EVALUATION OF DIFFERENT VIETNAMESE SOILS AS POTENTIAL SOURCE OF ARBUSCULAR MYCORRHIZAL FUNGAL INOCULUM IN *CAPSICUM FRUTESCENS*

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Abstract: Consumption of chili peppers (*Capsicum frutescens* L.) represents an important aspect of the daily diet for Vietnamese population because of its high content for antioxidant compounds. To increase the economic benefits related to chili peppers cultivation and reduce negative impacts of the high input agriculture on the environment, biological alternatives to chemical fertilizers are strongly demanded. Arbuscular mycorrhizal fungi (AMF) are well-known soil microorganisms of great interest for their potential application in agriculture as 'bio-enhancers' of plant performance. However selection of suitable AMF strains is time-costing and the outcome of field inoculation can be affected by the weak ability to compete among the native AMF population. In the present study we proposed a "bulk" approach to identify soil hosting AMF strains suitable for the development of inocula for *C. frutescens*. Three different soils were tested as source of AMF inoculum in bi-compartmented pot cultures. All the inoculated treatments performed significantly better, in terms of plant growth, compared with the non-inoculated control plants. Pots inoculated with soil from tropical forest showed the best growth performances. Molecular characterization of the AMF root assemblages highlighted differences in the composition among treatments, with the "tropical forest soil" treatment characterized by the highest number of AMF taxa colonizing the roots.

Keywords: arbuscular mycorrhizal fungi; chili pepper; *Capsicum frutescens* L.; Vietnam; inoculation; bi-compartmented pot culture

Introduction

Chili peppers belong to the plant genus *Capsicum* (family Solanaceae) and are among the most largely consumed spices throughout the world. In Vietnam, chili peppers (*Capsicum frutescens* L.) play an important role in the daily diet of the population because of the high content of vitamins, minerals such as Ca, P, K, Fe, and the antioxidant capsaicin responsible for the pungent taste (Malik et al., 2011). Vitamins such as C, E, pro- vitamin A and B are present in high concentrations in various chili pepper types (Howard et al., 2000; Bae et al., 2012). The intake of these antioxidant compounds in food is an important health-protecting factor when they are taken daily in adequate amounts (Sies, 1991).

Because of their importance on the market chili peppers provide high economic returns to farmers and thereby contribute to the Gross Domestic Product (GDP) of Vietnam, with 4% of the agricultural GDP (Office of Ministry of Agriculture and Rural Development). To increase yield of chili peppers most farmers use chemical fertilizers, but their high price significantly reduces their profit. Furthermore high input agriculture practices have a considerable negative impact on the environment and represent a matter of concern for the health of consumers (Van Bruggen, 1995; Atkinson et al., 2002).

An increasing demand for biological alternatives has promoted the interest on application studies of the subset of soil microorganisms known to improve plant growth and health (Willis et al., 2013). Some of them play an important role in the rhizosphere, influencing the nutrient acquisition by the plant. Among the soil biota responsible for key ecological services, arbuscular mycorrhizal fungi (AMF) occupy a

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special ecological niche because they represent the most ancient and widespread symbiosis involving 70–90% of land plant species (Parniske, 2008). The majority of agricultural crops have the potential to host AMF as root symbionts (FAO, 2012), with benefits from improved mineral nutrient uptake in exchange of photosynthetates. In addition to enhanced plant productivity AMF can provide increased resistance to soil pathogens, to abiotic stress factors like drought, salinity and heavy metal toxicity and contribute in improving soil structure (Jeffries et al., 2003).

Over the last decades, application of AMF in agriculture has increased greatly and a significant effort has been dedicated to develop suitable formulations for fungal propagules (Gianinazzi and Vosatka, 2004). However the application of AMF inocula on a field scale can be problematic for the unpredictable outcome of the establishment among the native AMF community in concurrence with unfavourable edaphic factors (Berruti et al., 2014). The use of an inoculum based on locally sourced AMF might be a suitable choice because of a better adaptation to the environmental conditions (Lambert et al., 1980), avoiding at the same time the ecological risks of the introduction of foreign species (Schwartz et al., 2006). Furthermore even though experiments with cultured isolates suggested AM fungi had very low host plant specificity (Klironomos, 2000), recent findings highlighted preferential host-symbiont associations as a factor to consider for a successful outcome of inoculation (Magurno et al., 2015). In the present study we propose a "bulk" approach to identify soil hosting AMF strains suitable for the development of inocula for *C. frutescens*. The use of AMF natural consortia from soils of different origin, instead of single AMF isolates, was chosen in order to:

- avoid the time-costing step of isolation of single AMF strains
- associate the beneficial effects on the plant growth to AMF strain/strains competitive in the root colonization

Materials and methods

Pot design

Three types of soils were chosen as a source of AMF inoculum, following a land-use gradient. Soil from tropical forest (FS) was sampled at Nam cat Tien (11°23'15.0"N; 107°28'05.0"E) in Dong Nai province, Vietnam. The soil was described as sandy and clay-rich. The dominant resident plant was *Tetrameles nudiflora*, a large deciduous tree species widespread in Southeast Asia. Agricultural (AS)and grassland (GS) soils, both sandy, were sampled at the district of Ho Chi Minh City (10°53'17.2"N; 106°40'03.1"E), Vietnam, from a field cultivated with cassava (*Manihot esculenta*) and from an adjoining meadow. All samplings were performed on March 2013, by collecting five soil cores per soil type at a depth of 30 cm. Cores from the same soil type were grinded and mixed together homogeneously.

In order to evaluate the contribution of the AMF consortia to the plant growth the tests were carried out in plastic pots (18cm x 18cm x14cm) divided by a nylon mesh filter (40µm pore size) in two compartments (Figure 1). Peat, mixed with sand, was used for its ability to retain moisture and as source of mineral nutrients at low concentration. The mixture of sand-peat was autoclaved at 121˚C for two hours and its pH was adjusted to 6.5. The upper part was filled with sand and peat with a ratio 5:1, while the bottom part was filled with sand and peat with a ratio 2:1. The bottom compartment was inoculated with 45 g of soil (1.5% w/w) close to the interface with the upper compartment. Because of the size of its pores the nylon mesh filter was representing a physical barrier for the roots to spread into the bottom compartment where peat was present at higher concentration while AMF hyphae could have access to both compartments (Smith and Read, 2008).

Five pots were prepared for each soil type and five pots without soil inoculation were added to the trials as control (CON). Totally 20 pots were used in the experiment.

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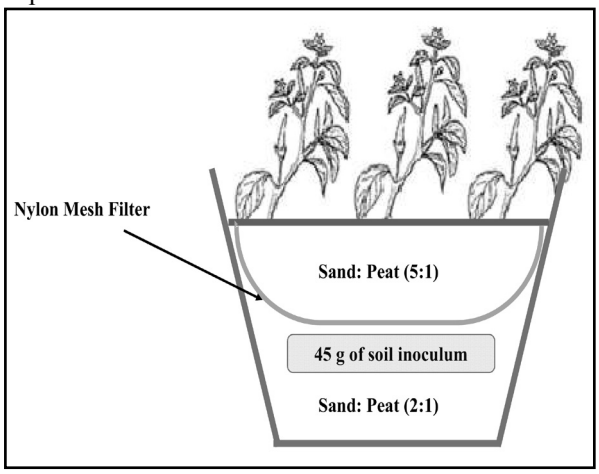
Figure 1. Schematic representation of the bi-compartmented pot culture system adopted in the experiments



A nylon mesh filter (pore size 40 µm) was used to separate two compartments with different amounts of peat as source of nutrients. The soil, source of AMF inoculum, was mixed with the substrate in the top part of the bottom compartment. Pots were planted with 3 seedlings/pot of *Capsicum frutescens* L. in a way that roots could spread only in the upper compartment with low peat content. The access to the peat present in higher concentration in the bottom compartment was achieved by the establishment of symbiosis with AMF hyphae (diameter 2-10 µm) free to move across the mesh membrane.

Plant growth conditions

Seeds of *Capsicum frutescens* L. were collected at The Institute of Biotechnology and Food Technology, Industrial University of Ho Chi Minh City (Vietnam). Seeds were surface sterilized with alcohol 80% and pre-germinated in Petri dishes under moist conditions. When two cotyledons appeared, after about three days, seedlings were transplanted into pots (three seedlings per pot). After transplanting seedlings were watered with Long Ashton nutrient solution for 7 days and subsequently with distilled water. Pots were kept in a climatic chamber EKOCHL 1500 (18/24°C, 60% RH, 16h light) for 14 weeks.

Spore counts and assessment of root colonization

Spore abundance was measured in the soils used for inoculation (FS, AS and GS) and in the substrate of the pots after plant harvesting. AMF spores were isolated in 3 replications from 35 g of air-dried soil/pot-substrate by wet sieving through 200 and 30 µm sieves, followed by sucrose gradient centrifugation (Ianson and Allen, 1986). After centrifugation, spores were transferred into Petri dishes and counted under stereomicroscope at 100X magnification. Spore abundance was expressed as the number of AMF spores per gram of soil.

To evaluate the level of root colonization, fifteen root fragments (1 cm long) from each plant were collected, washed with tap water and stained with trypan blue (Trouvelot et al., 1986). Root pieces were observed under stereomicroscope at 100X magnification and the root colonization was determined according to Trouvelot et al. (1986), using MYCOCALC software.

Agronomic variables

During the growth period, shoot length was measured at an interval of two weeks. After 14 weeks, plants were harvested paying attention to not damage the root system. Because of the extremely low nutrient conditions it was not possible to reach the flowering stage. The following variables were recorded: a) shoot length, b) root length, c) shoot dry weight, d) root dry weight.

Root DNA extraction, PCR and cloning

Molecular analyses were performed on one plant per treatment (FS, AS, GS, CON) chosen according to the best growth performances. DNA was extracted from three root fragments (1 cm long) for plant following the protocol described by Khan et al. (2007).

The AMF species composition inside the roots was analyzed by a PCR approach targeting a portion of the ribosomal Short Sub Unit (SSU). PCR was performed using the primers AML1 (5'-ATC AAC TTT CGA TGG TAG

GAT AGA-3') and AML2 (5'-GAA CCC AAA CAC TTT GGT TTC C-3'), specific for Glomeromycota (Lee et al., 2008).

Amplifications were carried out using the Phusion High-Fidelity DNA Polymerase (Thermo Scientific) with the following thermal profile: initial denaturation at 98°C for 1 min, followed by 35 cycles at 98°C for 10 sec (denaturation), 64°C for 15 sec (annealing), 72°C for 24 sec (extension), followed by a final extension at 72°C for 5 min.

PCR products were analyzed by gel electrophoresis. Bands at the expected size of 800 bp were cut out and DNA was extracted with the Illustra GFXTM PCR DNA and Gel band purification kit (GE Healthcare Life Sciences) according to the manufacturer's instructions.

Purified DNA fragments were cloned into CloneJET™ PCR Cloning Kit (Thermo Scientific) and transformed into *Escherichia coli* DH5α according to the manufacturer's instructions.Transformants were checked by PCR for the presence and size of the insert.

Restriction fragment length polymorphism analysis

Positive clones were analyzed for restriction fragment length polymorphism (RFLP) by digestion with Hinf*I* (Promega) and electrophoretic run on 2.5% TBE agarose gel. Representative clones were selected for each restriction profile found. Plasmids were extracted with the Wizard® Plus SV Minipreps DNA Purification System Kit (Promega) and sent for sequencing to Biomi Ltd (Agricultural Biotechnological Center, Gödöllő).

Phylogenetic analysis

Sequence similarities were determined using the blastn sequence similarity search tool provided by GenBank. Only sequences belonging to Glomeromycota were selected for the subsequent analyses and the others were discarded. Sequence editing was conducted manually using MEGA 4.0 (Tamura et al., 2007) and Chromas Lite 2.01. Sequences were aligned by MUSCLE with reference sequences identified with blastn and sequences representing the major taxonomic groups of Glomeromycota. Phylogenetic tree inference, using neighbour-joining method, was computed with MEGA 4.0 software assessing Kimura-2p model as distance method and 1000 replicates of non-parametric bootstrapping.

Statistical analysis

The data about the agronomic variables measured, spore abundance and root colonization were analyzed by SPSS software version 20 (IBM). The homogeny of variance of data were verified using Levene's test. When the p-value of the Levence's test was found to be less than 0.05, data were analyzed with Kruskal-Wallis test for non-parametric test.

In addition, Bonferroni test was used for post-hoc testing for multiple comparisons of the data measured. When the p-value of the Levence's test was found to be higher than 0.05, data were analyzed with ANOVA one way test and Tukey test was used as post-hoc test for multiple comparisons of the data measured.

Results

Spore counts and assessment of root colonization

Before substrate inoculation spore abundance was measured in the three different soils chosen as source of AMF inoculum. The amount of spores (average of three independent replications) observed per gram of soil was 4.34±1 in the tropical forest soil (FS), 6.26± 1 in the agricultural soil (AS) and 8.57±2 in the grassland soil (GS).

After plant harvesting the sand-peat substrate was collected and the spore abundance measured. In the control pots no spores were detected. Similar spore abundance was found among the different treatments (1.2 spores/g). Bonferroni test confirmed no significant differences among the three sets of pots

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inoculated with different soils.

After staining no root colonization was found in the roots from the control plants. Fungal mycelium and structures were observed in roots from all inoculated pots. Among the treatments FS showed the highest percentage of root colonization (81.78%) followed by GS (63%) and AS (56%). According to Bonferroni test FS treatment was significant different from AS and GS (Table 1).

Table 1. Percentage of AMF colonization in chili pepper roots. The percentages represent the average of the values measured for all the plants in every treatment. Standard deviation is provided with the values. Shared uppercase letters indicate no statistical difference (p>0.05) between the treatments, as determined by Bonferroni test. CON: control plants; FS: plants inoculated with forest soil; AS: plants inoculated with agricultural soil; GS: plants inoculated with grassland soil.

Treatment	Root colonization (%)
CON	0^{c}±0
FS	81.78^{a}±13.91
AS	56^{b}±11.75
GS	63.4^{b}±14.43

Table 1. Percentage of AMF colonization in chili pepper roots. The percentages represent the average of the values measured for all the plants in every treatment. Standarddeviation is provided with the values. Shared uppercase letters indicate no statistical difference (p>0.05) between the treatments, as determined by Bonferroni test. CON: control plants; FS: plants inoculated with forest soil; AS: plants inoculated with agricultural soil; GS: plants inoculated with grassland soil.

Plant growth

Shoot length. The plant growth after two weeks started to show a perceptible change among the treatments. At the moment of the plant harvesting, after 14 weeks, the average shoot length of FS plants was the highest (14.32 cm) followed by GS (11.57 cm), AS (11.01 cm) and CON (6.18 cm) plants respectively (Table 2). Bonferroni test showed that the CON plants were significantly different ($p< 0.05$) from the inoculated plants belonging to the FS, AS and GS treatments. According to the test the FS plants were significantly higher than AS and GS plants. No significant differences were observed between AS and GS treatments (Table 2).

Table 2. Growth of chili pepper plants measured in the assay after 14 weeks. Values are given as average of measures collected among all the plants for each treatment. Standard deviation is provided with the values. Shared uppercase letters indicate no statistical difference (p>0.05) between the treatments, as determined by Bonferroni test. CON: control plants; FS: plants inoculated with forest soil; AS: plants inoculated with agricultural soil; GS: plants inoculated with grassland soil.

Table 2. Agronomic variable of chili pepper plants measured in the assay after 14 weeks. Values are given as average of measures collected among all the plants for each treatment. Standard deviation is provided with the values. Shared uppercase letters indicate no statistical difference (p>0.05) between the treatments, as determined by Bonferroni test. CON: control plants; FS: plants inoculated with forest soil; AS: plants inoculated with agricultural soil; GS: plants inoculated with grassland soil.

Treatment	Shoot length (cm)	Root length (cm)	Dry shoot weight (mg)	Dry root weight (mg)
CON	5.18^{c}±1.9	9.88^{a}±0.09	20^{c}±7	10^{c}±3.5
FS	14.32^{a}±1.15	13.43^{a}±0.02	74^{a}±13	26^{a}±3.3
AS	11.01^{b}±3.09	12.70^{a}±0.02	47^{b}±14	20^{b}±2.2
GS	11.57^{b}±2.69	12.44^{a}±0.02	45^{b}±12	18^{b}±1.9

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Root length. The assessment of root length after 14 weeks showed a positive effect of the substrate inoculation on the root growth. The average root length of FS plants was the highest (13.43 cm) followed by AS (12.70 cm), GS (12.44 cm) and CON (9.88 cm) plants respectively (Table 2).Bonferroni test showed no significant difference among the different types of inoculation ($p>0.05$).

Dry shoot and root biomass. The dry shoot and root weight averages measured after 14 weeks confirmed the trend seen on the shoot length measurements. The weight of inoculated plants were significantly higher compared to the control plants. FS plants had significantly higher shoot and root dry biomass (74 and 26 mg respectively) than the plants belonging to the AS and GS treatments (Table 2). No significant differences were observed between AS and GS treatments.

Molecular analysis

DNA was extracted successfully from all the root samples (4).

Amplification from FS, AS and GS DNA gave an expected product of approximately 800bp while no amplicons were detected for CON DNA. After cloning a colony screening was performed to obtain 20 clones per treatment, positive for the PCR insert. The PCR products digested with Hinf*I* resulted in a total of six restriction profiles (RP1-6). The restriction profile 5 (RP5) was the most abundant, with 52 clones of 60 clones analyzed. All six restriction patterns were represented among the clones analyzed for the FS treatment. In the treatment AS only two restriction patterns were found as well as in the treatment GS. Fourteen clones, representative of the six restriction profiles, were sent for sequencing. The sequences were deposited at the National Center for Biotechnology Information (NCBI) GenBank with accession numbers KU195704-KU195714.

Phylogenetic analyses

After editing, sequences were analyzed by blastn. Three sequences, representing two restriction profiles (RP3 and RP4), were discarded because they were related either to plant or Basidiomycota DNA.

All four AMF restriction patterns were represented by the FS clones. Among the GS and AS clones only two (RP2 and RP5) and one (RP5) restriction patterns, respectively, were found. Eleven AMF sequences were used to build a phylogenetic tree (Figure 2). Sequences related to the restriction patterns RP5 and RP6 clustered inside the Claroideoglomeraceae family with the exception of one clone RP5 clustering with RP2 inside the *Gigaspora* genus. RP1 clustered with the reference sequence from *Glomus macrocarpum* in the family of Glomeraceae.

Discussion

The pot culture system adopted in the present study represented a challenge for the plants to survive. With the exception of the first week, when Long Ashton nutrient solution was provided, the only source of nutrients was provided by peat mixed to sand with different ratio in the two compartments (sand-peat 5:1 and sand-peat 2:1 respectively). Nevertheless, all the control and inoculated plants survived for the complete duration of the experiments. As expected, because of the extreme deficiency of nutrients, it was not possible to reach the reproductive stage of the plants, even after 14 weeks of growth.

The nylon mesh filter was used with the aim to give access to the high-peat-content compartment only to those plants able to establish a symbiotic relationship with AM fungi whose hyphae (diameter = 2-10 μm) could cross the membrane.

In the inoculation treatments AMF spores of new formation were found in both compartments of the pots and the plant roots showed a certain degree of mycorrhization, indicating that the experiment was successful in the establishment of AMF symbiosis. Even

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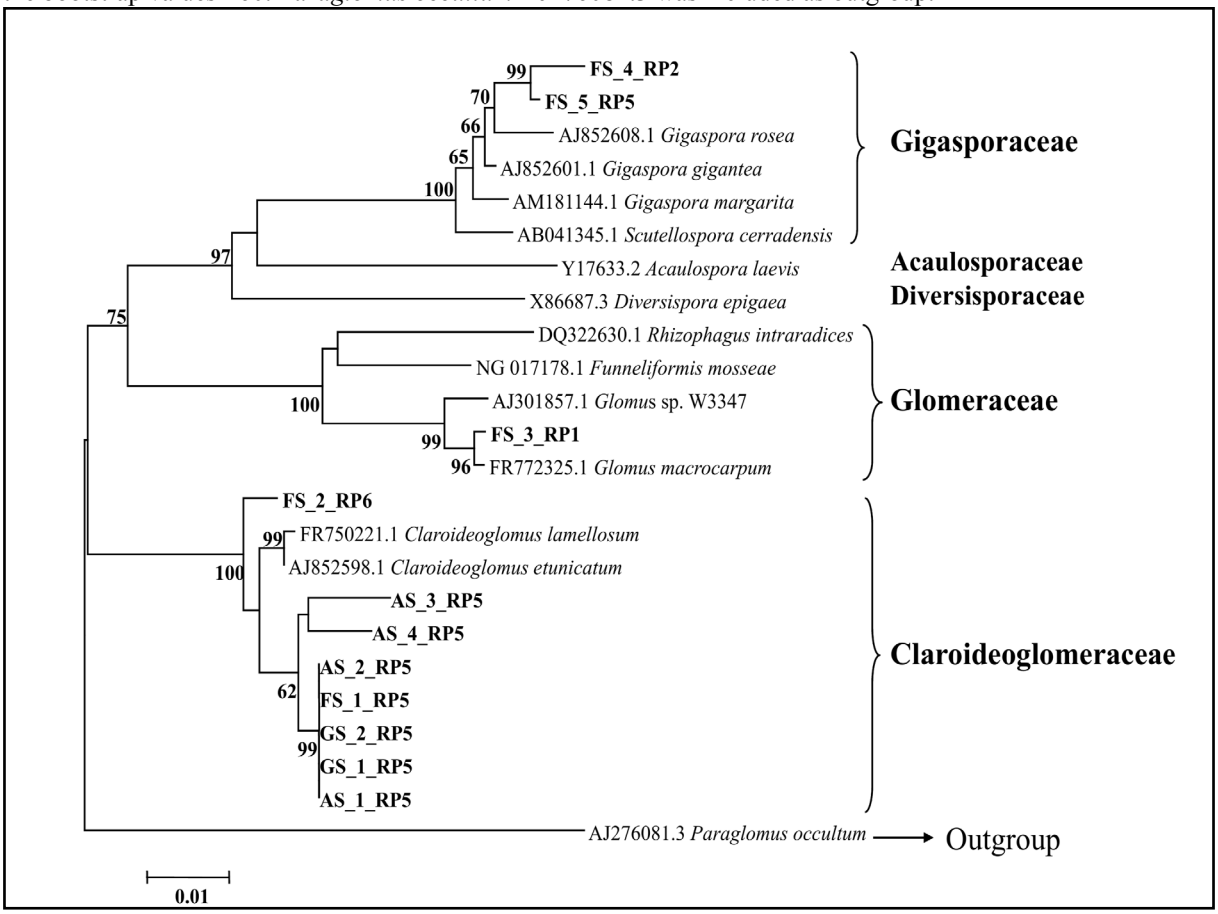
Figure 2. Neighbour-joining phylogenetic tree displaying the relationship between the 11 AMF sequences recovered from chili pepper roots and 13 reference sequences from GenBank, representing some of the main Glomeromycota families. Sequences obtained in this study are in bold and labelled with the corresponding restriction pattern and soil of provenience (FS: forest soil, AS: agricultural soil, GS: grassland soil). Numbers next to the nodes indicate the bootstrap values >60. *Paraglomus occultum* AJ276081.3 was included as outgroup.



if the soil source of AMF inoculum was placed in the bottom compartment the distance from the developing roots was not preventing the establishment of symbiosis. In a previous study extensive mycelial growth was observed in the absence of the host plant with germinating hyphal length up to 5 cm 15 days after spore germination (Logi et al., 1998).

During the 14 weeks, a significant difference in plant growth between the inoculation treatments and the control was observed.

This result indicated that the plants in the FS, AS and GS treatments could receive a better nutrition through the AMF mycelium spread in the compartment rich in peat content, where the plant roots could not have had access.

Plants from the FS treatment showed overall a better development compared to the plants from other treatments, directly correlated with the highest percentage of root colonization (≈ 82%). Generally a strong mycorrhization is associated with a reduced root system because the plant, supplied with nutrients by the AMF mycelium, does not need to invest resources in the roots development (Bonfante and Perotto, 1995). In the present study this was not observed probably due to the deficit of nutrients in the pot system.

Considering the spore abundance measured in the different soils before inoculation, the best growth performances of FS plants appeared not to be correlated merely with the number of spores present in the inoculum. Therefore the identity of AMF strains colonizing the roots could have played an important role in the outcome of the symbiosis.

Specific primer AML1 and AML2 were used to verify the presence and identify of the AMF taxa in the plant roots. All the treatments inoculated gave a positive PCR product and, as expected, no amplification was detected in the control plant roots. The phylogenetic analysis showed a good correlation between the restriction patterns observed and the phylotypes identified. Sequences were distributed in three families even if most of them, associated with two restriction patterns, clustered in the family of Claroideoglomeraceae.

In the FS treatment (inoculated with tropical forest soil) the highest number of taxa (4) was found, followed by GS (2) and AS (1) treatments. These results are in agreement with the data reported by Öpik et al. (2006), where the number of AM fungal taxa per host species differed between habitat types: the highest richness belonged to tropical forests (18.2 AMF taxa per plant species), followed by grasslands (8.3), temperate forests (5.6) and habitats under anthropogenic influence (arable fields and polluted sites, 5.2). In our assay molecular analyses were limited to a low number of clones (20 per treatment) thus our data could just resemble the richness gradient, not the taxa numbers, mentioned above.

Considering the distribution of clones among the phylotypes detected, it was not possible to recognize exclusive dominant taxa associated with the best plant growth performances observed in FS plants. In fact the same phylotype belonging to the Claroideoglomeraceae family was strongly dominant in all the treatments. However, the molecular target used for AMF identification does not allow a deep resolution at species/strain level. It was demonstrated that some functional traits could be different among strictly related species and among strains belonging to the same species (Takács et al., 2006). On this point of view, it could be hypothesized that the highest root colonization, as well as the growth response observed in the FS plants could be explained by species or strains different from those present in the AS and GS plants, even if belonging to the same AMF genus.

Furthermore, one of the two FS sequences clustering in the Gigasporaceae was associated to the dominant restriction profile RP5, mainly related to the Claroideoglomeraceae family. As a consequence an underestimation of the abundance of FS clones related to the Gigasporaceae family could have occurred. Members of the *Gigaspora* genus colonize soil more extensively than plant roots, thus their contribution to plant nutrition should not be correlated with the percentage of root colonization (Hart and Reader, 2002).

Conclusion

We described here an effective methodology to test and select useful AMF strains under controlled conditions. Contrary to the classical procedures of developing formulations for AMF propagules, we proposed an approach involving uncharacterized AMF assemblages with the aim to evaluate beneficial effects for the plant growth and ability of the AMF strains to compete in the root colonization in the same trial. Therefore this experimental system, when applied on a large scale with the purpose of commercial inocula development, could be suitable to test a high number of AMF strains at the same time, avoiding the time-costing step of strain isolation and propagation. The system we proposed could be applied also for other tests beyond those related to nutritional benefits of the symbiosis.

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