African Journal of Biotechnology Vol. 11(98), pp. 16422-16426, 6 December, 2012
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB12.1163
ISSN 1684–5315 ©2012 Academic Journals

Full Length Research Paper

Inoculation of *Ceratonia siliqua* L. with native arbuscular mycorrhizal fungi mixture improves seedling establishment under greenhouse conditions

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Accepted 16 November, 2012

The potential benefits of inoculation with arbuscular mycorrhizal (AM) fungi were investigated on carob tree *Ceratonia siliqua*, a Mediterranean legume in Morocco. The parameters under study were the effect of an inoculation on growth, mineral nutrition and roots mycorrhizal colonization of the plant under nursery conditions. *C. siliqua* growth was measured after six months of culture in plastic bags arranged in a randomised complete block under greenhouse conditions. Fungal inoculation consisted of a mixture of native AM fungi propagated on *Zea mays* roots. Results show that the fungal symbionts were effective to improve the growth of *C. siliqua*, confirming the requirement of mycorrhizal symbiosis for the successful establishment of *C. siliqua* in a degraded soil. The approach used with indigenous AM fungi complex isolated under *C. siliqua* appeared to be effective in promoting growth and nutrition of *C. siliqua.* After 6 months of culturing in nursery conditions, height, shoot and root biomass, total biomass, phosphorus and nitrogen foliar contents of the plants inoculated with native AM fungi were significantly higher than in the control. Glomus spores were extracted from the soil under *C. siliqua* and were observed on permanent slides under a microscope connected to a computer with digital image analysis software. Seven spore morphotypes were detected under *C. silqua* in the Ourika Valley, Morocco. Five *Glomus* species were classified as *Glomus aggregatum*, *Glomus intraradices and Glomus constrictum,* whereas, two other *Glomus* species were not identified. The analysis of this spore community revealed the presence of two other species belonging to *Gigaspora* genera. The use of a mixture of native AM fungi as fungal inoculum improves clearly growth, nutrition and roots colonization of *C. siliqua* seedling.

Key words: Arbuscular mycorrhizal fungi, diversity, growth, soil microbial activity, *Ceratonia siliqua.*

INTRODUCTION

Mycorrhizal symbiosis is known to be key components of natural systems (Carpenter and Allen, 1988; Brundrett, 1991), they are involved in governing cycles of major plant nutrients and in sustaining the vegetation cover in natural habitats (Requena et al., 2001). Disturbances generally result in the loss or reduction of mycorrhizal propagules in the soil and, consequently, decrease the mycorrhizal potential in the degraded areas (Jasper et al., 1991; Herrera et al., 1993; Mc Lellan et al., 1995).

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Consequently and because of these main ecological functions , loss or diminution of fungal symbionts propagules from degraded ecosystems can limit natural and artificial processes of revegetation (Requena et al., 2001).

In Morocco as in the other Mediterranean areas, ecosystems are subjected to desertification processes having occurred after scarce and irregular rainfall, long dry and hot summers. These environmental conditions frequently limit the performance of reforestation tasks. Numerous studies have focused on the optimization of nursery practices to produce high-quality seedlings (Caravaca et al., 2005; Rincon et al., 2006; Ouahmane et al., 2007a, b). Among the tested cultural practices, early inoculation with autochthonous mycorrhizal fungi has shown a promising nursery cultural practice to improve the quality of the seedlings and their performance in field conditions (Ouahmane et al., 2007a).

The carob tree (*Ceratonia siliqua* L.), is a sclerophyllous leguminous belonging to the Cesalpinaceae sub-family. The carob is largely distributed around the world and the Mediterranean region has been one of its mean domestication centres (Zohary, 1973), mainly in Spain, Italy, Portugal and Morocco, notably in marginal and calcareous soils (Martins-Loucao, 1999). Recently, this species has attracted much attention and became economically important (Sidina et al., 2009). Pods and seeds are used as row material in food, pharmaceutical and cosmetic industries (Batista et al., 1996; Vourdoubas et al., 2002; Barracosa et al., 2007).

In Morocco, the production of carob was estimated only to 8% of the world production. This production considered as the fourth range in the world is mainly from natural domesticated trees in agroforestry systems (Batlle and Tous, 1997).

Ecologically, the carob distribution in Morocco is centered in the north sides of the Atlas chain, the Rif Mountain and in some valleys of the south-west of the Anti-Atlas in arid and semi-arid bioclimates with an extension to sub-humid bioclimate in some stands (Emberger and Maire, 1941; Aafi, 1995). Carob tree present a high resistance to warm and cold bioclimates, and its ecophysiological behavior has been described as more resistant to water stress compared to other Mediterranean species (Winer, 1980; Nunes et al., 1992; Rejeb, 1992; Sakcali and Ozturk, 2004). Furthermore carob appears to grow successfully in saline soils (tolerance to a soil salt content of up to 3% of NaCl (Batlle and Tous, 1997; Cruz et al., 1996). Recently the commercial value of carob has increased and carob became a plant of multipurpose use (Roukas, 1994; Corsi et al., 2002; Makris and Kefalas, 2004; Sandolo et al., 2007). In fact, *C. siliqua* is used in Morocco in reforestation program serving both environmental and economic objectives. *C. siliqua* is used to valorize marginal lands or as substitute for drought sensitive species. The ability of this species to grow in such contrasting environment supposes a high degree of adaptability, and a highly benefit interaction with soil microbial components such as mycorrhizal fungi and associated microbial community established in rhizospheric soil surrounding carob tree roots. In fact, analysis of literature related with *C. siliqua* had shown that the mycorrhizal status of this legume and the importance of the symbiosis with mycorrhizal fungi in its performance is not reported. Therefore, this research program is aiming at the evaluation of the mycorrhizal status of carob tree and the importance of an association with mycorrhizal fungi in the performance and adaptability of carob seedlings under nursery conditions.

This paper provides an investigation of mycorrhizal status of *C. siliqua* throughout: (i) analysis of the diversity of mycorrhizal fungi community associated to carob, and (ii) assessment of the effect of an artificial inoculation with autochthonous mycorrhizal fungi on the improvement of growth and mineral nutrition of seedlings under greenhouse conditions,

MATERIALS AND METHODS

Study site

The experimental site was located in the Ourika valley (Haut Atlas, Morocco). The climate is semi-arid Mediterranean, with an annual rainfall of 460 mm. The plant cover is sparse due to overgrazing. In this area, *C. siliqua* is associated with various shrub species such as *Quercus rotundifolia Lamk., Pistacia atlantica L., Lavandula dentata L., Lavandula stoechas L., Cistus villosus Coss.* and *Cistus salviifolius Coss.*

Soil physico-chemical characteristics were as follows: pH (H_2O) 8.01; clay (%) 29.6, fine silt (%) 27.4, coarse silt (%) 16, fine sand (%) 13.1; coarse sand (%) 13.9; carbon (%) 2.33; total nitrogen (%) 0.155; C/N ratio 14.8; Olsen phosphorus 19.5 mg kg^{-1}soil.

Field sampling and arbuscular mycorrhizal (AM) fungi diversity assessment

Soil samples were collected from the rhizosphere of *C. siliqua* at 2 m from the trunk, under the canopy. They were taken from about 10 individual trees. Each sample consisted of five 100 g sub-samples collected at the 20 cm depth. All the soil samples were carefully mixed and the Glomus spores were extracted from the soil using the Gerdemann and Nicholson method (Gerdemann and Nicholson, 1963). One hundred grams of dry soil was wet sieved on 500 to 50 µm mesh sieves and centrifuged in a water sucrose solution (50% w/v) for 10 min at 1500 rpm. Then the supernatant was poured through a 50 µm sieve and rinsed with tap water. Spores were counted under a stereomicroscope and grouped according to their morphological characteristics. Spore size and colour were assessed in water under a stereomicroscope (Olympus SZ H10 research stereomicroscope) whereas, spore wall structures and other attributes were observed on permanent slides prepared according to the study of Azcon-Aguilar et al. (2003) under a microscope connected to a computer with a digital image analysis software. Morphotypes classification to the genus level and, when possible to the species, was mainly based on morphological features such as colour, size, wall structure and hyphal attachment (INVAM, 1997).

Table 1. Diversity and relative abundance of arbuscular mycorrhizal fungi collected under *C. siliqua* trees in natural area.

Total number of AMF spores 100^{-1}g soil	No. of spores
Glomus intraradices	170[a]
Glomus aggregatum	410[f]
Glomus constrictum	490[g]
Glomus sp1	205[b]
Glomus sp2	280[e]
Gigaspora sp1	270[c]
Gigaspora sp2	275[d]
Total	2100

Data followed by the same letter are not significantly different according to the Student-Newman-Keuls's test ($p < 0.05$).

Plant and mycorrhizal treatments

Seed of *C. siliqua* were immersed in a sulphuric acid solution 36N for 15 min, then transferred to distilled water for 4 h and, then transferred into Petri dishes on humid filter paper. The plates were incubated for 48 h at 24°C. The germinating seeds were used when rootlets were 1 to 2 cm long. Native AM fungi spores isolated from the rhizosphere soils previously collected under *C. siliqua* as described before, were surface sterilized with a solution of chloramine T (0.2 $g.l^{-1}$) and Streptomycine (0.2 $g.l^{-1}$) (Mosse, 1973) in order to eliminate the mycorhizosphere microflora. Then, in order to enrich the fungal inoculum, this mixture of native AM fungi was propagated on maize (*Zea mays* L.) for 12 weeks on a sterilized soil. The soil used was collected under *C. siliqua* in Ourika valley as described before, crushed, passed through a 2 mm sieve and autoclaved (120°C, 40 min). AM fungal inoculum consisted of infected maize root pieces (average length 0.5 cm). Non mycorrhizal maize roots were used for the control treatment.

Mycorrhizal inoculation of *C. siliqua* seedlings and plant analysis

Ceratonia siliqua seedlings were grown in 1 l pots filled with the same disinfected soil as before. One hole (1 x 5 cm) was made in the soil of each pot and filled with 1 g of fresh maize root. The observation of this maize root showed a very high rate of colonization with different mycorrhizal structures like arbuscules, vesicles and spores, with an intensity of almost 300 vesicles per cm. The uninoculated control received non-mycorrhized maize roots. The holes were then covered by the same autoclaved soil. The plants were arranged in a randomized, complete bloc design with 40 replicates per treatment. They were screened from the rain and grown under natural light in the Forest Research center greenhouse (Marrakesh, Morocco) (mean daylight approximately 12 h, mean temperature 24°C day).

After six months of culturing, 10 plants were randomly sampled from each treatment. They were uprooted and their root systems gently washed. Height and dry weight of the shoot and root (one week at 65°C) were measured. After drying, plant tissues were ground, ashed (500°C), digested in 2 ml HCL 6N and 10 ml HNO_3 N and then analysed by colorimetry for P (John, 1970). For N (Kjeldhal) determination, they were digested in 15 ml H_2SO_4 36N containing 50 g l^{-1} salicylic acid. Roots were cleared and stained according to the method of Phillips and Hayman (1970) modified (clearing of roots in KOH 10 g per 100 ml for 3 h at 90°C). The root pieces were placed on a slide for microscopic observation at 250 x magnification (Brundrett et al., 1985). About fifty 1-cm root pieces were observed per plant. Extent of mycorrhizal colonization was expressed in terms of fraction of root length with mycorrhizal internal structures (vesicles or hyphae): (length of root fragments colonized / total length of root fragments) x 100. The mycorrhizal frequency is calculated on all the *C. siliqua* plants examined. This parameter represents the percentage of mycorrhized plants compared to all the examined seedlings. The mycorrhizal dependency of *C. siliqua* is the calculation of the contribution of mycorrhizal fungi in plant growth. The ratio between the difference in shoots or root biomass between inoculated and non inoculated plants, and the related biomass in inoculated plants expresses the mycorrhizal dependence of a plant species to mycorrhizal fungi.

Statistical analysis

All data were subjected to a one way analysis of variance and the mean values were compared using Student-Newman-Keuls's "t" test ($p < 0.05$). SPSS for windows.10 software was used in this analysis.

RESULTS

Morphological analysis of arbuscular mycorrhizal fungi community associated with *C. siliqua* in the Ourika valley revealed seven spore morphotypes. Five *Glomus* species were classified as *G. aggregatum, G. intraradices, Glomus constrictum* and two non identified morphotypes, *Glomus* sp1 *and Glomus* sp2. Two different species belonging to *Gigaspora* genera were encountered in this analysis. Extraction of spores from soil samples showed an average of 2100 spores per 100 g dry soil. This number ranged from 600 to 4000 spores per 100 g of dry soil in different sampled soils (Table 1).

After six months of culturing under greenhouse conditions, height, stem diameter to the collar, shoots and roots biomass, total biomass, total leaf number, total shoots number, shoots and roots phosphorus and nitrogen contents of the *C.siliqua* seedlings inoculated with native AM fungi mixture were strongly improved comparing to the disinfected soil (control) (Tables 2 and 3).

Table 2. Growth of *C. siliqua* seedlings inoculated with mixture of autochthonous arbuscular Mycorrhizal fungi after six months culture under glasshouse conditions.

Parameter	Control	Mixture AM fungi
Height (cm)	$22^{a} \pm 1$	$29.25^{b} \pm 3$
Stem diameter to collar (mm)	$4.48^{a} \pm 0.3$	$6.43^{b} \pm 0.4$
Shoots biomass dry weight (g)	$5.68^{a} \pm 0.3$	$7.66^{b} \pm 0.5$
Total leaf number	$35 .75^{a} \pm 1$	$55.25^{b} \pm 1.5$
Total shoots number	$13.5^{a} \pm 3$	$17^{b} \pm 4$
Roots biomass dry weight (g)	$2.45^{a} \pm 0.25$	$3.24^{b} \pm 0.6$
Total biomass (g)	$8.13^{a} \pm 1$	$10.90^{b} \pm 1.4$

Data in the same line followed by the same letter are not significantly different according to the Student-Newman Keul's test ($p < 0.05$).

Table 3. Mineral nutrition of *C. siliqua* seedlings inoculated with mixture of autochthonous Arbuscular Mycorrhizal fungi after 6 months culture under glasshouse conditions.

Parameter	Control	Mixture AM fungi
Shoots P mg/plant	$25 .7^{a} \pm 2.6$	$49.41^{b} \pm 1.4$
Roots P mg/Plant	$6.37^{a} \pm 1.5$	$11.91^{b} \pm 0.7$
Total P mg/plant	$32.07^{a} \pm 2.6$	$61.32^{b} \pm 3.4$
Shoots N mg/plant	$94.43^{a} \pm 11$	$233.63^{b} \pm 6$
Roots N mg/plant	$32.53^{a} \pm 4.6$	$122.31^{b} \pm 18$
Total N mg/plant	$126.96^{a} \pm 13$	$355.94^{b} \pm 4.1$

Data in the same line followed by the same letter are not significantly different according to the Student-Newman Keul's test ($p < 0.05$).

Table 4. Effect of inoculation with a mixture of native AM fungi on root colonization and plants dry weight increase of *C. siliqua* after six month's culture under greenhouse conditions.

Parameter	Mixture AM fungi treatment
Mycorrhizal frequency (%)	100
Colonized root length (%)	84.5
Arbuscules (%)	33.25
Vesicules (%)	70.25
Shoots total dry weight increase (%)	25
Shoots mycorrhizal dependancy (%)	34
Roots total dry weight increase (%)	24.38
Roots mycorrhizal dependancy (%)	32.24

Compared with the control, shoots and root phosphorus contents of mycorrhizal plants were stimulated by 1.9 and 1.8 times, respectively, shoots and root nitrogen contents of mycorrhizal plants were stimulated by 2.47 and 3.7 times (Table 3). Furthermore and according to shoot and root growth, mycorrhizal inoculation of *C. siliqua* seedling had improved considerably this parameter, the mycorrhizal dependencies calculated were 34 and 32.24, respectively (Table 4).

Staining and microscopic observation of roots collected from *C. siliqua* seedling showed a large extent of AM colonization and a colonization rate of 84.5% was recorded (Table 4).

DISCUSSION

A high AM fungal diversity is associated with *C. siliqua* at the Ourika valley (Morocco). We have numbered about 2100 AM fungal spores per 100 g of soil collected under

trees of *C. siliqua* in natural area. This spore abundance was significantly higher than those recorded under other Mediterranean species such as *Cupressus atlantica* (Ouahmane et al., 2006b), *Tetraclinis articulata* (Abbas et al., 2006) and *Anthyllis cytisoides*, *Stipa tenacissima*, *Retama sphaerocarpa* (Requena et al., 1996). Under *C. atlantica* for example, Ouahmane et al. (2006) have numbered about 600 AM fungal spores per 100 g of soil collected.

In this study, it is well demonstrated that the growth of *C. siliqua* under greenhouse conditions was very dependent to AM symbiosis. Furthermore, the inoculation with a mixture of native AM fungi significantly stimulated nitrogen and phosphorus contents in shoot and root tissue of *C. siliqua* in a disinfected soil. This growth and mineral nutrition improvement was linked to a great colonization rate of roots. This result confirmed the high mycorrhizal dependency of *C. siliqua.* It is well known that AM fungi improved nutrient uptake, especially phosphorus and nitrogen, by increasing the abilities of the host plants to explore a larger volume of soil than roots alone and to mobilize phosphate from a greater surface area (Jakobsen et al., 1992; Joner et al., 2000).

These results are in accordance with previous studies where it was shown that the composition of AM communities was an important biological factor to plant species development (van der Heijden et al., 1998). It has been previously reported that AM plants have access to nitrogen forms that are unavailable to non-AM plants (Azcon-Aguilar et al., 1993; Subramanian and Charest, 1998). For instance a special attention is given to the relationship between mycorrhizal dependency of plants and phosphatase activity (Azcon et al., 1982; Khalil et al., 1994).

Conclusion

In conclusion to this work, a positive effect of inoculation with arbuscular mycorrhizal fungi was found in the first stages of growth that are usually considered as the most critical for revegetation, particularly in Mediterranean semi-arid areas. Native inoculum potential of AM fungi in arid and semi-arid Mediterranean ecosystems is generally limited. Hence the selection of efficient AM fungi is a key factor to ensure the success of soil revegetation programmes. However, these fungal symbionts have to be well adapted to the environmental conditions in order to show a high level of effectiveness in improving the performance of the host plant species. From the present study, the use of native AM fungi as a source of AM inoculum could be of great interest to accelerate the process of reafforestation in arid and semiarid degraded soils. *C. siliqua* is reported as non noduling cesalpineacea, hence the importance of mycorrhizal fungi inoculation to improve growth, phosphate and nitrogen nutrition of seedling and their resistance to environmental conditions, particularly in the field where the transplantation shock is the first cause of seedlings mortality.

ACKNOWLEDGEMENTS

The CRF, Centre de Recherche Forestière, Rabat, Morocco funded this research program. The authors thank the technical staff members of the Regional Forest Research Center in Marrakech for their helpful physical effort while implementing the experimental protocol.

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