertilization was registered on K, Mn and Fe levels.

Table 2 shows the p-values for the interactions between the two fixed factors (inoculation and fertilization). In roots, strongly significant interactions were detected for the accumulation of all elements, apart from P. These are explained in figure 2 and 3. Ca, Mg, Cu and Mn uptake in BM inoculated plants was characterized by a decrease in presence of high fertilization and shows a different trend comparing to the other two inoculation types (SI and NI). The same was visible for K and Zn uptake in plants inoculated with SI. Fe uptake was boosted by the BM inoculum and depressed by the SI inoculum in low-fertilized conditions with respect to high-fertilized conditions, while non-inoculated plants showed a constant low uptake.

In leaves, significant interactions were highlighted for Ca, Cu, Mn, and Zn. The interactions are given in figure 4. Ca uptake showed the lowest value after inoculation with SI in low-fertilized conditions, while the highest in high-

fertilized conditions, with respect to the other two inoculation types (BM and NI). Cu uptake in the three different inoculation types was reduced and homogenized by high fertilization. Mn uptake was enhanced by the SI inoculum and depressed by the BM inoculum by high fertilization with respect to low-fertilized conditions, while non-inoculated plants showed a constant low uptake. High input fertilization equalized the effect of NI and SI inoculation on Zn uptake in high-fertilized conditions.

3.2 Inoculum characterization

The AMF community of the mixed beneficial microorganisms (BM) inoculum was analyzed in detail using two primer combinations that target two different rDNA regions. The NS31/AM1 primer combination yielded a clone library, with 550bp SSU fragments. We screened 59 clones and sequenced 29 of them that were chosen as representative of the 14 RFLP types that were found. BLAST searches in fact revealed that, out of the 29 SSU sequences obtained using the NS31/AM1 primers, 28 sequences featured remarkable high similarity with sequences from taxa belonging to the Glomeromycota phylum, while only one was related to *Ascomycota* sequences. No ITS sequences were obtained with the GOCC primer set.

3.3 Phylogenetic analyses of AMF sequences

The 14 RFLP types that were found clustered in 4 OTUs. Phylogenetic analyses were performed using 1-3 representative sequences per OTU in order to assign their cognate Glomeromycotan groups. Sequences were aligned with reference sequences of well-characterized AMF isolates retrieved from a recent study by Krüger et al. (2012). The resulting phylogenetic tree of the SSU rRNA sequences is shown in figure 5. By far, the most abundant and diverse AMF taxa in the inoculum sample belonged to the Glomeraceae family, with 28 clones grouped in OTU1 and 25 clones grouped in two OTUs (OTU2 and OUT3). The Claroideoglomeraceae family was also represented, with 5 clones that clustered in OTU4. Only 2 RFLP types of the 59 considered clones prevailed. The first counted 22 clones, which clustered in OTU1 with sequences belonging to the *Rhizophagus* genus. The other one, with 20 clones, belonged to OTU3, and did not group with any of the reference sequences and was therefore allocated to the uncultured Glomeraceae group.

3.4 Mycotrophic status of the roots

All the sampled camellia roots that had been inoculated exhibited AMF colonization. Traces of mycorrhization were also reported in the control. In general, AMF inoculation resulted in the formation of small infected patches at the root level and arbuscules were seldom found. The percentages of AMF colonization and the abundance of arbuscules in the mycorrhizal roots are shown in table 3. The frequency of mycorrhizal fungi in the roots (F%) was significantly higher after inoculation with BM than after inoculation with SI. The colonization intensity (M%) was generally very low, but significantly higher than the non-inoculated control. Arbuscules formation was rare and no statistical difference was detectable between inoculated and control pots.

High input fertilization decreased mycorrhization levels and, in particular, a significantly stronger intensity of colonization was highlighted in the low-fertilized plants. No interactions were highlighted between the two fixed factors (inoculum and fertilization regime).

The NS31/AM1 primer combination successfully amplified only samples coming from plants inoculated with AMF, yielding 4 SSU fragment clone libraries. A total number of 46 clones (8-15 per clone library) were screened and 2 RFLP-types were detected. Further comparisons with our RFLP-type database led to their affiliation with OTU1 (*Rhizophagus* genus) and *F. mosseae* BEG12. These 2 RFLP-types were retrieved from clone libraries created with amplicons of samples of plants incoculated with BM and SI, respectively.

4. Discussion

To our knowledge, besides a recent work by our group (Berruti et al. 2013), limited research has been published on the investigation of the symbiosis between AMF and *C. japonica* (Mejstrik 1974; Ahulu et al. 2005). More information is available on other species belonging to the *Camellia* genus, and above all on *C. sinensis* (L.) O. Kuntze, i.e. the tea plant (Kahneh et al. 2006; Phukan et al. 2008; Singh et al. 2008a, 2008b; Gupta and Sharma 2010; Singh et al. 2010; Aliasgharzad et al. 2011). Our study has uncovered an apparently very low symbiosis profile. The low mycorrhization frequencies detected in this study were close to those found in *C. japonica* trees and shrubs occurring in natural, semi-natural and anthropic environments in New Zealand (Mejstrik 1974). Other works describing natural, semi-natural and post-inoculation AM colonization percentages in Indian *C. sinensis*, instead, showed higher mycorrhization frequencies (Singh et al. 2008a, 2008b). However, a recent study highlighted no mycorrhization at all in tea plants after pot inoculation and in open gardens (Aliasgharzad et al. 2011). In our study, after inoculation, the colonization intensity was very low (max 1.5%) while the presence of arbuscules was negligible, suggesting a very low exchange activity (Smith and Read 2008). These very low colonization levels could be due to many factors, such as the substrate

properties, the fertilization regimes and the AMF species composition of the applied inoculum (Verbruggen and Kiers 2010). Using peat-based substrates may be favorable to achieve rapid plant growth in containers (Larcher and Scariot 2009; Berruti and Scariot 2012), but the effects on mycorrhizal colonization are not yet well understood. A recent study has pointed out that the less decomposed light peat, rather than dark peat, can be inhibitory of AMF colonization (Vestberg and Kukkonen 2009). However, when reported, the inhibitory effect of *Sphagnum* peat amendments has never caused a dramatic AMF colonization decrease (Biermann and Linderman 1983; Vestberg et al. 2005). Other studies described the occurrence of AMF colonization in high volume *Sphagnum* peat mixtures (Corkidi et al. 2004; Matysiak and Falkowski 2010) and assessed the presence of high AMF colonization in several tree species seedlings in a peat swamp ecosystem (Tawaraya et al. 2003). A cultivation practice that has been reported to be detrimental for AMF is the use of high-input fertilization regimes. AMF occurrence and abundance have been negatively correlated with phosphorous (Treseder 2004) and nitrogen (Antonininka et al. 2011) enrichments in field conditions. Greenhouse studies have also revealed how controlled-release fertilizers with high P levels are unfavorable for AMF establishment, and that generally low P levels let mycorrhization successfully occur (Charron et al. 2001; Linderman and Davis 2004). Our results indicate that high fertilization reduced AMF colonization, since mycorrhization intensity was higher in low-fertilized plants. However, in another inoculation trial on the same plant cultivar cultivated with the same high fertilization regime in a high volume peat (70%) substrate mixture, we found high degree of mycorrhization frequency (82-95%), intensity (40-61%), and high presence of arbuscules (35-59%) after inoculation with field-collected AMF propagules (data not shown). In another recent experiment carried out by our group (Berruti et al. 2013), the relationships between *C. japonica* 'Nuccio's Pearl', cultivated with the same growing conditions, and AMF found associated to camellia specimens in semi-natural ecosystems (field-collection), was investigated and validated through a laser microdissection approach aimed at the detection and molecular identification of the intra-cellular arbuscules. Other comparisons conducted by other authors found that commercial inocula often underperformed inocula from field-collection (Gaur et al. 1998; Rowe et al. 2007). Interestingly, a recent review by Verbruggen and Kiers (2010) forwarded key remarks about the debate on low AMF specificity, which no longer seems to be well supported. The need to take specificity into account when designing and applying a beneficial AMF inoculum was underlined in this review. Host-specificity is rarely taken into account by manufacturers willing to produce an AMF-based inoculum and this fact could represent a bias in the correct formulation of a potentially successful inoculum.

In our study, the inoculation with AMF-based commercial inocula failed to replace high input fertilization, which was fundamental for good plant health, development and flowering. Low-fertilized plants showed an extreme depression of growth and a marked foliar chlorosis. Only minor effects were observed after application of both single AMF isolate (SI) and consortium (BM) inocula. The height restraint registered after inoculation with SI eventually

resulted in a slight enhancement of flowering. The BM inoculum favored an increase in the total foliage diameter instead. Chlorophyll increases were seen on plants inoculated with BM. This is likely to be correlated with an improved plant nitrogen status (Gianquinto et al. 2004) and also due to the fact that other helper bacteria and fungi were co-inoculated along with AMF. However, in support of the importance of AMF, data on plant nutrition revealed a generally improved uptake in roots inoculated with both inocula. Generally, increases in some important macroelements (Ca, Mg, K) and microelements (Cu, Mn, Fe, Zn) were detected in the root system of inoculated camellias, while only K, Cu and Mn accumulation was increased in the leaves. The most significant results regarded Mn and Fe, whose uptakes were up to 5 and 10 times higher in inoculated roots. Our study revealed a significant interaction between the inoculum type and the fertilization regime adopted in the root and leaf accumulation of many nutrients. This often meant that nutrient uptake was potentiated in low fertilization after inoculation with BM (root Ca, Mg, Fe, Cu, and Mn; leaf Cu, Mn, and Zn). The diversity of the functional traits that characterize different AMF species able to trigger an agriculturally significant response on a given host is still under investigation (Munkvold et al. 2004; van der Heijden and Scheublin 2007). It is clear how biodiversity at the species, group and family level can increase the number of functional traits in a community and therefore support ecosystem functioning (van der Heijden et al. 1998). Since different AMF isolates are not always capable of fulfilling the expected benefits for the plant (Klironomos 2003), biodiversity and species composition play a direct role on the quality of an applied inoculum (Vosatka and Albrechtova 2008; Ijdo et al. 2011). Hence, in order to assess the inoculum composition (on the basis of label indications) and purity, a high fidelity molecular species composition analysis is crucial (Siddiqui and Kataoka 2011; Vosatka et al. 2012). For this reason, we decided to molecularly characterize the AMF species carried in the BM inoculum. Thus, for the first time, a standard phylogenetic analysis has been performed directly on an inoculum, as a quality control step. This kind of analysis was not carried out on SI, as the isolate present in this inoculum had already been identified. Our study has revealed the presence of 4 OTUs which clustered in only two families, i.e. Glomeraceae, known for being very generalist, non-specific and widespread (Helgason et al. 2007; Oehl et al. 2010), and Claroideoglomeraceae. In particular, 3 OTUs highlighted a strong predominance of isolates from the Glomeraceae family, clustering with the *Rhizophagus* genus (OTU1), or forming an uncultured Glomeraceae clade (OTU2 and OTU3). These results partly support the BM inoculum label indications, which include an isolate of *Rhizophagus intraradices* (ex-*Glomus intraradices*, Glomeraceae, OTU1) and two isolates of *Viscospora viscosum* (ex-*G. viscosum*, Claroideoglomeraceae, OTU4) in the inoculum. The only exception was the not reported presence of two OTUs (OTU2 and OTU3), which were probably allocated to the wrong species during the product formulation. These results are not surprising since isolates carried in the BM inoculum are characterized morphologically and only few experts worldwide can reliably assign an AMF isolate to a species on the basis of spore morphology (Young 2012). As highlighted by this study, it is

easier to spot different species using molecular techniques than morphological observation. For this reason, we strongly suggest that a molecular characterization of the AMF isolates carried in the applied inocula should be conducted on commercially available inocula.

Molecular traceability studies of the AMF isolates are also pivotal for the assessment of the success of inoculation and provide precise information on which AMF are persisting and prevailing in the inoculated roots (Vosatka et al. 2012). In our study, we found that two single isolates, a *Rhizophagus* member (OTU1) and *F. mosseae* BEG12, were present in plant roots inoculated with BM and SI, respectively. A recent study by Kiers et al. (2011) proved how the best plant-AMF combinations are stabilized by reciprocal benefits in vitro. Given this, we can assume that among the AMF biodiversity present in the BM inoculum, OTU1 was the most suitable to the host and the environmental conditions provided (peat-based substrate and greenhouse conditions). Nevertheless, both isolates did not result in acceptable degrees of mycorrhizal colonization of *C. japonica* roots, confirming that the AMF set present in the two commercial inocula was inadequate for this particular plant species.

5. Conclusions

On the basis of our results, we conclude that the applied nonspecific AMF inocula poorly colonized the root system of *C. japonica* 'Dr. Burnside' and failed to replace fertilization. Given that *C. japonica* can be highly mycorrhizal (Berruti et al. 2013), the failure of the two tested commercial inocula may suggest the need to reconsider the way such inocula are formulated, especially when dealing with plant species with particular rhizospheric conditions (Vosatka et al. 2012). Natural or semi-natural ecosystems, where the desired host plant is well established, represent a valid source of naturally selected AMF. Unfortunately, field samples carry a great number of soil biota and cannot be inoculated in pots as is. Further studies will be aimed at the standardization and formulation of AMF inocula from selected field soils able to fully express their potential benefits.

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Tables legend

Table 1

Growth and ornamental value of camellias grown under the three biofertilization regimes (NI, SI and BM), and in high or low fertilization regimes (HFE and LFE) at the end of cultivation. The statistical relevance (p-value, $p < 0.05$ = significant, $p < 0.001$ = highly significant, ns = non-significant) of the effect of the two fixed factors (inoculum and fertilization regime) and their interaction is provided.

Table 2

Macroelement and microelement uptake in the root and leaf tissues under the three biofertilization regimes (NI, SI and BM), and in high or low fertilization (HFE and LFE) at the end of cultivation. The statistical relevance (p-value, $p <$

0.05 = significant, $p < 0.001$ = highly significant, ns = non-significant) of the effect of the two fixed factors (inoculum and fertilization regime) and their interaction is provided.

Table 3

Mean values of the two experimental points of mycorrhization frequency (F%), AMF colonization intensity (M%) and the presence of arbuscules (A%) in the control (NI) and inoculated pots (SI and BM), and in high or low of fertilization (HFE and LFE). Values are calculated according to the freeware Mycocalc, available online (http://www2.dijon.inra.fr/mychintec/Mycocalc-prg/download.html). The statistical relevance (p-value, $p< 0.05$ = significant, $p < 0.001$ = highly significant, ns = non-significant) of the effect of the two fixed factors (inoculum and fertilization regime) and their interaction is provided.

Figures legend

Figure 1

Mean plot profiles for foliage diameter, height/diameter ratio and branches dry weight explaining the two-way interactions between the two fixed factors (inoculation and fertilization regime). Means followed by the same letter do not differ significantly at $p < 0.05$, according to REGW-F test. Lower-case and capital letters are used for low fertilization (LFE) and high fertilization (HFE), respectively. NS or ns indicate no significance.

Figure 2

Mean plot profiles for macroelements (Ca, Mg, and K) accumulation in roots explaining the two-way interactions between the two fixed factors (inoculation and fertilization regime). Means followed by the same letter do not differ significantly at $p < 0.05$, according to REGW-F test. Lower-case and capital letters are used for low fertilization (LFE) and high fertilization (HFE), respectively. NS or ns indicate no significance.

Figure 3

Mean plot profiles for microelements (Fe, Zn, Cu, and Mn) accumulation in roots explaining the two-way interactions between the two fixed factors (inoculation and fertilization regime). Means followed by the same letter do not differ significantly at $p < 0.05$, according to REGW-F test. Lower-case and capital letters are used for low fertilization (LFE) and high fertilization (HFE), respectively. NS or ns indicate no significance.

Figure 4

Mean plot profiles for macro and microelements (Ca, Cu, Mn, and Zn) accumulation in leaves explaining the two-way interactions between the two fixed factors (inoculation and fertilization regime). Means followed by the same letter do not differ significantly at $p < 0.05$, according to REGW-F test. Lower-case and capital letters are used for low fertilization (LFE) and high fertilization (HFE), respectively. NS or ns indicate no significance.

Figure 5

Neighbor-joining phylogenetic analysis of the arbuscular mycorrhizal fungal SSU rDNA sequences (~500bp) obtained from the BM inoculum. The phylogenetic tree constructed on a portion of the arbuscular mycorrhizal fungal SSU rDNA (~500bp) using the Neighbor-Joining method (Saitou and Nei 1987). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches (Felsenstein 1985) and branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The evolutionary distances were computed using the Kimura 2-parameter method (Kimura 1980) and are in the units of the number of base substitutions per site. The analysis involved 136 nucleotide sequences (126 reference sequences and 10 sequences representative of the OTUs found, labeled in bold). Evolutionary analyses were conducted in MEGA5 (Tamura et al. 2011). Brackets on the right and bold black text indicate diverse taxonomic groups, as described by Krüger et al. (2012). Branches corresponding to the main genera, families and orders that clustered with no OTU sequences were compressed for ease of visualization.

Tables

Table 1

treatment	replicates[w]	height (cm)	foliage diameter (cm)	height/diameter ratio	leaves dry weight (g)	branches dry weight (g)	chlorophyll content (SPAD unit)	number of flowers	flower depth (cm)
NI	70	28.4a	21.4b	1.34a	14.8a	7.4a	46.4b	1.7	4.5b
SI	70	25.3b	20.6b	1.22b	12.5b	5.7b	44.5b	2.4	4.2b
BM	70	27.9ab	24.2a	1.15b	15.0a	6.9a	49.6a	2.1	5.3a
inoculum		<0.05	<0.001	<0.01	<0.05	<0.05	<0.01	ns	<0.05
HFE	105	36.5	25.4	1.50	20.7	10.5	74.1	4.1	4.5
LFE	105	18.2	18.8	0.98	7.7	2.9	20.4	0.0	nd[j]
fertilization		<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	nd
*inoculum*fertilization*		ns	<0.001	<0.001	ns	<0.05	ns	ns	nd

[w]Number of replicates varied for leaves and branches dry weights (40 for the inoculum factor and 60 for the fertilization factor) and flower depth (20 for the inoculum factor). [z]Means followed by the same letter do not differ significantly at $p < 0.05$, according to REGW-F test. [y]ns = non-significant. [j]nd = not determinable.

Table 2

tissue	treatment	replicates	P (%)	Ca (%)	Mg (%)	K (%)	Cu (mg/kg)	Mn (mg/kg)	Fe (mg/kg)	Zn (mg/kg)
Root	NI	6	0.36a[z]	0.55c	0.21c	0.70c	12.6b	20.5b	239.8c	204.2b
	SI	6	0.29b	0.73b	0.26b	1.00a	11.0c	98.8a	2655.0b	146.8c
	BM	6	0.37a	0.93a	0.33a	0.75b	25.1a	98.5a	3642.2a	216.7a
	inoculum		<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
	HFE	9	0.47	0.63	0.26	0.82	17.0	59.5	2134.8	212.4
	LFE	9	0.21	0.84	0.27	0.81	15.4	85.6	2223.2	166.0
	fertilization		<0.001	<0.001	<0.01	ns	<0.001	<0.001	<0.05	<0.001
	*inoculum*fertilization*		ns	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Leaf	NI	6	0.09a	2.46b	0.14	1.07b	15.5c	154.8c	106.7	94.4a
	SI	6	0.04b	2.24a	0.14	1.22a	22.5a	331.5a	111.8	90.4b
	BM	6	0.04b	2.17a	0.12	1.07b	19.4b	292.8b	105.8	82.92c
	inoculum		<0.001	<0.001	ns	<0.05	<0.001	<0.001	ns	<0.001

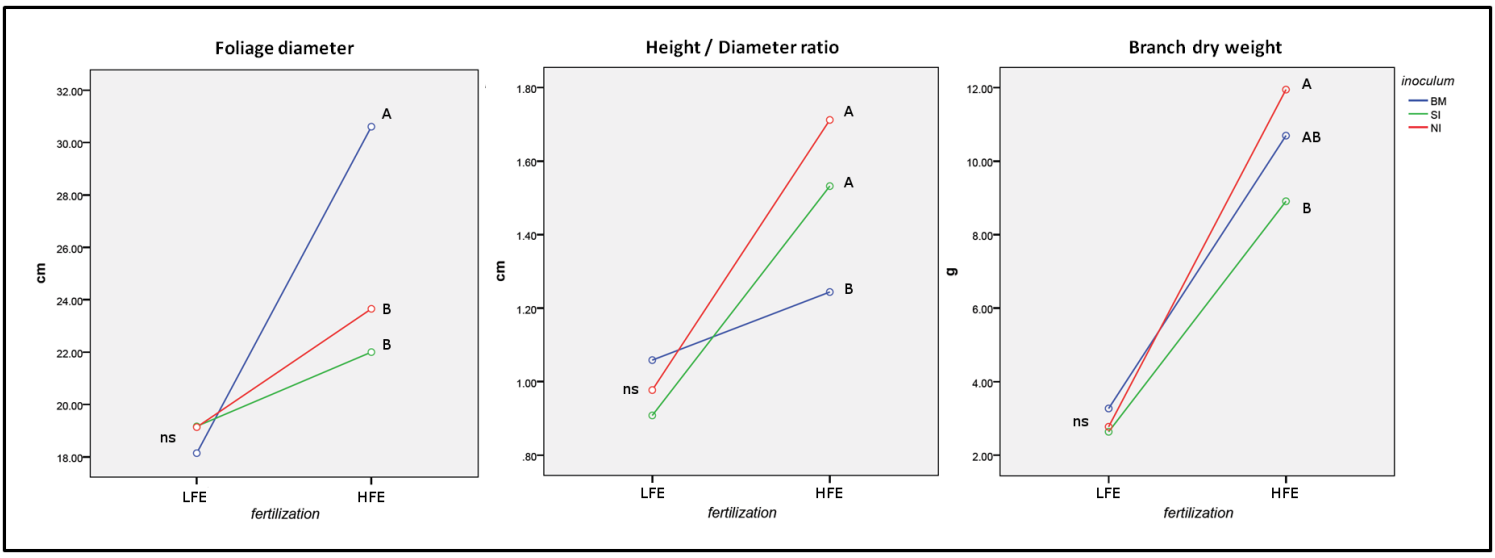
HFE	9	0.07	2.78	0.17	1.14	14.0	260.4	107.1	71.6
LFE	9	0.04	1.80	0.10	1.10	24.3	259.0	109.1	107.0
fertilization		<0.001	<0.001	<0.001	ns	<0.001	ns	ns	<0.001
*inoculum*fertilization*		ns	<0.001	ns	ns	<0.001	<0.001	ns	<0.05

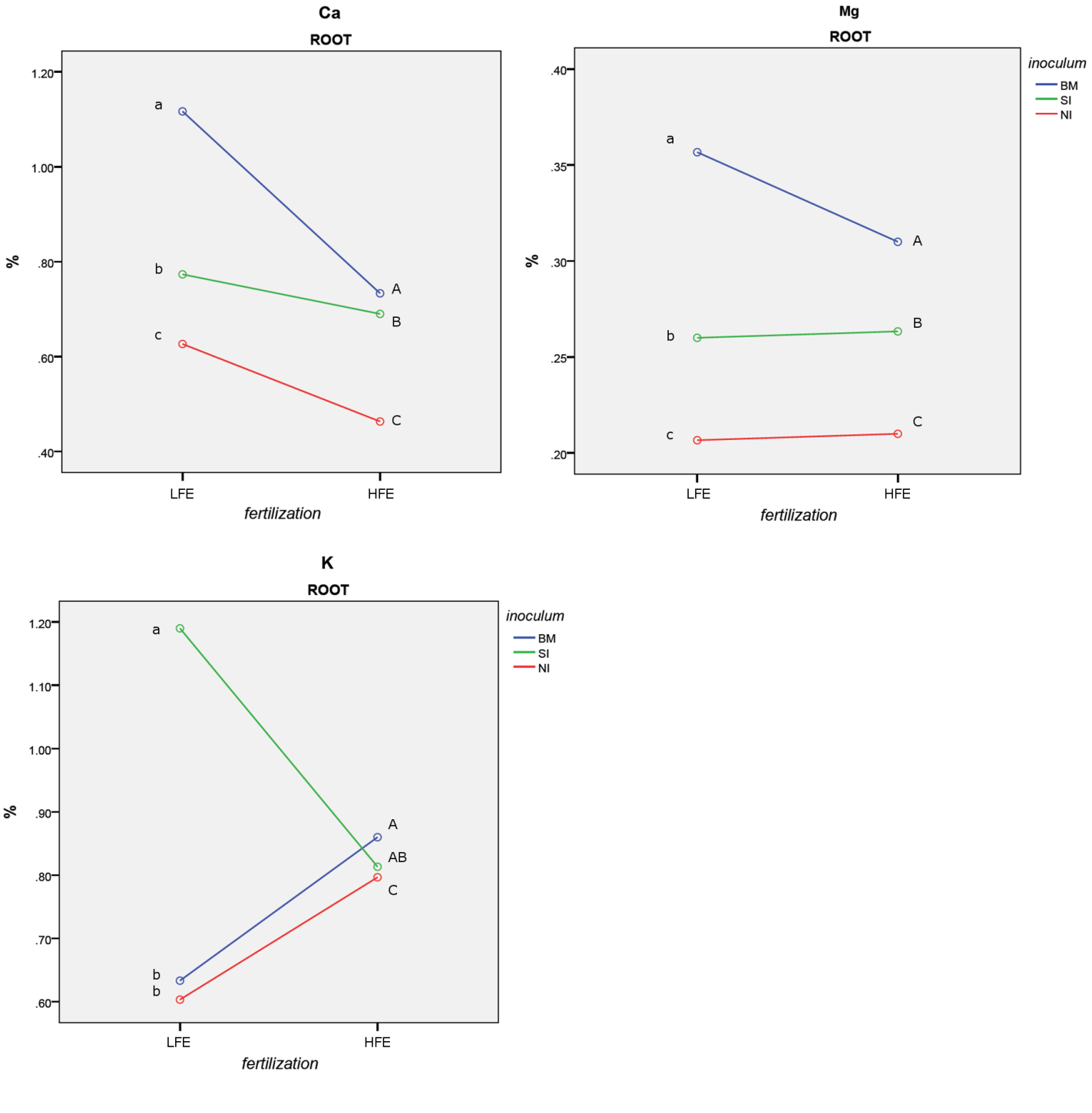
[z]Means followed by the same letter do not differ significantly at P<0.05, according to the REGW-F test

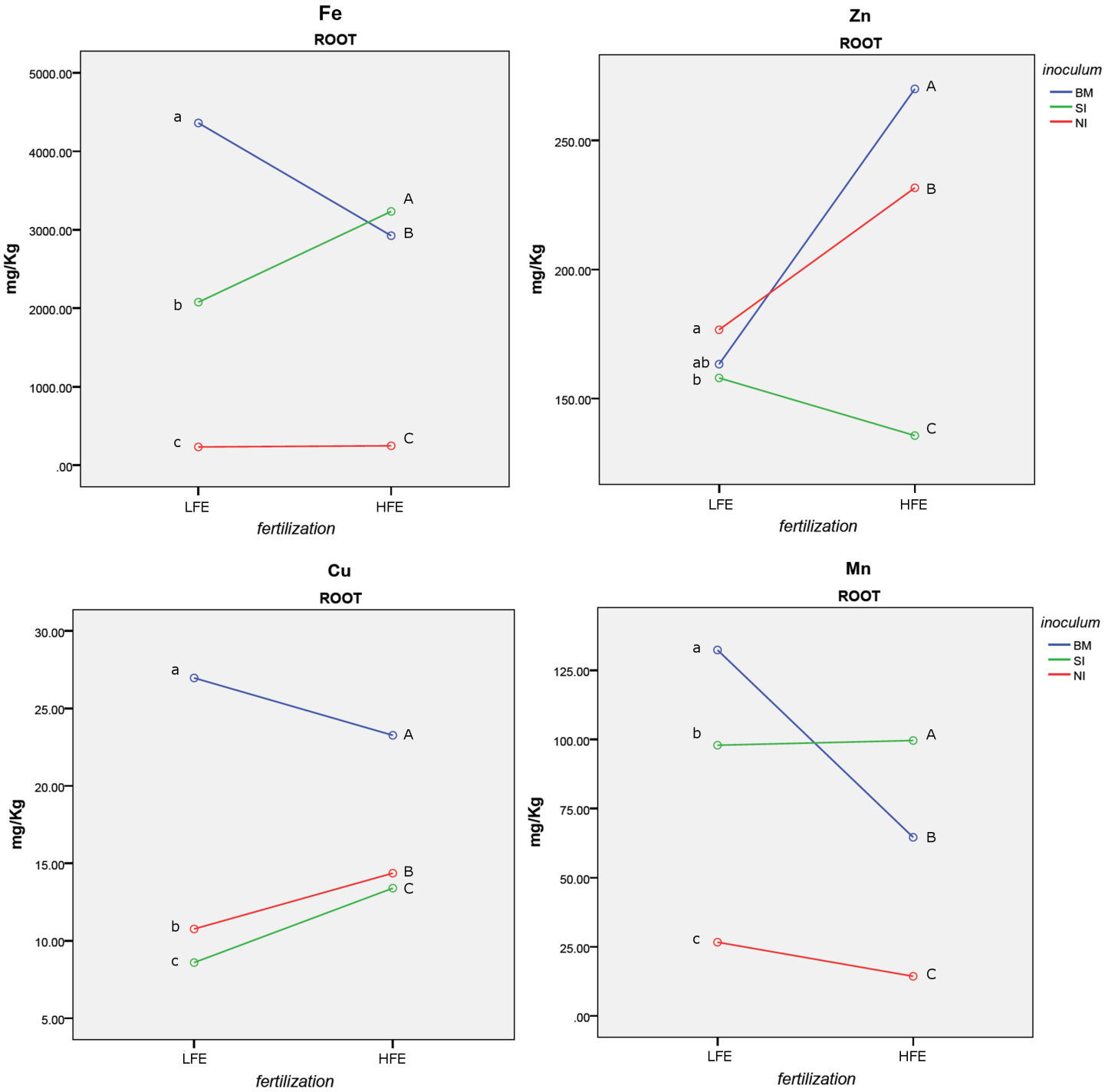
Table 3

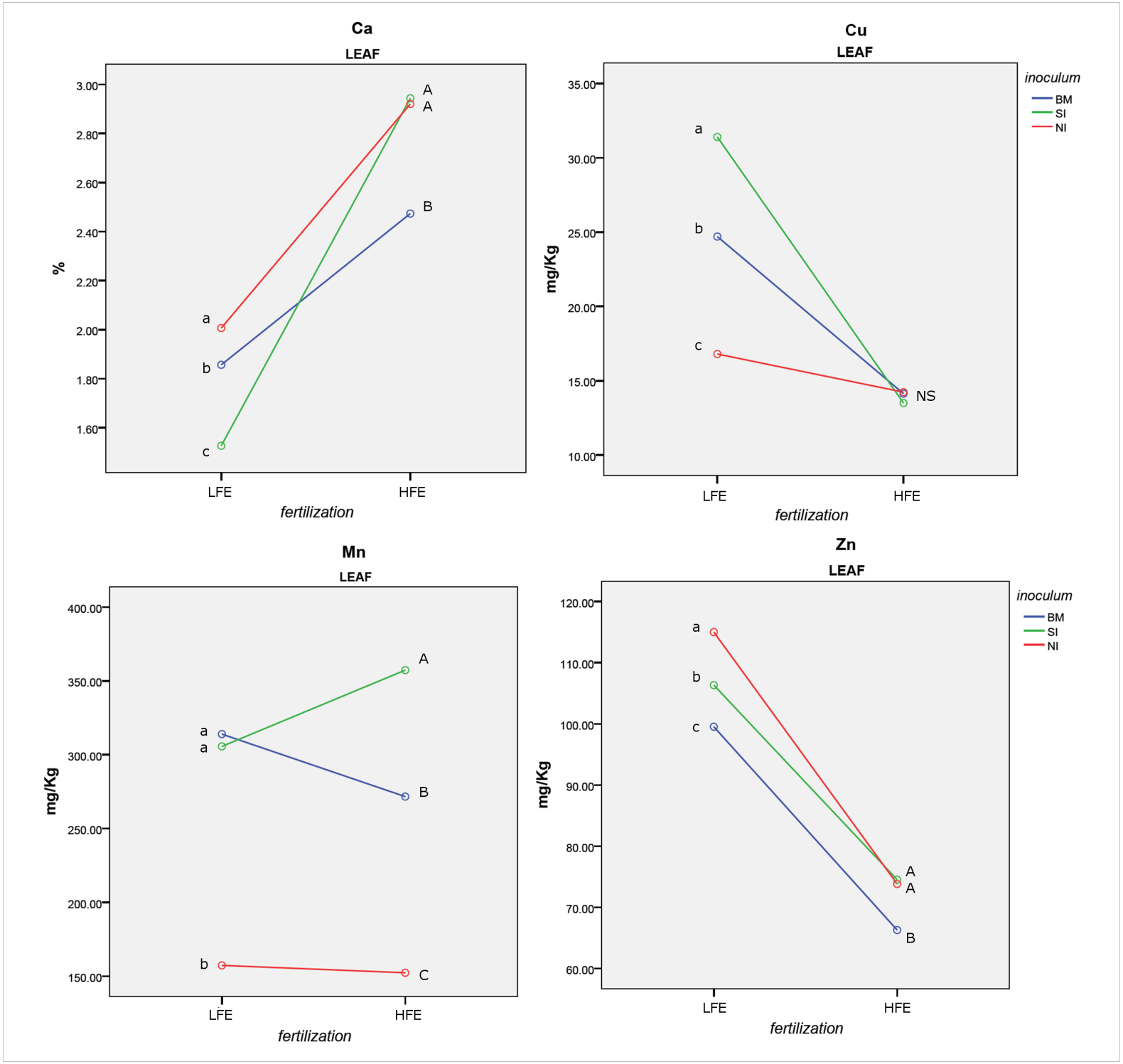
factor	replicates	mycorrhization frequency (%)	mycorrhization intensity (%)	arbuscule abundance (%)
NI	4	2.41c[z]	0.04b	0.00
SI	4	13.70b	0.80a	0.08
BM	4	37.22a	1.46a	0.17
inoculum		<0.01	<0.01	ns
LFE	6	11.72	0.31	0.04
HFE	6	23.83	1.22	0.13
fertilization		ns[y]	<0.05	ns
*inoculum*fertilization*		ns	ns	ns

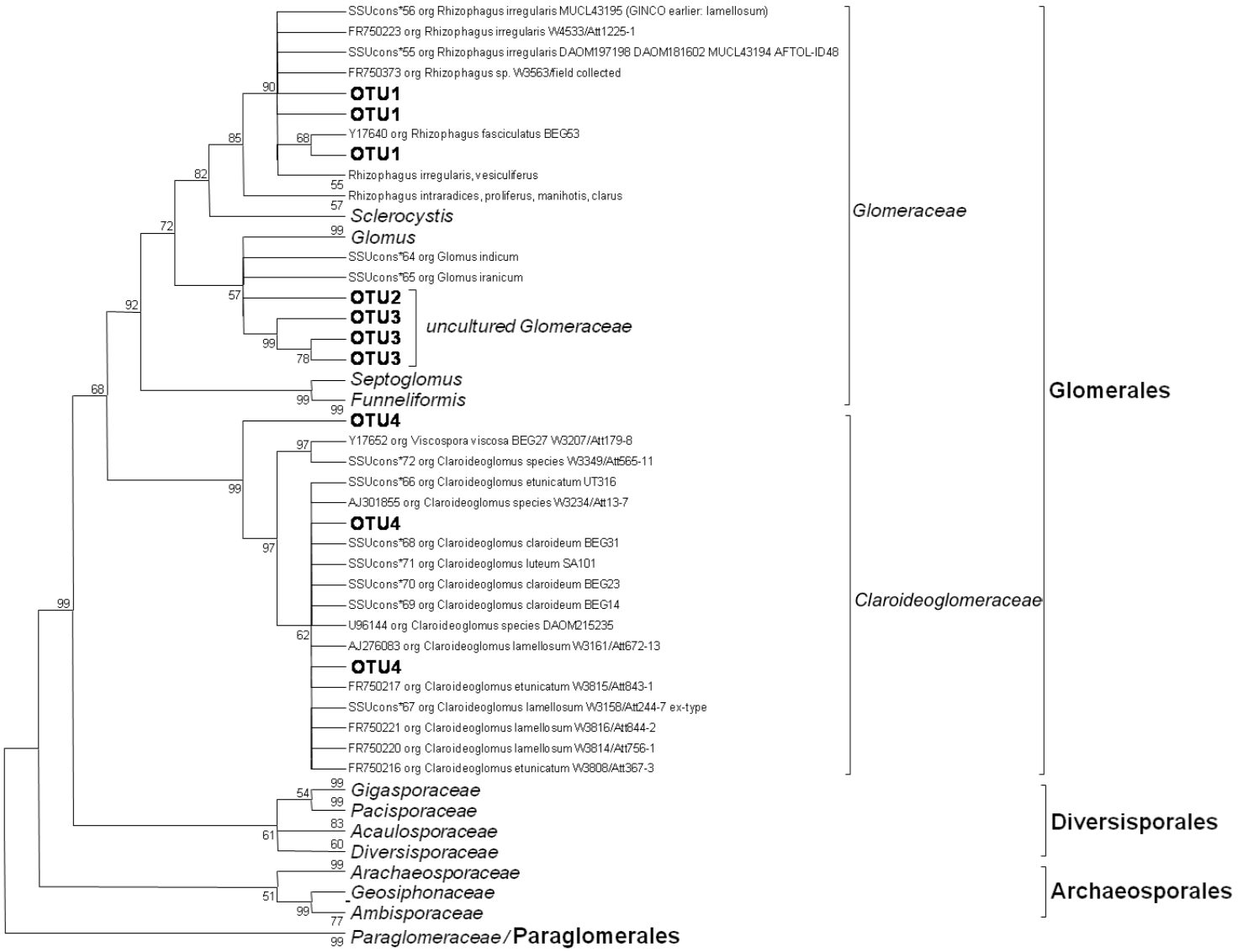
[z]Means followed by the same letter do not differ significantly at $p < 0.05$, according to the REGW-F test. [y]ns = non-significant.


Foliage diameter
cm
32.00
30.00
28.00
26.00
24.00
22.00
20.00
18.00
A
B
B
ns
LFE
HFE
fertilization
Height / Diameter ratio
cm
1.80
1.60
1.40
1.20
1.00
.80
A
A
B
ns
LFE
HFE
fertilization
Branch dry weight
g
12.00
10.00
8.00
6.00
4.00
2.00
A
AB
B
ns
LFE
HFE
fertilization
inoculum
BM
SI
NI


Ca
ROOT
1.20
1.00
.80
.60
.40
%
a
b
c
A
B
C
LFE
HFE
fertilization
Mg
ROOT
.40
.35
.30
.25
.20
%
a
b
c
A
B
C
LFE
HFE
fertilization
inoculum
BM
SI
NI
K
ROOT
1.20
1.10
1.00
.90
.80
.70
.60
%
a
b
b
A
AB
C
LFE
HFE
fertilization
inoculum
BM
SI
NI


Fe
ROOT
5000.00
4000.00
3000.00
2000.00
1000.00
.00
mg/Kg
a
b
c
A
B
C
LFE
HFE
fertilization
Zn
ROOT
250.00
200.00
150.00
mg/Kg
a
ab
b
A
B
C
LFE
HFE
fertilization
inoculum
BM
SI
NI
Cu
ROOT
30.00
25.00
20.00
15.00
10.00
5.00
mg/Kg
a
b
c
A
B
C
LFE
HFE
fertilization
Mn
ROOT
125.00
100.00
75.00
50.00
25.00
.00
mg/Kg
a
b
c
A
B
C
LFE
HFE
fertilization
inoculum
BM
SI
NI


Ca
LEAF
%
3.00
2.80
2.60
2.40
2.20
2.00
1.80
1.60
A
A
B
a
b
c
LFE
HFE
fertilization
Cu
LEAF
mg/Kg
35.00
30.00
25.00
20.00
15.00
10.00
a
b
c
NS
LFE
HFE
fertilization
inoculum
BM
SI
NI
Mn
LEAF
mg/Kg
400.00
350.00
300.00
250.00
200.00
150.00
A
a
a
B
b
C
LFE
HFE
fertilization
Zn
LEAF
mg/Kg
120.00
110.00
100.00
90.00
80.00
70.00
60.00
a
b
c
A
A
B
LFE
HFE
fertilization
inoculum
BM
SI
NI


SSUcons*56 org Rhizophagus irregularis MUCL43195 (GINCO earlier: lamellosum)
FR750223 org Rhizophagus irregularis W4533/Att1225-1
SSUcons*55 org Rhizophagus irregularis DAOM197198 DAOM181602 MUCL43194 AFTOL-ID48
FR750373 org Rhizophagus sp. W3563/field collected
OTU1
OTU1
Y17640 org Rhizophagus fasciculatus BEG53
OTU1
Rhizophagus irregularis, vesiculiferus
Rhizophagus intraradices, proliferus, manihotis, clarus
Sclerocystis
Glomus
SSUcons*64 org Glomus indicum
SSUcons*65 org Glomus iranicum
OTU2
OTU3
OTU3
OTU3
uncultured Glomeraceae
Septoglomus
Funneliformis
OTU4
Y17652 org Viscospora viscosa BEG27 W3207/Att179-8
SSUcons*72 org Claroideoglomus species W3349/Att565-11
SSUcons*66 org Claroideoglomus etunicatum UT316
AJ301855 org Claroideoglomus species W3234/Att13-7
OTU4
SSUcons*68 org Claroideoglomus claroideum BEG31
SSUcons*71 org Claroideoglomus luteum SA101
SSUcons*70 org Claroideoglomus claroideum BEG23
SSUcons*69 org Claroideoglomus claroideum BEG14
U96144 org Claroideoglomus species DAOM215235
AJ276083 org Claroideoglomus lamellosum W3161/Att672-13
OTU4
FR750217 org Claroideoglomus etunicatum W3815/Att843-1
SSUcons*67 org Claroideoglomus lamellosum W3158/Att244-7 ex-type
FR750221 org Claroideoglomus lamellosum W3816/Att844-2
FR750220 org Claroideoglomus lamellosum W3814/Att756-1
FR750216 org Claroideoglomus etunicatum W3808/Att367-3
Gigasporaceae
Pacisporaceae
Acaulosporaceae
Diversisporaceae
Arachaeosporaceae
Geosiphonaceae
Ambisporaceae
Paraglomeraceae/Paraglomerales
Glomeraceae
Claroideoglomeraceae
Glomerales
Diversisporales
Archaeosporales