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ORIGINAL ARTICLE

Improvement of *Cupressus atlantica* Gaussen growth by inoculation with native arbuscular mycorrhizal fungi

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Abstract

Aims: The study aimed to determine whether inoculation with native arbuscular mycorrhizal (AM) fungi could improve survival and growth of seedlings in degraded soils of Morocco.

Methods and Results: Soil samples were collected from the rhizosphere of *Cupressus atlantica* trees in the N'Fis valley (Haut Atlas, Morocco). AM spores were extracted from the soil, identified and this mixture of native AM fungi was propagated on maize for 12 weeks on a sterilized soil to enrich the fungal inoculum. Then *C. atlantica* seedlings were inoculated with and without (control) mycorrhizal maize roots, cultured in glasshouse conditions and further, transplanted into the field. The experiment was a randomized block design with one factor and three replication blocks. The results showed that a high AM fungal diversity was associated with *C. atlantica*; native AM fungi inoculation was very effective on the growth of *C. atlantica* seedlings in glasshouse conditions and this plant growth stimulation was maintained for 1 year after outplanting.

Conclusions: Inoculation of *C. atlantica* with AM fungi increased growth and survival in greenhouse and field.

Significance and Impact of the Study: The data indicate that use of native species of AM fungi may accelerate reforestation of degraded soils. Further studies have to be performed to determine the persistence of these mycorrhizae for a longer period of plantation and to measure the effects of this microbial inoculation on soil biofunctioning.

Introduction

Lack or scarcity of plant cover largely contributed to the acceleration of soil degradation and desertification processes that involved a loss or reduction of major physico-chemical and biological soil properties (Requena *et al.* 2001). Hence, soil erosion increased whereas water infiltration and soil organic matter content as well as microbial activities decreased (Garcia *et al.* 1997). Numerous studies have shown that in such conditions, indigenous inoculum levels of arbuscular mycorrhizal (AM) fungi were significantly reduced (Duponnois *et al.* 2001a; Palenzuela *et al.* 2002; Azcon-Aguilar *et al.* 2003). AM fungi are considered essential key components of sustainable soil-plant systems (van der Heijden *et al.* 1998; Requena *et al.* 2001; Schreiner *et al.* 2003), particularly in semi-arid ecosystems (Carpenter and Allen 1988; Requena *et al.* 2001). This symbiotic process mobilizes and transports nutrients to roots (Smith and Read 1997), improves soil aggregation in eroded soils (Querejeta *et al.* 1998; Caravaca *et al.* 2002) and reduces water stress (Augé 2001). During the past decade, considerable research has

been made by using specific mycorrhizal fungal strains to enhance outplanting performances with forest tree species on various reafforestation sites (Brundett *et al.* 1996; Caravaca *et al.* 2005). The identification of efficient AM fungi is usually considered as a prerequisite to inoculation programs as the efficiency of mycorrhizal inoculation on the plant growth depends on the species involved (Roldan *et al.* 1992; Herrera *et al.* 1993; Duponnois *et al.* 2001b). It has been shown that survival rates and early growth performance of various softwood and hardwood species was significantly improved in the field after specific AM fungal inoculation (Mosse *et al.* 1982; Herrera *et al.* 1993; Jasper 1994; Caravaca *et al.* 2003; Pattinson *et al.* 2004). However, inoculation of plants with AM fungi still only occurs at a small scale in revegetation schemes and few studies have clearly demonstrated the benefits of fungal inoculation in the field. The degree of mycorrhizal responses on a reafforestation site depends on the status of fungal colonization at planting, persistence of introduced fungi and other biotic and abiotic factors to the planting site (Dodd and Thomson 1994). Hence, the use of AM fungi and plants adapted to the local environmental conditions may be a prerequisite to ensure the success of reafforestation programmes (Boddington and Dodd 2000).

In Morocco, overgrazing, deforestation caused by demographic pressure, irregularity of rainfall distribution and changes in cultural practices have severally altered the natural diversity of plant cover. In particular, the area of natural and introduced cypress stands (one native species *Cupressus atlantica* and two introduced species: *Cupressus sempervirens* and *Cupressus arizonica*) has declined as well as their natural regeneration. Reafforestation programmes have been realized but without success. Although it has been demonstrated that *C. atlantica* was representative of highly mycorrhizal-dependent plant species (Ouahmane *et al.* 2006a), the potential effect of mycorrhizal inoculation on cypress growth has not been assessed, more particularly on the survival rates and early growth performance in the field.

The objectives of this study were: (i) to identify the native AM fungi associated with *C. atlantica* and (ii) to determine the effect of inoculation with native AM fungi on the establishment of this tree species in a degraded site in Morocco.

Materials and methods

Study site

The experimental site was located in the N'Fis valley (Haut Atlas, Morocco) at the Idni station (8°17′02″W, 31°54′34″N, 1700 m above sea level). The climate is semi-arid Mediterranean, with an annual rainfall of 634 mm. The plant cover is sparse as a result of overgrazing. In this area, *C. atlantica* is associated with various shrub species (*Cistus salviifolus* L., *Lavandula dentata* L., *Lavandula stoechas* L., *Thymus pallidus* Coss., *Thymus satureioides* Coss., *Polygala balansae* Coss., *Globularia alypum* L.) and grasses (i.e. *Stipa nitens* Ball.). Soil 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 (%), 0·11; Olsen phosphorus (P), 16·1 mg kg^{-1}.

Field sampling and AM fungi diversity assessment

Soil samples were collected from the rhizosphere of *C. atlantica* at 2 m from the trunk, under the crown. They were taken from 10 individual trees. Each sample consisted of five 100-g subsamples collected at 20-cm depth. All the soil samples were carefully mixed and the Glomalean spores were extracted from the soil using the Gerdemann and Nicholson method (Gerdemann and Nicolson 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 rev min^{-1}. 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. The relative abundance of each fungal type was calculated per 100 g of dry soil. 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 Azcon-Aguilar *et al.* (2003) under a microscope connected to a computer with digital image analysis software. Morphotype 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 (Morton and Benny 1990; INVAM 1997).

Plant and mycorrhizal treatments

Seeds of *C. atlantica* were immersed in distilled water at 4°C for 24 h and, then transferred into petri dishes on humid filter paper. The plates were incubated for 1 week at 20°C. The germinating seeds were used when rootlets were 1–2-cm long.

Native AM fungi spores isolated from the rhizosphere soils previously collected under *C. atlantica* 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) 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. atlantica* in Idni station 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 *Cupressus atlantica* seedlings

Cupressus atlantica seedlings were grown in 1-l pots filled with the same disinfected soil as before. One hole (1 cm × 5 cm) was made in the soil of each pot and filled with 1 g of fresh maize root. The uninoculated control received nonmycorrhized 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 protected with a screen from the rain and grown under natural light in the University Cadi Ayyad (Marrakech. Morocco) (mean daylight 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. Height and dry weight of the shoot (1 week at 65°C) were measured. After drying plant tissues were ground, ashed (500°C), digested in 2 ml of 6 N HCL and 10 ml of HNO_3 for nitrogen and then analysed by colorimetry for phosphorus (John 1970). For nitrogen (Kjeldhal) determination, they were digested in 15 ml H_2SO_4 (36 N) containing 50 g l^{-1} of salicylic acid. Roots were cleared and stained according to the method of Phillips and Hayman (1970). The root pieces were placed on a slide for microscopic observation under 250× 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 (arbuscules, vesicles or hyphae): (length of root fragments colonized/total length of root fragments) × 100.

Field procedure and host plant analysis

The experiment was a randomized block design with one factor and three replication blocks. The factor had two levels: noninoculated and inoculated with the mixture of native AM fungi. In April 2005, an area of 1000 m^2 was established in the Idni station. It was cleaned from trees and shrubs. *Cupressus atlantica* seedlings were planted in individual holes at 3 m apart. There were at least 30 seedlings per treatment and 20 seedlings per replication block (10 plants × 2 treatments in each block). The tree height was measured monthly. The dead plants were replaced in both treatments during the first month of plantation. After 1 year of plantation, subsamples of leaf tissue were collected from three plants, randomly chosen in each block and in each treatment. After drying (1 week at 65°C), their nitrogen (Kjeldhal) and phosphorus contents were determined using the methods described before. Roots were sampled from inoculated and noninoculated plants (about 500 mg fresh weight per plant) in each block. Then, they were treated as describe before to evaluate their AM colonization index.

Statistical analysis

All data were subjected to a one-way analysis of variance and the mean values were compared using Student's *t* test ($P < 0{\cdot}05$). Growth of trees from inoculated and noninoculated pots was compared with an analysis of covariance (regression lines slope comparison) taking into account the block effect, with the R software (R Development Core Team 2006). R is a free software environment for statistical computing and graphics that can be downloaded at http://www.r-project.org/. It is made of a dozen base packages, plus hundreds of contributed packages covering all areas of statistics.

Results

Ten spore morphotypes were detected under *C. atlantica* in the Idni station (Table 1). Four *Glomus* species were classified as *Glomus fasciculatum*, *Glomus manihotis*, *Glomus aggregatum* and *Glomus monosporum* whereas four species were not identified (Table 1). The analysis of this spore community revealed the presence of two other genera, *Acaulospora* and *Scutellospora*, which were only represented by one species (Table 1). Above 700 spores per 100 g of dry soil were extracted and *G. fasciculatum* was the most abundant followed by *G. manihotis* and *Glomus* sp. 4 (Table 1).

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 (Table 2). Compared with the control, shoot and root growth of inoculated plants was stimulated by 1·62× and 1·59×, respectively (Table 2).

In the field experiment, the effect of blocks was first tested using the 'lm' function of the R software. The corresponding *P*-value was not significant ($P = 0{\cdot}26$). The effect of inoculation on tree height over time was significant meaning that inoculation had a statistically

Table 1 Diversity and abundance of arbuscular mycorrhizal (AM) fungal spores associated with *Cupressus atlantica* in the Idni station (N'Fis valley, Morocco)

AMF species	Colour	Size (μm)	Relative abundance (%)
Glomus fasciculatum	Dark red	133 (112–152)	23·8 (1·7)* a†
Glomus manihotis	Red to yellow	149 (124–171)	16·7 (1·6) b
Glomus aggregatum	Red to yellow	57 (22–84)	7·1 (0·1) d
Glomus monosporum	Black	156 (147–163)	3·1 (0·1) f
Glomus sp. 3	Yellow	87 (51–99)	9·3 (1·2) cd
Glomus sp. 4	Yellow	184 (170–202)	13·4 (1·4) bc
Glomus sp. 5	Yellow	127 (125–153)	11·3 (0·2) c
Glomus sp. 6	Black	124 (116–134)	3·0 (0·04) f
Acaulospora sp.	Black	184 (163–192)	7·6 (0·04) d
Scutellospora sp.	Black	253 (245–261)	4·7 (0·02) e

*Standard error of the mean.
†Data in the same column followed by the same letter are not significantly different according to the Newman Keul's test ($P < 0·05$).

Table 2 Effect of inoculation with a mixture of native arbuscular mycorrhizal (AM) fungi on growth variables, shoot mineral contents and root colonization in *Cupressus atlantica* plants after 6 month's culture in nursery conditions

	Treatments	
	Control	Mixture AM fungi
Height (cm)	12·3 (0·46)* a†	16·1 (0·71) b
Shoot biomass (mg dry weight)	1163 (20) a	1893 (264) b
Root biomass (mg dry weight)	740 (64) a	1183 (132) b
Total biomass (mg dry weight)	1903 (62) a	3077 (392) b
Shoot phosphate content (mg g^{-1} dry weight)	0·09 (0·01) a	0·167 (0·03) b
Shoot nitrogen content (mg g^{-1} dry weight)	2·23 (0·23) a	3·50 (0·1) b
Colonized root length (%)	–	41·2 (2·3)

*Standard error of the mean.
†Data in the same line followed by the same letter are not significantly different according to the Newman Keul's test ($P < 0·05$).

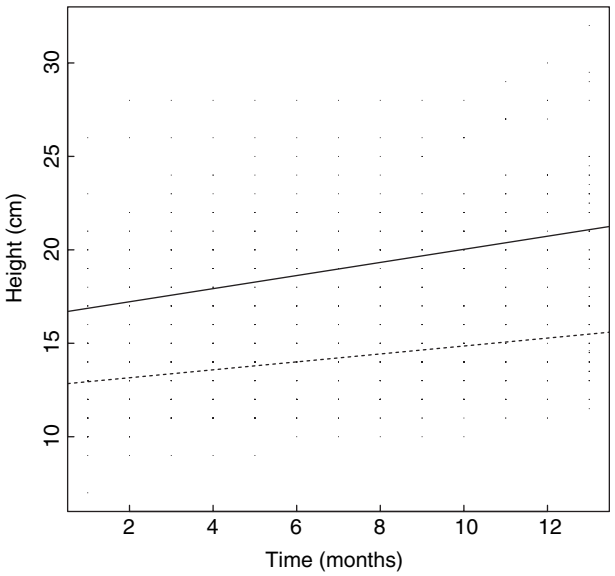

Figure 1 Comparison of growth rates for inoculated (*dashed line*) and noninoculated *Cupressus atlantica* seedlings (*solid line*) during the first months of plantation in field conditions.

significant effect on the slope of the growth regression line (nonparallelism test $P = 0·046$). The slope of the regression line of inoculated seedlings ($a_i = 0·351$) was clearly higher than that of the noninoculated plants ($a_{ni} = 0·212$) (Fig. 1). The intercept was also higher for inoculated seedlings ($b_i = 16·9$ *vs* $b_{ni} = 12·9$), suggesting that seedling growth was already faster before outplanting in the field. After 1 year of plantation, height of inoculated trees was stimulated 1·3× compared with the control treatment. During the first year of plantation, mean growth increment of inoculated plants was significantly higher than that recorded with noninoculated plants (Fig. 2). However, the increase of height growth markedly varied depending on the season (Fig. 2). The lowest data were recorded between July and October whereas the highest were measured between February 2005 and May 2006 (Fig. 2). No significant differences were recorded within each treatment during the dry season but, after October 2005, growth rates of inoculated plants were significantly higher than those recorded in the control treatment (Fig. 2).

The rate of mortality reached 36·7% in the control treatment whereas it was drastically reduced (16·7%) in the native AM fungi treatment (Fig. 3). The nitrogen and phosphorus mineral contents were significantly higher in the foliar tissues of inoculated plants than in those of the noninoculated cypress plants (Table 3). After 1 year of plantation, the extent of mycorrhizal colonization was significantly higher for the inoculated plants (62%) than for the noninoculated plants (25%).

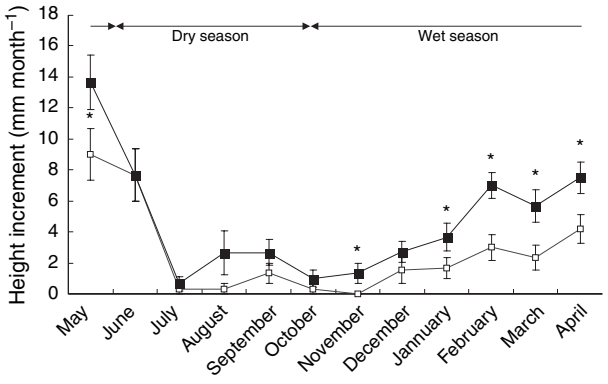

Figure 2 Time course changes in height increment (expressed in mm per month) of *Cupressus atlantica* outplants in the field under natural conditions, either noninoculated (*open squares*) or inoculated with a mixture of native arbuscular mycorrhizal (AM) fungi (*closed squares*). An *asterisk* indicates that the difference between the two treatments is significant at the 0·05 probability level in the corresponding month.

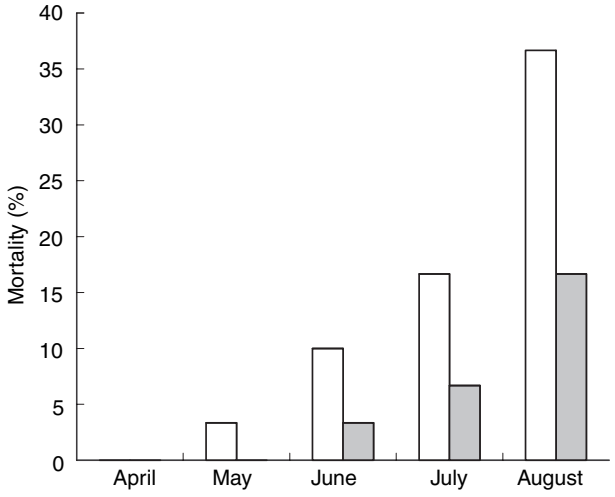

Figure 3 Cumulative mortality of *Cupressus atlantica* outplants in the field under natural conditions, either noninoculated (control □) or inoculated with native arbuscular mycorrhizal (AM ■) fungi during the first months of plantation.

Table 3 Effect of native arbuscular mycorrhizal (AM) fungi inoculation on leaf mineral content after 1 year plantation in the field

	Treatments	
	Control	Native AM fungi
Nitrogen (mg g^{-1} dry weight)	2·01 (0·13)* a†	3·66 (0·19) b
Phosphate (mg g^{-1} dry weight)	0·077 (0·004) a	0·114 (0·004) b

*Standard error of the mean.

†Data in the same line followed by the same letter are not significantly different according to the Newman Keul's test ($P < 0·05$).

Discussion

From this study, discussion could be based on three main points: (i) whether a high AM fungal diversity is associated with *C. atlantica* at the Idni station, (ii) whether native AM fungi inoculation could be effective on the growth of *C. atlantica* seedlings in a disinfected soil and (iii) whether this plant growth stimulation could be maintained in field conditions.

It has been previously assessed that AM fungi were abundant under *Cupressus* spp. (Michelsen *et al.* 1993; Ouahmane *et al.* 2006b). For instance, Ouahmane *et al.* (2006b) numbered about 600 AM fungal spores per 100 g of soil collected under *C. atlantica*. This spore abundance was significantly higher than those recorded under other Mediterranean species such as *Anthyllis cytisoides*, *Stipa tenacissima*, *Retama sphaerocarpa* (Requena *et al.* 1996) or *Tetraclinis articulata* (Abbas *et al.* 2006). Soil-borne spores of AM fungi are usually only very low numbers of viable spores in soil from eroded ecosystems (Sieverding 1991; Requena *et al.* 1996; Stutz and Morton 1996). This is not supported by the present study as a high number of spores were found in all samples and as many of these spores appeared viable. This suggests that this propagule could be the main source of a mycorrhizal inoculum in this ecosystem.

Our investigations highlighted the dominance and diversity of *Glomus* species over *Acaulospora* and *Scutellospora* species. The species recorded are typical of semi-arid Mediterranean ecosystems (Dodd and Krikun 1984; Jeffries *et al.* 1988; Requena *et al.* 1996; Ferrol *et al.* 2004; Abbas *et al.* 2006). The most abundant *Glomus* species associated with *C. atlantica* was *G. fasciculatum*. It has been demonstrated that this AM fungal species promoted growth of a broad range of plant species such as *Tetraclinis articulata* in forest nurseries in the southeastern part of the Iberian Peninsula (Diaz and Honrubia 1993), fruit tree species (Bâ *et al.* 2000) or fast growing plants (Tarafdar and Praveen-Kumar 1996; Bhoopander *et al.* 2005).

The inoculation with a mixture of native AM fungi significantly improved the growth of *C. atlantica* and stimulated its nitrogen and phosphorus assimilation in shoot tissues in a disinfected soil. This result confirmed the high mycorrhizal dependency of this plant species as it has been previously demonstrated with an allochtonous AM fungus, *Glomus intraradices* (Ouahmane *et al.* 2006a). Mycorrhizal inoculation appeared effective in improving nutrient content. It is well known that AM fungi enhanced nutrient uptake, especially nitrogen and phosphorus, as this fungal symbiosis increased the abilities of the host plants to explore a larger volume of soil than roots alone and to take up phosphate from a greater surface area (Jakobsen *et al.* 1992; Joner *et al.* 2000).

In field conditions, the positive effect of native AM inoculation on the plant growth was maintained and its magnitude was increased during the first year of plantation. Hence, the effectiveness of microsymbionts in improving outplanting performance of *C. atlantica* seedlings was evident. The mycorrhizal endophytes were locally isolated from the plantation site; they were well adapted to the ecosystem and appeared effective in improving nutrient foliar content. In addition, this positive effect was found in the first stages of growth that are usually considered as the most critical for revegetation, particularly in Mediterranean semi-arid areas (Caravaca *et al.* 2003). Inoculation with native AM fungi significantly decreased the seedling mortality during the first months of the plantation. It is well known that the transplanting shock and the damage suffered by the seedlings could be critical to the success of the plantation. As inoculated seedlings were stronger than the noninoculated ones, they could survive outplanting by showing better resistance to the environmental conditions (Duponnois *et al.* 2005). In addition, whereas no growth differences were found between both treatments during the dry season (June–October), a significant positive effect of native AM fungi inoculation was found at the beginning of the wet season. It is well known that the size of a plant could affect its water relation (Augé 2001). Larger plants with larger root systems might have access to more extensive soil water reserves (Fitter 1985; Koide 1993). In addition, our results showed that inoculated plants were highly colonized by AM fungi that could explain the increased survival over summer by AM cypress plants. It has been previously established that levels of AM root colonization was positively linked with higher plant growth and higher plant uptake of minerals (Duponnois, unpublished data).

Native inoculum potential of AM fungi in arid and semi-arid Mediterranean ecosystems is generally limited which, in turn, prevents plant establishment and growth. Hence, the selection of efficient AM fungi is a key prerequisite 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 relevance to accelerate the process of reafforestation in arid and semiarid degraded soils.

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