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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;

carbon (%) 2.33; total nitrogen (%) 0.01; total phosphorus 39 mg kg^{-1}; soluble phosphorus (Olsen; mg kg^{-1} soil) 4.8.

Soils sterilization is well known as affecting soil physical and chemical properties such as extractable Mn levels (Wolf et al., 1989), but because the same procedure was applied for both conditions before planting, the impact of these changes on the biological comparison between AM-inoculated and non-inoculated plants should be avoided. One hole (1 cm × 5 cm) was performed in the soil of each pot and filled with 1 g of fresh maize mycorrhized root. The control treatment received the same amount of fresh maize non-mycorrhized root. After covering the holes with the same autoclaved soil, the pots were arranged in a randomized complete block design with 40 replicates per treatment. Seedlings were grown under natural light (day length approximately 10 h, mean temperature 25 °C during day). After 6 months of culturing, 10 plants were randomly sampled from each treatment. They were uprooted and their root systems gently washed with tap water. For each *C. siliqua* seedlings, height and collar diameter were measured and the oven dry weight (1 week at 65 °C) of the aerial part was determined. Nitrogen and Phosphorus determination from plant tissues were performed as described in Boudiaf et al. (2013). AM colonization was assessed as described in Ouahmane et al. (2006) by using trypan blue staining and mycorrhizal dependency of *C. siliqua* was expressed as the percentage of the difference between the total dry weight of a mycorrhizal plant and the total dry weight of a nonmycorrhizal plant divided by the total dry weight of the mycorrhizal plant (Plenchette et al., 1983). Remaining roots were oven-dried (1 week at 65 °C) and weighed.

2.4. Field experimental design, tree growth measures and soil analyses

The study site was located at Outguente in the Ourika watershed (Morocco). The climate is semi-arid Mediterranean, with an annual rainfall of 400 mm and a mean annual temperature of 18 °C. The physico-chemical characteristics of the soil were as follows: pH (H_2O) 6.92; clay (%) 29.6; fine silt (%) 27.4; coarse silt (%) 16; fine sand (%) 13.1; coarse sand (%) 13.9; carbon (%) 2.1; total nitrogen (%) 0.375; total phosphorus (mg kg^{-1} soil) 4.46; soluble Olsen phosphorus (Olsen; mg kg^{-1} soil) 1.74.

The experiment was arranged in a randomized block design with one factor and three replications block. The factor had two levels: non-inoculated (control) and AM inoculated. *C. siliqua* seedlings were planted in individual holes at 3 m apart. There were at least 30 seedlings per treatment and 20 seedlings per replication block (ten plants × two treatments). The tree height and their collar diameter (taken at 10 cm over the ground) were measured annually. During the first months of plantation, the dead seedlings were replaced. After three years of plantation, soil cores (0.5 kg) were collected at the basis of the tree trunk and at 0- to 10-cm depth. Soil samples were crushed, passed through 2-mm sieves and kept at 4 °C for further analysis. Sub-samples of leaf tissues were collected from three plants randomly chosen in each block and in each treatment. After drying (65 °C, 1 week), their N and P contents were determined using the methods described above.

All the soil samples were characterized by measuring total soil organic C after dichromate oxidation, total organic N by the Kjeldahl method, and soluble P by the Olsen method. The functional diversity of soil microflora was assessed by measuring the patterns of *in situ* catabolic potential (ISCP) of microbial communities (Degens and Harris, 1997). A set of 16 carboxylic acids (α-ketobutyric acid, α-ketoglutaric acid, fumaric acid, oxalic acid, tartric acid, gluconic acid, ascorbic acid, malic acid, malonic acid, quinic acid, 3-OH-butyric acid, formic acid, gallic acid, succinic acid, uric acid, citric acid), 8 amino-acids (L-serine, L-glutamic acid, L-phenylalanine, L-asparagine, L-lysine, L-cysteine, L-tyrosine, L-histidine), 2 amines (D-glucosamine, L-glutamine), two amides (*N*-methyl-D-glucosamine, L-succinamide) and 3 carbohydrates (D-mannose, D-sucrose, D-glucose) were screened for differences in Substrate Induced Respiration (SIR) responsiveness between the two soil treatments according Duponnois et al. (2005a) and Dilgo et al. (2012). Carbon dioxide measurements were subtracted from the CO_2 basal production and were expressed as μg CO_2 g^{-1} soil h^{-1}. Catabolic evenness (a measure of relative variability in the catabolic functions) was stated with the Simpson–Yule index, $E = 1/p_i^2$ with p_i = [respiration response to individual substrates]/[total respiration activity induced by all substrates for a soil treatment] (Magurran, 1988). Microbial biomass C (MBC) was calculated with the substrate-induced respiration (SIR) method according to Sparling (1995) and expressed as μg C g^{-1} soil [=50.4 × respiration rate (μl CO_2 g^{-1} soil h^{-1})]. The metabolic quotient (qCO_2) was calculated dividing the CO_2 basal respiration by the MBC content.

2.5. Statistical analysis

Data were treated with two-way analysis of variance (ANOVA). Means were compared with the Newman–Keul's test ($P < 0.05$). The percentages of the mycorrhizal colonization were transformed by arcsin(sqrt) prior statistical analysis.

3. Results

3.1. Native mycorrhizal inoculum characterization

AM fungal community associated to carob trees were characterized in two agricultural sites in Morocco. AM fungal LSU rDNA sequences from both sites (A and B) were analyzed together since carob roots from both origins were pooled for inoculum formulation. Overall, a subset of 114 sequences obtained by cloning/sequencing was analyzed. Alpha diversity analysis revealed similar results based on 98% and 97% sequence similarity threshold for data clustering (Table S1). Because the latter threshold is also widely used as cut-off point for fungal species delimitation (Brock et al., 2009), it was then selected for the following analyses. Thirteen molecular operational taxonomic units (MOTUs) were observed, reaching 96% of the estimated diversity and with less than 2 additional MOTUs to sample according Boneh estimation (Table S1). AM fungal community showed a very low evenness with three dominant MOTUs counting for 84% of the total sequence number. Phylogenetic analyses revealed their affiliation to *Rhizophagus* spp. (Glomeraceae) (Fig. 1). Rare MOTUs were also affiliated to *Rhizophagus* spp., but also to *Sclerocystis* and *Glomus* genera. Only one rare MOTU was affiliated to Claroideoglomeraceae.

3.2. Plant development in glasshouse and field conditions

After 6 months of culture under glasshouse conditions, the growth of AM-inoculated carob seedlings was significantly higher than the control plants, i.e., 1.42, 3.81 and 6.48-fold increase in height, shoot and root dry weights (Table 1). Mycorrhizal dependency was estimated at 77.7% (Table 1). No mycorrhizal structure was observed for the control plants. The phosphorus (P) and nitrogen (N) foliar and root contents of the plants inoculated with native AM fungi were also significantly higher than in the control (Table 1). The extent and the intensity of AM colonization were 100% and 67.8%, respectively, whereas the percentage of arbuscules and vesicles were 54.2% and 14.83%, respectively (Table 1).

In field conditions, the difference between AM-inoculated and non-inoculated carob outplants increased during the 3 years of

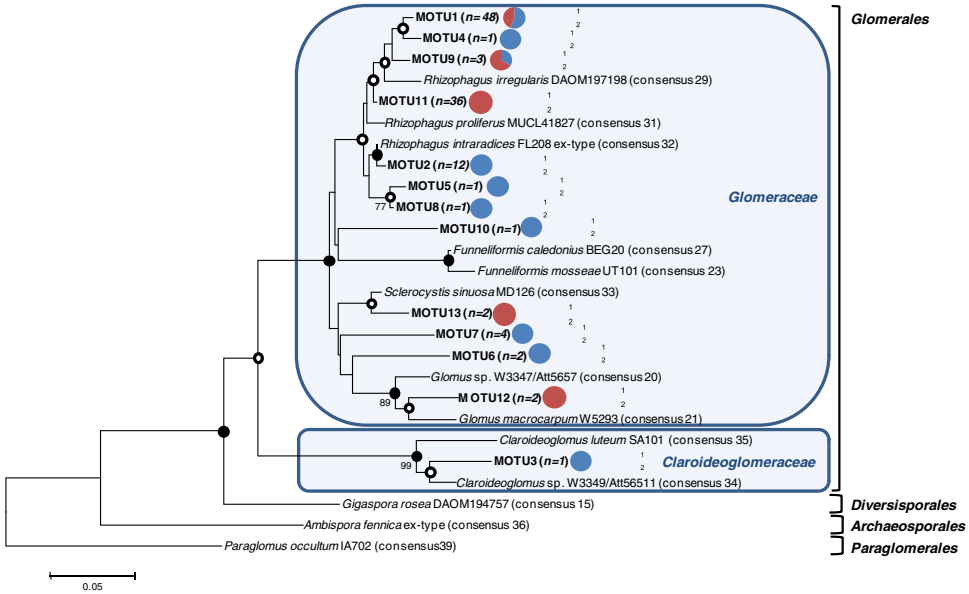

Fig. 1. Phylogenetic tree of Arbuscular Mycorrhizal Fungal sequences obtained from *C. siliqua* roots. MOTU were obtained based on a threshold of 98% similiraity (MOTHUR), and the circles at the right indicates the % of sequences from site A (blue) and B (red) in each MOTU. The phylogenetic tree was constructed using the Kimura-two parameter for distance correction and the neighbour-joining method. Levels of bootstrap value are indicated by black circles (if >80%) or open circles (if between 50% and 80%). The reference sequences are based on LSU consensus sequences of the Glomeromycota from Krüger et al. (2012). *Paraglomus* was used as an outgroup as it represents the most basal glomeromycotan branch. The scale bar shows the number of base changes per nucleotide sequence position. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

plantation, with a significant higher height (Fig. 2) for AM-inoculated outplants. Significant results were observed for collar diameter (data not shown). The rate of mortality reached 65.5% for the non-inoculated outplants whereas it was drastically reduced (25.5%) for the AM-inoculated outplants (Fig. S1). The N and P nutrition was also significantly higher for the AM-inoculated outplants after 3 years of plantation (Table 1).

Table 1
Effect of mycorrhizal inoculation on carob functional traits after in glasshouse and plantations experiments.

	Treatments	
	non-inoculated	AM-inoculated
6-month-old glasshouse cultures		
Height (cm)	9.67 ± 0.88 a	13.75 ± 1.18 b
Collar diameter (mm)	1.12 ± 0.81 a	1.93 ± 0.72 b
Root length (cm)	13.3 ± 2.03 a	27.1 ± 2.97 b
Shoot biomass (g dry weight)	0.91 ± 0.21 a	3.47 ± 0.47 b
Root biomass (g dry weight)	0.31 ± 0.06 a	2.01 ± 0.57 b
Total biomass (g dry weight)	1.22 ± 0.25 a	5.48 ± 0.99 b
Mycorrhizal dependency (%)		77.7
Shoot N content (mg g^{-1} dry weight)	2.25 ± 0.07 a	3.08 ± 0.21 b
Root N content (mg g^{-1} dry weight)	0.81 ± 0.03 a	1.02 ± 0.05 b
Shoot P content (mg g^{-1} dry weight)	0.11 ± 0.02 a	0.41 ± 0.01 b
Root P content (mg g^{-1} dry weight)	0.27 ± 0.01 a	0.32 ± 0.004 b
Colonized root length (%)	–	100
Mycorrhizal intensity (%)	–	67.8 ± 9.09
Arbuscules (%)	–	54.2 ± 7.23
Vesicules (%)	–	14.83 ± 3.65
3-year-old tree plantations		
Leaf nitrogen content (mg g^{-1} dry weight)	3.12 ± 0.32 a	4.14 ± 0.18 b
Leaf phosphorus content (mg kg^{-1} dry weight)	6.92 ± 0.16 a	8.71 ± 0.59 b

Average ± standard error. For each parameter, significant differences between treatments, according to the Newman Keul's test ($P < 0.05$), are indicated with different letters.

3.3. Soil chemical characteristics and microbial functionalities in field condition

Concerning soil characteristics, N, soluble P and C contents were significantly higher under the AM-inoculated outplants than under the non-inoculated ones (Table 2). A positive effect was also recorded for AM inoculated treatment for the organic matter soil content, the specific respiration rate of the soil microbial biomass (qCO_2) and the MBC/TOC ratio (Table 2). On the opposite, the microbial biomass was significantly reduced in the soil sampled under the AM-inoculated outplants compared to the non-inoculated ones (Table 2).

Concerning microbial functionalities, the soil catabolic evenness was significantly higher under AM-inoculated outplants than under non-inoculated plants (Table 3). Whereas no difference recorded between the treatments for the average SIR responses

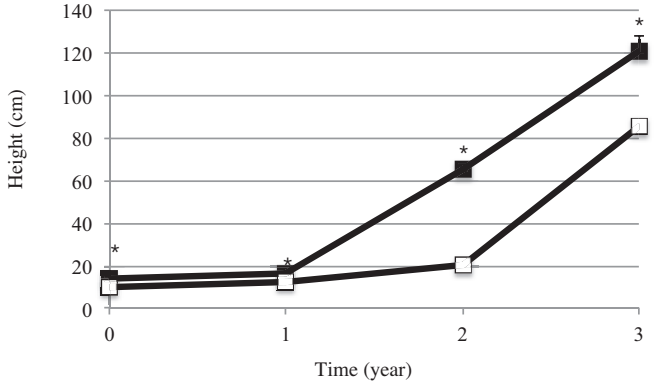

Fig. 2. Height of carob outplants in the field, either inoculated with AM fungi (*n*) or non-inoculated (control □). An asterisk indicates a significant ($P < 0.05$) difference between the two treatments for a given year.

Table 2
Microbial biomass and chemical characteristics of the soils sampled under non-inoculated and AM-inoculated carob trees after 3 years of plantation.

	Treatments	
	non-inoculated	AM-inoculated
Total nitrogen (%)	0.33 ± 0.006 a	0.42 ± 0.01 b
Soluble phosphorus (mg kg^{-1} soil)	1.51 ± 0.07 a	1.97 ± 0.08 b
Organic matter (%)	2.48 ± 0.08 a	4.15 ± 0.24 b
TOC (%)	15.7 ± 0.052 a	26.3 ± 0.16 b
Microbial biomass (μgC g^{-1} soil)	195.9 ± 27.53 a	91.6 ± 6.61 b
q CO_2 (μg C—CO_2 g^{-1} MBC h^{-1})	0.012 ± 0.001 a	0.028 ± 0.002 b
MBC: TOC (%)	12.1 ± 1.73 a	3.5 ± 0.25 b

Average ± standard error. For each parameter, significant differences between treatments, according to the Newman Keul's test ($P < 0.05$), are indicated with different letters. MBC = Microbial Biomass Carbon, TOC = Total Organic Carbon.

Table 3
Catabolic evenness and average SIR responses (μg CO_2 g^{-1} soil g^{-1}) with each substrate group (carbohydrates, amides, amino-acids and carboxylic acids) from the soils sampled under non-inoculated and AM-inoculated carob trees after three years of plantation.

	Treatments	
	non-inoculated	AM-inoculated
Catabolic evenness	20.7 ± 0.25 a	23.1 ± 0.15 b
Carbohydrates	84.4 ± 7.28 a	61.1 ± 8.01 a
Amino-acids	134.4 ± 1.65 a	136.7 ± 3.71 a
Amines	136.7 ± 16.4 a	131.6 ± 14.2 a
Amides	98.3 ± 14.5 a	166.7 ± 4.41 b
Carboxylic acids	188.5 ± 5.26 a	230.2 ± 6.54 b

with carbohydrates, amino-acids and amines, significant differences were observed with carboxylic acids and amides (Table 3). A range of substrates were preferentially used in soils under non-inoculated carob outplants, i.e., D-glucose, L-tyrosine, 3 OH-butyric acid and L-lysine (Fig. 3). By contrast, L-glutamic acid, L-asparagine, L-histidine, *N*-methyl-D-glucosamine and most of the carboxylic acids were preferentially used in soils under AM-inoculated carob outplants (Fig. 3).

4. Discussion

The success of reforestation and afforestation strategies is strongly dependant of seedlings quality, which depends of two crucial steps, (i) the plant growth in nursery and (ii) the transfer in field conditions. The current results clearly shows that (i) the AM inoculation stimulates the growth of carob plants and improves its nutrient status in nursery conditions, (ii) this beneficial effect is increasing during time in field conditions and (iii) the AM inoculation induces significant changes in soil microbial functions.

As previously demonstrated by Ouahmane et al. (2012), a high mycorrhizal dependency of carob trees was observed in our conditions. The AM fungal diversity recorded from the carob root samples also confirmed the predominance of Glomeraceae (Ouahmane et al., 2012), but a more reliable identification has been provided in the current study by combining molecular and phylogenetic methods, with sequences affiliated/related to six different genera in Glomeraceae (6; mainly *Rhizophagus*) and Claroideoglomeraceae (1). Members of Glomeraceae are the most widely distributed in ecosystems (Öpik et al. 2010). Glomeraceae fungi related to *Rhizophagus intraradices/irregularis* are described as the predominant members in various ecosystems, notably in

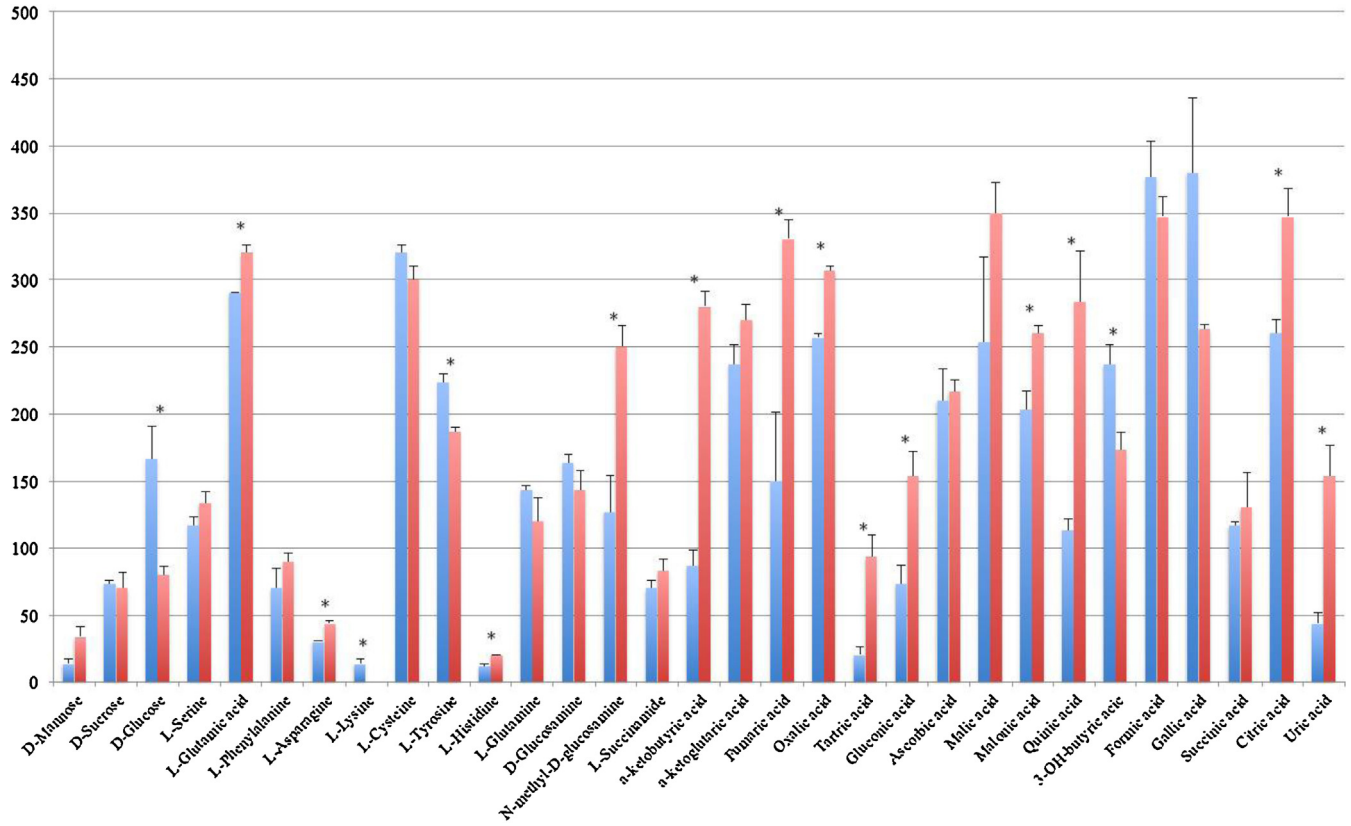

Fig. 3. Catabolic response profiles for each soil origin. Error bars represent standard errors ($n = 3$). Blue bars, soil under non-inoculated carob trees; Red bars, soil under AM-inoculated carob trees. An asterisk indicates a significant difference between the two treatments, according to the Newman Keul's test ($P < 0.05$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

semi-arid environments (Sanchez-Castro et al., 2012; Carter et al., 2014), similarly to the current results.

Contrary to Ouahmane et al. (2012), no AM fungi belonging to *Gigaspora* was identified. The type of samples analyzed may explain this discrepancy between both studies. Indeed, the characterization of carob-associated AM fungal community was based on soil samples in Ouahmane et al. (2012), whereas roots were used in the current study. Such biases between soil and root-associated AM fungal community were described in various environments (Hempel et al., 2007; Hempel et al., 2007) strengthening the hypothesis of a certain host specificity in AM symbiosis (Montesinos-Navarro et al., 2012; Scheublin et al., 2004; Davison et al., 2011). In addition, spatial and seasonal effects are well-known drivers of mycorrhizal communities, limiting the comparison between studies (Davison et al., 2012; Dumbrell et al., 2011).

The ecological engineering strategy developed in the current study has been thus based on the formulation of a mycorrhizal inoculum composed of native AM fungi actually colonizing carob roots. The use of native fungi is highly recommended as an effective strategy for efficient mycorrhizal inoculation in natural ecosystems (Requena et al., 2001; Caravaca et al., 2003; Ouahmane et al., 2007a; Johnson et al., 2010; Duponnois et al., 2011) but remains poorly applied. The current results demonstrate the high potential of such approach since it has drastically sustainably improved the growth of carob seedlings and their nitrogen and phosphorus assimilation through nursery cultivations and the 3-year-old plantation. This facilitation effect of AM fungi on plant growth and nutrient uptake is well documented (Smith and Read, 2008) but was mainly assessed in nursery, and few AM-inoculation field experiments were performed in arid and semi-arid ecosystems (Caravaca et al., 2003; Duponnois et al., 2005b, 2007; Ouahmane et al., 2007a; Bilgo et al., 2012; Barea et al., 2011). All these field experiments reported that the AM positive effect was convincing in the first stages of plantation where the plants are very sensitive to the environmental conditions (e.g., drought, mineral deficiencies). The better resistance of native AM-inoculated plants to environmental stresses is partly due to the direct effect of AM inoculation, i.e., a higher root system robustness, but also probably to the pre-colonization by a well-adapted AM community specific to the plant host, reducing the colonization by "cheaters" (Johnson et al., 2012) after the transfer of seedlings in natural environments. Although the AM fungal community was isolated from a different region in Morocco, the sites selected for the AM fungal sampling is a long term traditional agricultural site where scattered adult carob trees have been conserved and thus considered as native since no exotic AM fungus was introduced, and adapted to similar semi arid conditions encountered on the plantation site.

In addition to the impact of AM fungi on plant nutrient uptake, an increase of soil soluble P and soil N content was also observed. Whereas it has been largely demonstrated that AM fungi actively contribute to phosphate cycling (phosphate solubilisation and phosphatase activity), evidences on their ability to directly contribute to N-mineralization and to transfer N to the host plant remain controversial (Veresoglou et al., 2012). Indirect fungal effect through modification of soil microbial community was observed to increase degradation ability of organic matter (Toljander et al., 2006). Surprisingly, AM-inoculated carob outplants induced a decrease in soil microbial biomass. Such effect is poorly described or inconsistent results have been obtained (Edwards et al., 1998; Marschner et al., 1997; Meyer and Linderman, 1986), but a similar impact was reported with AM-inoculated *Acacia* in a Sahelian plantation (Bilgo et al., 2012).

Several microbial activities indicator of organic matter decomposition efficiency (qCO_2, metabolic quotient; Anderson and Domsch, 1990) and resistance to various environmental stresses (catabolic evenness; Degens et al., 2001) were improved under AM-inoculated carob outplants. Major changes in the catabolic potential of soil microbial communities were mainly observed for carboxylic acids. Interestingly, low molecular weight (LMW) organic acids, are known to influence soil processes including nutrient mobilization (Lilienfein et al., 2004) and uptake (Marschner, 1995), dissolved metal and contaminant mobility (Li et al., 2006), and mineral weathering (Oelkers et al., 2011). The AM fungi are involved in the P mobilization from organic and inorganic compounds by producing phosphatases, organic acids, phenolic compounds, protons and siderophores (Drever and Vance, 1994; Joner and Johansen, 2000). The production of organic acids in AM fungi is however poorly documented, but malate and citrate have been detected in hyphal exudates of these fungi (Tawaraya et al., 2006; Plassard and Fransson, 2009). The current results suggest that the establishment of the native AM community-inoculated carob trees may also impact the functional diversity of soil microflora by facilitating soil microorganisms able to catabolise organic compounds (e.g., carboxylic acids), confirming previous observation in field experiments (Bilgo et al., 2012). The impact of AM on soil microbial amide catabolism has been previously described in AM-inoculated plant experiments performed on Moroccan Atlas Cypress with native AM fungi (Ouahmane et al., 2007b) but no clear biological relationship was evidenced.

In conclusion, the current study demonstrates the high potential of ecological engineering strategies based on native AM fungal community inocula for efficient carob-based plantations and more largely in reforestation and afforestation strategies in arid and semi-arid degraded soils, improving sustainably the growth and nutrient status of AM-inoculated plants and inducing a positive soil microbial environment for nutrient cycling and environmental stress resistance. Further studies have to be performed to test the long-term sustainability of this cultural practice and the influence of native AM inoculation on the carob pod production.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ecoleng.2015.03.007.

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