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Potentialities of ecological engineering strategy based on native arbuscular mycorrhizal community for improving afforestation programs with carob trees in degraded environments

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ABSTRACT

Efficient afforestation programs are crucial to limit soil degradation in various arid and semi-arid ecosystems. However, the success of these programs is dependant to the plant type selected for revegetation and the methods used for seedling production. Exotic fast-growing trees have been largely planted but their use is currently controversial because of their potential negative ecological impacts. Whereas the positive impact of arbuscular mycorrhizal (AM) fungal inoculation in nursery was demonstrated, few studies focused on the monitoring of mycorrhizal inoculation in arid and semi-arid ecosystems. In addition, the majority of studies are based on single-species inocula with non native AM fungal strains. The current study aims at evaluating the efficiency of mycorrhizal inoculation of the emblematic Mediterranean carob tree (*Ceratonia siliqua*) in a Moroccan degraded site, through an ecological engineering strategy based on the use of a complex native AM community (naturally associated to carob trees). Results demonstrate the high potential of this approach by improving sustainably the growth and nutrient status of carob trees in a 3-year-old plantation and also by inducing a positive soil microbial environment for nutrient cycling and environmental stress resistance.

1. Introduction

The carob tree (*Ceratonia siliqua* L.), a sclerophyllous leguminous belonging to the Caesalpinaceae sub-family, is a relevant component of the Mediterranean vegetation. Its cultivation is environmentally and economically valuable in marginal and prevailing calcareous soils of the Mediterranean region (Batlle and Tous, 1997). This multipurpose tree species was traditionally used for its pods providing fodder for ruminants (Louca and Papas, 1973) and non-ruminants (Hillcoat et al., 1980). More recently the products derived from the carob pods (pulp and seed) have also been considered in human food, pharmaceutical and cosmetic industries (Barracosa et al., 2007). For all these reasons, this crop is currently being re-emphasized for agriculture diversification and soil restoration in dryland areas (Janick and Paull, 2008).

A common characteristic of degraded soil is the lack of fertility leading to a low plant cover (Séré et al., 2008). Moreover, in semi-arid regions, the spontaneous processes of plant establishment and natural succession are largely slowed by low and variable precipitations and wind desiccation (Allen, 1989). Rehabilitation of degraded soils in semi-arid Mediterranean regions could be achieved through the reconstruction of their basic properties such as nutrient availability, organic matter content, soil structure, etc. These objectives can be succeeded with different practices such as the use of ameliorants (e.g., organic mulch), soil amendments with organic wastes and industrial by-products (e.g., compost). Another rehabilitation strategy could be based on the management of plant-microbe symbioses (Barea et al., 2011). It is now largely admitted that microbial interactions can drive ecosystems functions (e.g., plant biodiversity, productivity and variability) and that below-ground diversity of arbuscular mycorrhizal (AM) symbiosis is a key soil biological component that ensure the maintenance of plant biodiversity and ecosystem functioning (van der Heijden, 2002; van der Heijden et al., 2006). A loss of AM propagules is usually recorded following degradation of the plant

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cover that could further inhibit natural or artificial revegetation processes (Barea et al., 2011). In order to restore disturbed areas, one of the strategies is thus to enhance the indigenous inoculum levels of AM fungi, but the success of inoculation programs is dependent of the identification of efficient AM fungi (Roldan et al., 1992). It has been also reported that the use of native mycorrhizal potential as a source of AM inoculum was the most effective inoculation strategy to ensure the successful re-establishment of native plants in degraded soils (Caravaca et al., 2003).

Although it has been assessed that *C. siliqua* was representative of highly mycorrhizal-dependent plant species (Ouahmane et al., 2012), the effect of AM inoculation on carob growth in natural conditions during the time and its impact on soil microbial activities in its vicinity remains unknown. The objectives of this study were (i) to produce a mycorrhizal inoculum consisting of AM fungi naturally associated with *C. siliqua* and (ii) to determine the performance of this native inoculum on carob establishment in a degraded site in Morocco.

2. Materials and methods

2.1. Natural ecosystem sampling and AM fungal diversity assessment

2.1.1. Carob root sampling

Root samples (10-cm depth) were collected from two agricultural fields (sites A and B) located in the Essaouira region (Morroco) and distance of five kilometer, i.e., close to Bou Mkila (31°31′46.81″ N, 9°34′35.82″ W) and Ounara (31°31′46.81″ N, 9°34′35.82″ W), respectively. For information, no nodule was observed on the carob roots. In these areas, *C. siliqua* is traditionally associated with barley cultures. For each site, three root samples were collected from a carob tree and stored at 4 °C before further analysis.

2.1.2. DNA extraction, PCR amplification and cloning-sequencing of LSU rDNA sequences

For each site, *C. siliqua* root samples were washed with ultra pure water and the fine roots were selected (100 mg) for grinding in liquid nitrogen. Total DNA from plant tissue were then extracted using DNeasy Plant Mini Kit (QIAGEN, Courtaboeuf, France) according to the manufacturer's instructions. DNA integrity was checked on 1% agarose gel and quantified with a Nanodrop ND100 spectrophotometer (Thermo-scientific, Courtaboeuf, France), and stored at −20 °C.

DNA extracts were submitted to nested PCR reactions to enhance the efficiency of the amplification (Gollotte et al., 2004). Primers LR1 and FLR2, targeting the fungal 5′ end of LSU rDNA sequences, were used for the first nested PCR amplification, and primers FLR3 and FL4, targeting arbuscular mycorrhizal fungal (AMF) 5′end of LSU rDNA sequences, for the second nested PCR amplification. Reactions were performed in a final volume of 25 μl containing 1 × reaction buffer, 1.5 mM $MgCl_2$, 0.8 μM of each primer, 200 μM of each dNTP, 0.2 mg ml^{-1} BSA, and 0.625 U of GOTaq DNA polymerase (Promega, Charbonnieres, France). An aliquot (1 μl) of diluted DNA extract was added to each PCR reaction. PCR products were diluted 1/100 and used as templates for the second nested PCR amplification with the primers FLR3 and FLR4. Thermal cycling was carried out with a denaturation step of 94 °C for 3 min, 35 cycles with 30 s denaturation at 94 °C, 60 s annealing at 58 °C, 60 s or 40 s elongation (for LR1-FLR2 or FLR3-FLR4, respectively) at 72 °C, and a final elongation step for 10 min at 72 °C.

For each site, FLR3-FLR4 amplicon from DNA of *C. siliqua* root samples was used for cloning into the plasmid vector pGEM-T (pGEM-T Easy Vector System kit; Promega) in order to generate a LSU rDNA clone library. A total of 114 positive clones were sequenced (Genoscreen, Lille, France). All of the sequences described here have been submitted to the EMBL database under accession numbers LM643868-LM643981.

2.1.3. Operational taxonomic unit (MOTU) designation and alpha diversity analysis

Sequences from *C. siliqua* root samples were aligned using ClustalW implemented in MEGA version 5.05 (Tamura et al., 2011). The alignment was used as input for MOTHUR (Schloss et al., 2009) to cluster the sequences into MOTUs of a defined sequence identity. Distance matrices were constructed using the command *dist.seqs* (with each gap penalization option) and sequences were clustered into MOTUs using the command *cluster*. The MOTUs were defined at 98% sequence similarity. Diversity (inverse Simpson [1/D], coverage), richness (number of MOTUs and Chao) and evenness (Simpson index-based measures) indexes were estimated. Simpson index has been preferred to Shannon index because of stronger biases observed in the case of small sample size for the latter one (Mouillot and Leprêtre, 1999). The sequencing effort was evaluated by using Boneh calculator (Boneh et al., 1998) implemented in MOTHUR.

2.1.4. Phylogenetic assignment of AMF LSU rDNA sequences

For each MOTU, a reference sequence was selected for phylogenetic assignment with LSU rDNA consensus sequences of the Glomeromycota from Krüger et al., 2012. The deduced phylogenetic tree, and bootstrap values were all computed using the global gap removal option. The neighbour joining (NJ) method and Kimura 2-parameter were used. Nodal robustness of the tree was assessed using 1000 bootstrap replicates.

2.2. Preparation of the native AM fungal inoculum

Carob roots sampled from both sites were pooled together, cut into 0.5-cm pieces and surface-sterilized in 30% H_2O_2 for 10 min to eliminate the mycorrhizosphere microflora. Then they were thoroughly rinsed in sterile distilled water and kept at 4 °C before use. Seeds of maize (*Zea maize* L.) were surface-sterilized with 1% NaOCl for 15 min and rinsed with distilled water. They were pre-germinated for 2 days in Petri dished on humid filter paper at 25 °C in the dark. The germinating seeds were used when rootlets were 1–2 cm long. Maize seedlings were grown in 1-l pots filled with a calcined clay (particule size average 5 mm), Oil-Dri USspecial Ty/IIIR (Oil-Dri Company, Chicago, USA). Then 1 g of carob root fragments was placed in a hole (1 × 5 cm) in the plant growth substrate of each pot. For the treatment without AM fungi (control), carob root fragments prepared as before were autoclaved (120 °C, 20 min) and placed in the pots at the same rate. After 12 weeks of culturing in glasshouse conditions, the maize plants were uprooted, gently washed and roots were cut into 0.5 cm and then kept at 4 °C before use.

2.3. Controlled mycorrhization of carob seedlings in glasshouse conditions

Seeds of *C. siliqua* were surface-disinfected with 95% concentrated sulfuric acid for 15 min. Then, the acid solution was removed and the seeds were rinsed for 12 h four times in sterile distilled water. They were then aseptically transferred into Petri dishes filled with 1% (w/v) agar and germinated in the dark at 25 °C for 4 days. The germinating seeds were used when rootlets were 1–2 cm long. Carob seedlings were grown in 1-l pots filled with a soil previously crushed, passed through a 2 mm sieve and autoclaved (120 °C, 60 min). After autoclaving its physico-chemical characteristics were as follows: pH (H_2O) 7.3; clay (%) 4.6; fine silt (%) 30.8; coarse silt (%) 13.3; fine sand (%) 30.1; coarse sand (%) 20.9;