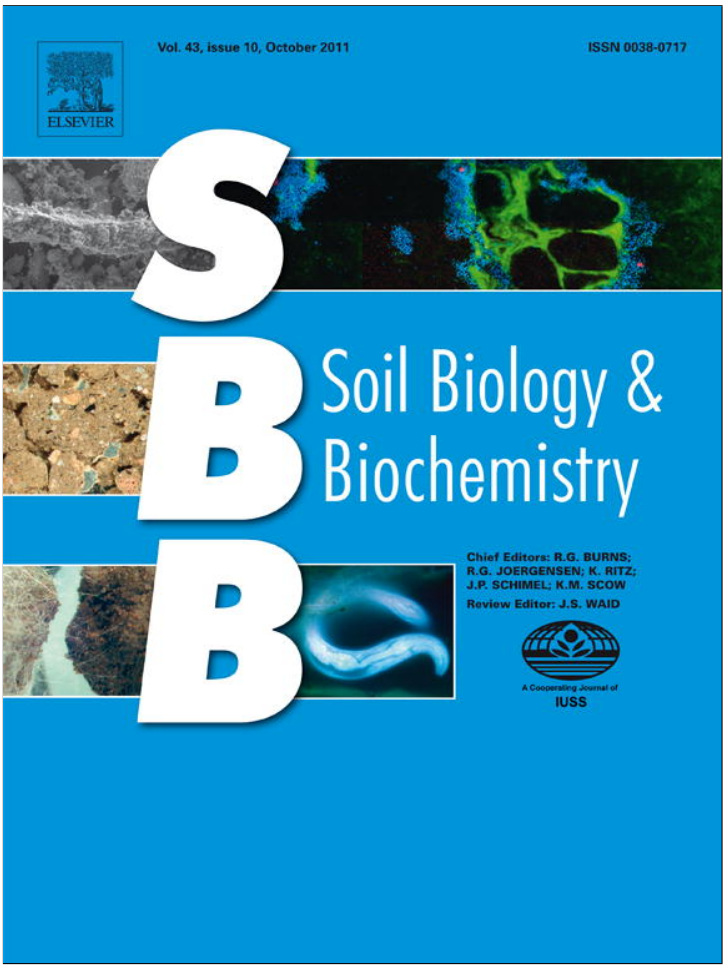

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Nurse shrubs increased the early growth of *Cupressus* seedlings by enhancing belowground mutualism and soil microbial activity

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ABSTRACT

The influence of shrubs used as nurse plants was tested on the growth of *Cupressus atlantica*, on microbial activity and on arbuscular mycorrhizal (AM) soil potential in a Mediterranean environment. An experimental plantation was conducted combining uninoculated, arbuscular mycorrhized Cypress seedlings and an association between *Lavandula stoechas* planted close to newly planted *C. atlantica* seedlings. After three years plantation, this association between *C. atlantica* and *L. stoechas* lead to a higher growth of *C. atlantica* and better soil microbial characteristics compared to the control treatment. AM mycelium network, total microbial activity, dehydrogenase activity, phosphate-solubilizing fluorescent pseudomonads and N, P nutrient uptake by *C. atlantica*, were significantly higher in the presence of *L. stoechas* than those recorded in the other treatments. This pioneer shrubs facilitates the early establishment of Cypress seedlings by improving soil microbial characteristics and AM fungus community development. Given that the facilitative effect of one plant species to another increases with abiotic stress, the benefits of this technique would be useful in reforestation programs undertaken to rehabilitate degraded areas in Mediterranean region.

1. Introduction

Shrub and tall-grass species grow following a patchy distribution are characteristic of the plant communities in semiarid ecosystems. The vegetation patches usually form “fertility islands” (Garner and Steinberger, 1989) or “resource islands” (Schlesinger et al., 1996) that could be involved in the development of native plant species (Callaway, 1995, 1997). This vegetation type can improve its own environment, for example by self-promoting changes in water infiltration, organic matter, etc (Bochet et al., 1999; Valladares and Pugnaire, 1999) and act as “nurse plants” through their positive impacts in the survival of other native plant species (Carrillo-Garcia et al., 2000; Bashan et al., 2009). It is well accepted that the spatial proximity among plants is beneficial in environments such as Mediterranean-type ecosystems that are characterized by abiotic stress and in particular by water stress (Boucher et al., 1982; Callaway and Walker, 1997; Gomez-Aparicio et al., 2004).

In arid Mediterranean ecosystems, desertification generally alters natural plant communities (population structure, succession pattern and species diversity) and physico-chemical and biological soil properties (nutrient availability, microbial activity, soil structure, etc) (Garcia et al., 1997a; Albaladejo et al., 1998; Requena et al., 2001).

Among components of soil microbiota, mycorrhizal fungi are considered essential key components of sustainable soil–plant systems, especially in arid ecosystems (Carpenter and Allen, 1998; Brundrett, 1991). The mycorrhizal symbiosis mobilizes and transports nutrients to roots (Smith and Read, 2008), reduces water stress (Augé, 2001) and improves soil aggregation in eroded soils (Caravaca et al., 2002). It has also been reported that arbuscular mycorrhizal (AM) fungi affect the diversity of plant communities (van der Heijden et al., 1998; Klironomos et al., 2000; O'Connor et al., 2002) and influence relationships between plants (West, 1996; Marler et al., 1999; van der Heijden et al., 2003). It is also

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well known that AM fungi and bacteria can interact synergistically that lead to stimulation of plant growth (Artursson et al., 2006). Specific interactions between AM fungi and Plant Growth Promoting Rhizobacteria (PGPR) most likely occur and numerous studies have demonstrated that some groups of bacteria such as fluorescent pseudomonads could be involved in the mycorrhizal establishment and functions (Meyer and Linderman, 1986; Linderman, 1997; Villegas and Fortin, 2001, 2002). In addition, these PGPRs can directly enhance plant growth by a variety of mechanisms as production of siderophores that chelate iron, solubilization of minerals such as phosphorus, synthesis of phytohormones, etc (Artursson et al., 2006).

The mycorrhizal potential of degraded semiarid Mediterranean ecosystems is generally low (Maremammani et al., 2003). Since a low level or a decrease of fungal symbiont abundance can limit both natural and artificial processes of revegetation, it is necessary to apply mycorrhizal inoculation technologies or to manage native AM fungus communities to replace or reinforce the mycorrhizal potential in these degraded areas (Requena et al., 2001; Ouahmane et al., 2006). It has been recently demonstrated that controlled mycorrhization of *Cupressus atlantica* with native AM fungi improved plant growth and survival in Mediterranean field conditions (Ouahmane et al., 2007b).

Lavandula spp. are representative plant species in Mediterranean shrublands and belong to the natural succession in arid Mediterranean ecosystems (Barea et al., 1992). They have been classified as "obligatory mycorrhizal" (Brundrett, 1991) or as "highly dependent on mycorrhiza" (Habte and Manjunath, 1991). Recent studies have shown that these plant species enriched their cultural soils with AM fungal propagules and that this positive contribution was linked to the total soil P contents (Ouahmane et al., 2006). These results also emphasized the potential role of "resource islands" and "nurse plants" of Lavender plants in the regeneration processes of Mediterranean tree species such as *Cupressus* spp. (Ouahmane et al., 2007a). All these data have been recorded in controlled conditions (glasshouse conditions) but from our knowledge, the effects of Lavender plants *in situ* on the growth of *Cupressus* sp. and on the soil microbial characteristics have not been tested.

According to the above theoretical and empirical framework, we hypothesize that the proximity between a nurse plant, *Lavandula stoechas* and a tree species, *C. atlantica*, would give better results in terms of tree growth than would standard reforestation techniques using open spaces without vegetation. To test this hypothesis, we carried out an experimental reforestation plantation in the Haut Atlas mountains in Morocco. Specifically, we addressed the following questions: (1) How does the use of shrubs as microsites for planting improve tree growth?, (2) this facilitative effect would be as positive as that resulting from a reinforcement of AM soil potential through controlled mycorrhization of *C. atlantica* in field conditions (Ouahmane et al., 2007b)?, (3) How does the magnitude of the positive interactions between shrubs and tree seedlings depend on seasonal conditions in the 3 first years of planting?

2. Materials and methods

2.1. Study site

The trial was established 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 arid Mediterranean, with an annual rainfall of 634 mm. The plant cover is mainly composed of shrub species (*Cistus salviifolus* L., *L. 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}.

2.2. Plant and mycorrhizal treatments

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

A mixture of AM fungi, isolated under *C. atlantica* in the Idni station, was propagated on maize (*Zea mays* L.) on a disinfected soil (Ouahmane et al., 2007a,b). The soil used was collected under *C. atlantica* in Idni station, crushed, passed through a 2-mm sieve and autoclaved (120 °C, 40 min). After 12 weeks of culture, maize plants were uprooted and gently washed. The roots were then cut into 0.5 cm pieces bearing around 100 vesicles and an average of 10 spores per cm. Non-mycorrhizal maize roots, prepared as above, were used for the control treatment without arbuscular mycorrhizal inoculation. Native AM fungal inoculums consisted of a mixture of *Glomus* species such as *Glomus fasciculatum*, *Glomus aggregatum*, *Glomus manihotis*, etc (Ouahmane et al., 2007a,b).

One seedling of *C. atlantica* was planted per plastic bag (1 l) filled with the same disinfected soil collected in the Idni station. One hole (1 cm × 5 cm) was made in the soil of each pot and filled with 1 g of fresh maize root (mycorrhizal or not for the control treatment without fungus). The holes were then covered with the same autoclaved soil.

Seeds of *L. stoechas* collected from the field were germinated on moistened disinfected (140 °C, 40 min) sand (Ouahmane et al., 2007a). After 1 week's culture, seedlings were individually transplanted to 1 liter pots filled with the same disinfected soil used as before.

The pots were arranged in a complete randomized block design. They were protected with a screen from the rain and grown under natural light in the University of Cadi Ayyad (Marrakech. Morocco) (mean daylight approximately 10 h, mean temperature 25 °C during day). After 6 month's culture, ten *C. atlantica* plants were randomly sampled from each treatment. They were uprooted and their root systems gently washed. Height and dry weight of the shoots (1 week at 65 °C) were measured. After drying, plant tissues were ground, ashed (500 °C), digested in 2 ml HCl (6 N) and 10 ml HNO_3 (1 N) and then analyzed by colorimetry for P (John, 1970). Other plant tissue samples were digested in 15 ml H_2SO_4 (36 N) containing 50 g l^{-1} of salicylic acid. One root sub-sample (0.5 g fresh weight) was collected from each plastic bag to quantify their internal colonization by AM fungi. Roots were cleared and stained according to the method of Phillips and Hayman (1970). About fifty 1 cm root pieces were randomly chosen from each root sub-sample and placed on a slide for microscopic observations under 250× magnification (Brundrett et al., 1985). Extent of mycorrhizal formation was expressed in terms of fraction of root length with mycorrhizal internal structures (vesicles or hyphae): (length of colonized root fragments/total length of root fragments) × 100. Then the dry weight of roots was measured (65 °C, 1 week).

2.3. Field experimental design and host plant analysis

The experiment had a randomized block design with two factors and three replication blocks. The first factor was the direct AM inoculation or not (control) of *C. atlantica* seedlings and the second was the dual cultivation of *C. atlantica* with *L. stoechas* (or without *L. stoechas* for the control). An area of 1000 m^2 was established in the Idni station and cleaned from trees and shrubs. The seedlings

were planted in individual holes (30 cm diameter and 30 cm depth), spacing of 3 m × 3 m. For the dual cultivation, four *L. stoechas* seedlings (mean height = 10 cm) were planted at 15 cm around each *C. atlantica* plant. There were at least 30 seedlings per treatment and 30 seedlings per replication block (10 plants × 3 treatments in each block). Tree height was measured each month. The dead plants were replaced in each treatment during the first months of plantation. After 3 year's plantation, sub-samples of leaf tissue were collected from three *C. atlantica* plants, randomly chosen in each block and in each treatment and pooled together. After drying (1 week at 65 °C), their nitrogen (Kjeldahl) and phosphorus contents were assessed using the methods described before. Some root samples (500 mg fresh weight per plant) were taken from three *C. atlantica* plants, randomly sampled from each treatment and from each block and pooled together. Then extent of AM formation was determined for each plant in each treatment according to the methods described before.

2.4. Soil microbial analysis

2.4.1. Description of AM fungus communities

Soil samples (1 kg) were collected from the rhizosphere of *C. atlantica* plants in each block and in each treatment. Each soil sample consisted of ten 100 g sub-samples collected at 0–10 cm depth at 10 cm from each *C. atlantica* plant in each block and each treatment. They were kept at 4 °C for further measurements. AM hyphal length was measured on membrane filters according to Jakobsen and Rosendahl (1990). AM fungal spores were extracted from soil samples by wet sieving and decanting, followed by sucrose centrifugation (Gerdemann and Nicolson, 1963). After centrifugation, the supernatant was poured through a 50-μm sieve and rinsed with tap water. Spores were counted under a stereomicroscope and grouped according to morphological characteristics. The relative abundance of each fungal type was calculated per 100 g of dry soil. Spore size and color 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 color, size, wall structure and hyphal attachment (Morton and Benny, 1990; INVAM, 1997). Relative abundance of each fungal species in each treatment was calculated. Mycorrhizal fungal diversity was then calculated as well as mycorrhizal fungal spore diversity using Simpson–Yule's diversity index (Krebs, 1989).

2.4.2. Enzyme activities

Dehydrogenase activity was assessed following the Skujins (1976) method modified by Garcia et al. (1997b). Dehydrogenase activity was determined in 1 g of soil at 60% of its field capacity, suspended in 0.2 ml of 0.4% INT (2-*p*-iodophenyl-3-*p*-nitrophenyl-5-phenyltetrazolium chloride) in distilled water for 20 h at 22 °C in darkness. The INTF formed (iodo-nitrotetrazolium formazan) was extracted by 10 ml of methanol by shaking vigorously for 1 min and filtered through a Whatman N° 5 filter paper. INTF was measured spectrophotometrically at 490 nm.

The fluorescein diacetate (3′, 6′-diacetylfluorescein [FDA], Sigma–Aldrich Chimie, France) hydrolysis assay was used to estimate the total microbial activity in soil samples (Schnürer and Rosswall, 1982). Soil samples (1 g equivalent dry weight) were suspended in 200 μl FDA and 15 ml of sterile 60 mM sodium phosphate buffer, pH 7.6. After an incubation at 25 °C for 1 h on a rotary shaker, FDA hydrolysis reaction was stopped by adding 750 μl acetone. After centrifugation of soil suspensions (2400*g*, 10 min), the supernatants were sampled, passed through a 45 μm filter and the absorbance readings were taken at 490 nm. There were three replicates for each treatment and a fourth received 15 ml of buffer without substrate that served as a control used to correct for background. The rate of fluorescein diacetate hydrolysis (μg of product corrected for background fluorescence per hour per gram of soil) was calculated to determine total microbial activity for each treatment.

2.4.3. Fluorescent pseudomonad efficacy for phosphorus mobilization

Soil sub-samples (1 g fresh weight) were suspended in 10 ml sterile magnesium sulfate solution (0.1 M) and shaken manually (10 times up and down). Then serial dilutions of homogenized suspensions were plated on King's B medium (King et al., 1954) and incubated at 30 °C for 2 days. The King's B medium plates were examined under UV light and fluorescent colonies were counted and randomly selected. The isolates of fluorescent pseudomonads (30 bacterial strains per treatment) were purified, sub-cultured on King's B medium and cryopreserved at −80 °C in glycerol 60%/TSB (tryptic soy broth, 3 g l^{-1}) culture (1:1; v/v). The TCP (tricalcium orthophosphate) medium was used to test the ability of fluorescent pseudomonads to solubilize tricalcium orthophosphate (Frey-Klett et al., 2005). Its composition was as follows: 4 g $Ca_3(PO_4)_2$, 10 g glucose, 5 g NH_4Cl, 1 g NaCl, 1 g $MgSO_4$, $7H_2O$ and 20 g agar per liter at pH = 7.2. Petri dishes (5.5 cm diameter) were filled with 10 ml of TCP agar medium per dish (Frey-Klett et al., 2005). Bacterial isolates were then picked up from their mother cultures and placed in the center of Petri dished on TCP agar medium. The plates were incubated at 25 °C for 5 days. All the bacterial isolates grew on TCP agar medium. Phosphate solubilization was indicated by clear zones around the bacterial colonies. Phosphate-solubilizing ability was classified as "0" or "+" depending on the presence of well defined clear zone produced by bacterial colony.

2.5. Statistical analysis

All data were subjected to a two-way analysis of variance and the mean values were compared using the Newman–Keul's test ($p < 0.05$). The percentages of mycorrhization were arcsin ($\sqrt{x}$) transformed before statistical analysis. A three-way analysis of variance was first used to check for triple interactions and test the influence of each factor separately (inoculation, block, time). Growth of *C. atlantica* from inoculated, uninoculated and associated with *L. stoechas* treatments 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, 2010). R is a free software environment for statistical computing and graphics that can be downloaded at http://www.r-project.org/. The distributions of AMF species and phosphate solubilizing bacteria were compared between each soil origin with 2 × 2 contingency tables and chi-square test (χ^2 test) and Yates correction for small numbers.

3. Results

As it has been previously reported and after 6 month's culture in nursery conditions, mycorrhizal inoculation of *C. atlantica* with native AM fungi has significantly improved height, shoot and root biomass, total biomass, phosphorus and nitrogen foliar contents (Ouahmane et al., 2007c).

In the field experiment, the effect of blocks was not significant according to the "lm" function of the R software ($p = 0.26$). The effects of AM fungus inoculation or dual cultivation on tree height were significant. During the three years of plantation, height

growth was significantly higher in the AM native inoculated treatment that that measured in the other treatments (Fig. 1). During the first year, the lowest tree height was recorded with *C. atlantica* trees associated with *L. stoechas* (Fig. 1). Then (second year), no significant differences were recorded between the height growth of the non inoculated plants and the *C. atlantica* seedlings associated with *L. stoechas* (Fig. 1). After 3 year's plantation, the treatment effects on the *C. atlantica* height growth were inverted and the height of *C. atlantica* trees associated with *L. stoechas* was significantly higher than that measured in the control treatment (uninoculated plants) but lower than AM inoculated soil. A three-way analysis of variance was used to check for triple interactions (which were not statistically significant) and test the influence of each factor separately (inoculation, block, time). Linear models were fit to *C. atlantica* growth curves (Fig. 2). The slope of the regression line of uninoculated *C. atlantica* trees ($a_{ni} = 0.214$) was significantly lower than those recorded in the other treatments ($p < 0.0001$) (Fig. 2). The slope of the regression line of *C. atlantica* trees cultivated with *L. stoechas* ($a_{lav} = 0.413$) was higher than that of the inoculated plants ($a_i = 0.329$) ($p < 0.0001$) (Fig. 2). The intercepts were also significantly different among the treatments and ranged from AM native mixture ($b_i = 16.9$) > uninoculated ($b_{ni} = 12.9$) > dual cultivation with *L. stoechas* treatments ($b_{lav} = 10.5$) (Fig. 2). After 3 year's plantation, height of inoculated trees and those associated with *L. stoechas* was stimulated 1.3× and 1.1×, respectively, compared to the control treatment. The increase of height growth markedly varied depending of the season (Fig. 3). The lowest data were recorded during the dry seasons (From July to October 2005 and from June to October 2006). During the second wet season (From November 2005 to April 2006), growth rates of *C. atlantica* cultivated in association with *L. stoechas* were generally higher than those recorded in the other treatments (Fig. 3). The highest growth was found at the end of the wet season in the dual cultivation treatment (Fig. 3).

After 3 year's plantation, shoot N and P contents were significantly higher in the inoculated and dual cultivated plants of *C. atlantica* (Table 1). The total microbial activities, dehydrogenase activities of soils collected from the inoculated and *L. stoechas*/*C. atlantica* treatments as well as the abundance of fluorescent pseudomonads, were significantly higher than those measured in the control treatment (Table 1). The frequency of fluorescent pseudomonads able to solubilize inorganic phosphorus was significantly higher in the soil collected in the dual cultivation treatment than that collected under the uninoculated *C. atlantica* ($\chi^2 = 4.65$, $p = 0.031$) (Fig. 4).

The number of AM spores was significantly higher in the soil collected under *C. atlantica*/*L. stoechas* dual cultivation than in both other treatments whereas the abundance of AM spores was significantly higher in the soil collected under inoculated *C. atlantica* than in the control (uninoculated trees) (Table 2). The same pattern was observed with the length of external hyphae ranged among the treatments as follows: *C. atlantica*/*L. stoechas* dual cultivation > inoculated *C. atlantica* > uninoculated *C. atlantica*. AM colonization of *C. atlantica* roots was significantly lower for the uninoculated seedlings than in the other treatments (Table 1). Ten AM species were detected in the soils: AMF 1: *G. fasciculatum*; AMF 2: *G. manihotis*; AMF 3: *Glomus* sp. 1; AMF 5: *Glomus* sp. 2; AMF 6: *Glomus* sp. 3; AMF 7: *Glomus* sp. 4; AMF 8: *Acaulospora* sp.; AMF 9: *G. aggregatum*; AMF 10: *Glomus* sp. 5; AMF 11: *Glomus* sp. 6 (Table 2, Fig. 5). Simpson–Yule's index was significantly higher in the soil collected in the *C. atlantica*/*L. stoechas* dual cultivation treatment than in the control (Table 2). Spores of *G. fasciculatum* were mainly recorded in the soils sampled from the inoculated and dual cultivation treatments (Table 2). The distribution of AM species within treatments was significantly different: uninoculated *C. atlantica vs* inoculated *C. atlantica* ($\chi^2 = 31.7$, $p < 0.0002$), uninoculated *C. atlantica vs C. atlantica*/*L. stoechas* dual cultivation ($\chi^2 = 32.3$, $p < 0.0002$) and the soil sampled from *C. atlantica*/*L. stoechas* dual cultivation *vs* inoculated *C. atlantica* ($\chi^2 = 21.8$, $p < 0.009$) (Fig. 5).

4. Discussion

From this study, the discussion could be based on two main points: (i) whether the growth of *C. atlantica* increased when it was associated with *L. stoechas* and (ii) whether this facilitative effect on tree performance depended to soil microbial activity and soil AM potential.

The positive effect of the native AM mixture has been kept and its magnitude was increased during the first years of plantation in field conditions. Controlled mycorrhization is based on the use of mycorrhizal strains best suited to host plant species that rapidly colonize their root systems and are well adapted to the environmental conditions of the planting site (Perry et al., 1987). In the present study, the mycorrhizal fungi were locally isolated from the plantation soil and are consequently well adapted to the ecosystem. They are also effective in improving nutrient foliar content. The

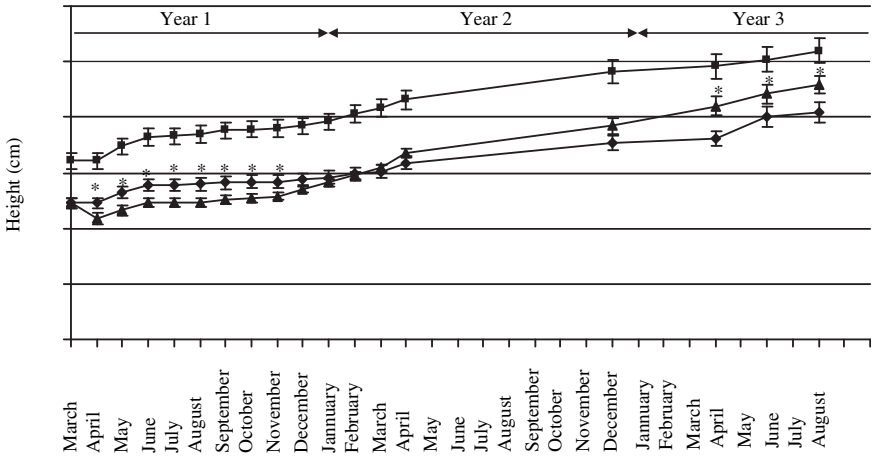

Fig. 1. Time course changes in plant height (expressed in cm) of *C. atlantica* outplants growing under natural conditions in Haut Atlas mountains (Morocco), either uninoculated (Control) (—◆—), inoculated with a mixture of native AM fungi (—■—) or associated with *L. stoechas* plants (—▲—). Symbols represent means (±standard error of the mean). An asterisk indicates that the difference between the height of uninoculated *C. atlantica* and *C. atlantica* associated with *L. stoechas* is significant in the corresponding month according to the Newman–Keul's test ($p < 0.05$).

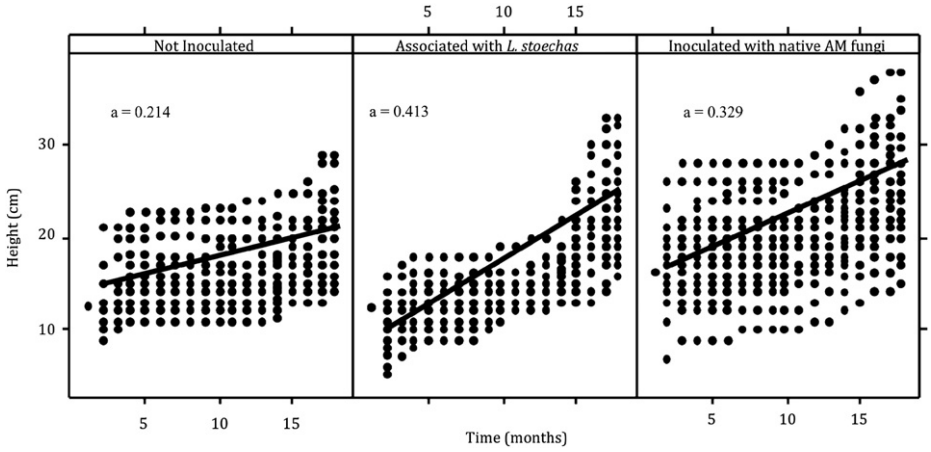

Fig. 2. Regression model between the height growth of *C. atlantica* outplants either uninoculated (Control), inoculated with a mixture of native AM fungi or associated with *L. stoechas* plants. The slope of the regression lines (a) is given in each graphic.

positive effect of mycorrhizal inoculation with selected AM fungi on plant growth is usually observed during the first steps of the plantation after outplanting in field conditions (Duponnois et al., 2005a, 2007) and height differences measured between inoculated and uninoculated plants at outplanting was kept during the plantation. In the present study, the magnitude of the mycorrhizal inoculation was increased during the 3 years of plantation. Moreover, the number of AM spores and hyphal length were significantly higher under *C. atlantica* inoculated plants than in the soil collected under uninoculated plants. These results are in accordance with previous studies where it was showed that the composition of AM communities was an important biological factor to plant species development (van der Heijden et al., 1998). Mycorrhizal inoculation also led to a significant increase of the length of mycorrhizal hyphae in the soil and of microbial enzymatic activities. Increasing hyphal length results in greater efficiency of nutrient absorption (Smith and Gianinazzi-Pearson, 1988). This process is particularly important for slowly diffusing mineral ions such as P (Jakobsen et al., 1992; Joner et al., 2000). In the present study, a significant effect of the mycorrhizal inoculation has been recorded in P and N nutrition of *C. atlantica* plants. Few studies have been carried out on the role of AM symbiosis in N nutrition of plants. But it has been reported that AM plants have access to nitrogen forms that are unavailable to non-AM plants (Azcón-Aguilar et al., 1993; Subramanian and Charest, 1998). External mycelium of AM fungi can transport NO_3^- from the hyphal compartment in soil to the host plant (Subramanian and Charest, 1998). Further, increasing hyphal length stimulates N assimilation and nutritional status of the host plant as it has been found in the present study.

In addition to increasing the absorptive surface area of their host plant root systems, the hyphae of AM fungi provide an increased area for interactions with other soil microorganisms (Johansson et al., 2004). Mycorrhizal symbiosis alters root functions and microbial equilibrium in the rhizosphere (Rambelli, 1973; Leyval and Berthelin, 1993). This zone influenced by both the roots and the mycorrhizal symbiont has been named "mycorrhizosphere" by Linderman (1988, 2008) and included the more specific term "hyphosphere" which only referred to the zone surrounding individual fungal hyphae (Johansson et al., 2004). Hence fungal activities could also modify the structure and the functionalities of soil microbial communities (Duponnois et al., 2005b). In the present study, mycorrhizal inoculation of *C. atlantica* with a mixture of native AM fungi has significantly increased the total microbial activity and the microbial dehydrogenase activity. Dehydrogenase is an indicator of general activity and quite easy to analyze (Caravaca et al., 2005). This enzymatic activity indicates the status of soil microbial activity in arid areas subjected to degradation and desertification processes and was very low in the most degraded soils (Garcia et al., 1994). For instance, Bastida et al. (2006) had found that the dehydrogenase activity of disturbed soil (15 years of devegetation) was significantly lower (about 7 μg INTF g^{-1} soil h^{-1}) than that measured in undisturbed soil (about 12 μg INTF g^{-1} soil h^{-1}). Hence the positive influence of

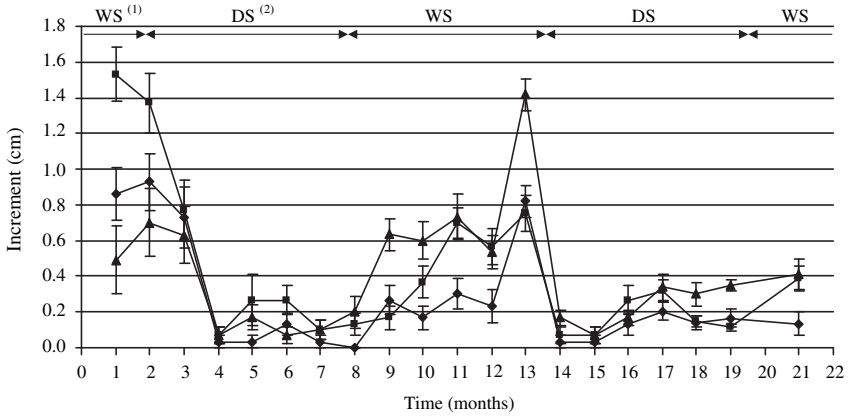

Fig. 3. Time courses changes in height increment (expressed in cm per month) of *C. atlantica* outplants in the field under natural conditions either uninoculated (Control), inoculated with a mixture of native AM fungi or associated with *L. stoechas* plants. For the legend, see Fig. 1. (1)WS: wet season. (2)DS: dry season.

Table 1
Biochemical properties of soil treatments and shoot nitrogen and phosphorus contents of inoculated or uninoculated *C. atlantica* and *C. atlantica* associated with *L. stoechas* plants.

	Not inoculated	Inoculated with native AM fungi	Associated with *L. stoechas*
Shoot N content ($g\,g^{-1}$ dry weight)	4.76 (0.32)[a]a[b]	6.16 (0.16)b	6.25 (0.19)b
Shoot P content ($g\,g^{-1}$ dry weight)	0.343 (0.028)a	0.457 (0.023)b	0.415 (0.036)b
Total microbial activity (μg of hydrolyzed fluorescein diacetate $h^{-1}\,g^{-1}$ of soil)	12.4 (0.33)a	18.8 (1.18)b	23.2 (2.5)b
Dehydrogenase activity (μg INTF g^{-1} soil h^{-1})	36.9 (1.69)a	43.6 (2.2)b	51.9 (6.5)b

[a] Standard error of the mean.
[b] Data in the same line followed by the same letter are not significantly different according to the Newman–Keul's test ($p < 0.05$).

mycorrhizal inoculation on dehydrogenase activity suggests that the symbiotic symbionts reinforce soil microbiological activity in an arid climate. AM inoculation has also stimulated fluorescent pseudomonad multiplication. Since soil samples have been collected under *C. atlantica* and were free of roots, it could be considered that measurements have been carried out on hyphosphere soils. It has been previously demonstrated that ectomycorrhizal mycelium increased the fluorescent pseudomonads growth (Ramanankierana et al., 2006). For AM symbiosis, Andrade et al. (1997) showed that the development of the AM mycelium in soil had little influence on the composition of the microflora in the hyphosphere but AM root colonization was positively linked with fluorescent pseudomonads in the rhizosphere as it has been found in the present study. On the opposite, *G. fasciculatum* in association with *Z. mays* or *Trifolium subterraneum* reduced the vital counts of fluorescent pseudomonads (Meyer and Linderman, 1986).

Dual cultivation of *C. atlantica* with *L. stoechas* has induced a better *C. atlantica* growth than that recorded in the control treatment (without *L. stoechas*) but no better than AM inoculated soil. This positive effect has only been recorded during the third year of plantation whereas, during the first year, an opposite effect of this plant association has been found (growth inhibition). Co-occurring facilitative and competitive effects between plant species often vary in time or space (Callaway, 1995, 1997; Callaway et al., 1996) but the biological factors that determine the balance between positive and negative are poorly understood. Some factors have been considered such as life stage, plant density, species-specific physiology, indirect interactions (i.e. mycorrhizal fungi) and abiotic stress (Callaway and Walker, 1997).

At outplanting, the height of *C. atlantica* and *L. stoechas* plants was similar and plant size was very limited and it could be considered that these two species did not compete for light and water. Hence other factors are involved in the biological processes that regulate both plant species coexistence. Several studies showed that AM fungi alter plant diversity (van der Heijden et al., 1998; Klironomos et al., 2000; O'Connor et al., 2002) and change competitive relationships between plants (West, 1996; Marler et al., 1999). It has been previously demonstrated that *Lavandula* species are very mycotrophic and enriched the soil in AM fungal propagules (Ouahmane et al., 2006). In particular they promoted the

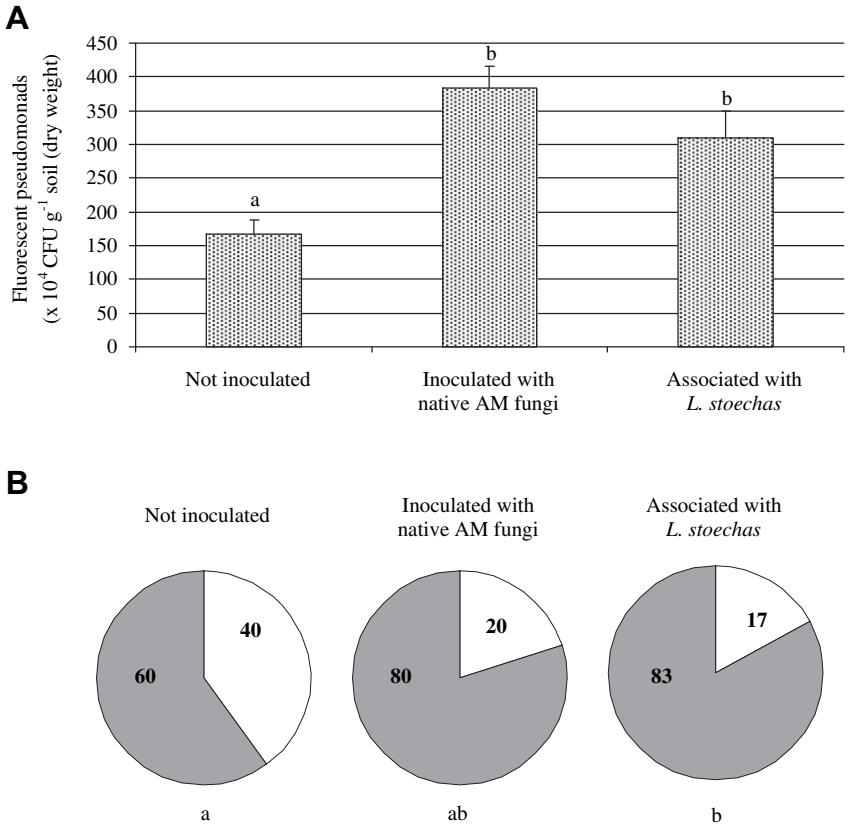

Fig. 4. Abundance of fluorescent pseudomonads from soils collected under inoculated or uninoculated *C. atlantica* and under *C. atlantica* associated with *L. stoechas* plants (A) and assessment of their distribution according to their capacity to solubilize tricalcium orthophosphate (B). (A) Columns indexed by different letters represent data significantly different according to the Newman–Keul's test ($p < 0.05$). (B) Proportions of bacterial isolates able to solubilize tricalcium orthophosphate in disks indexed by different letters are significantly different according to a χ^2 test ($p < 0.05$). No shading, no solubilization of inorganic phosphorus *in vitro*; solid, solubilization of inorganic phosphorus *in vitro*.

Table 2
Diversity and abundance of AM fungus species in soils collected under inoculated or uninoculated *C. atlantica* and under *C. atlantica* associated with *L. stoechas* plants.

	Not inoculated	Inoculated with native AM fungi	Associated with *L. stoechas*
Total number of AM spores per 100 g of soil	206 (32.5)[a]a[b]	323 (9.4)b	331 (17.9)c
Hyphal length ($m\,g^{-1}$ of soil)	1.153 (0.045)a	1.647 (0.084)b	2.245 (0.083)c
AM colonization (%)	44.2 (10.3)a	55.3 (9.8)ab	68.9 (5.4)b
Number of spores per AM species (per 100 g of soil)			
Glomus fasciculatum	63.7 (2.33)a	86.0 (6.43)b	96.0 (10.58)b
G. manihotis	37.0 (9.07)a	48.3 (9.53)a	34.7 (10.73)a
G. aggregatum	24.3 (10.68)a	37.0 (12.74)a	44.7 (13.48)a
Glomus sp. 1	19.0 (8.39)a	30.0 (7.57)a	41.3 (12.98)a
Glomus sp. 2	11.0 (5.19)a	19.3 (8.25)a	11.7 (1.76)a
Glomus sp. 3	38.7 (2.96)a	76.0 (22.7)a	52.3 (9.39)a
Glomus sp. 4	2.3 (2.33)a	0.0 (0.00)a	19.7 (10.49)a
Glomus sp. 5	0.0 (0.00)a	15.0 (4.73)b	18.0 (4.16)b
Glomus sp. 6	2.3 (2.33)a	0.0 (0.00)a	1.0 (0.58)a
Acaulospora sp.	2.3 (1.86)a	11.3 (4.33)a	11.3 (4.33)a
Simpson–Yule's index	4.36 (0.19)a	5.22 (0.43)ab	5.72 (0.08)b

[a] Standard error of the mean.
[b] Data in the same line followed by the same letter are not significantly different according to the Newman–Keul's test ($p < 0.05$).

development of the AM mycelium network that is considered as the main source of fungal inoculum in semiarid and arid ecosystems (Bashan et al., 2000). *Lavandula* species are also drought-tolerant native species able to cope with nutrient stress in the eroded soils and have been recommended to re-establish functional shrublands (Francis and Thorne, 1993). Hence it suggests that the influence of *L. stoechas* on AM communities and on fungal propagule multiplication (Soil mycorrhizal potential) was larger than that of *C. atlantica* at the first steps of the plantation. The presence of *L. stoechas* near *C. atlantica* plants enhances the abundance of AM spores and hyphal length. It is well known that mycorrhizal associations are diffuse and non-specific (Smith and Read, 2008). Mycorrhizal fungi form mycelial links in a common mycorrhizal network. *Lavandula* and *Cupressus* plants are probably connected through a mycorrhizal network after few months of plantation. One

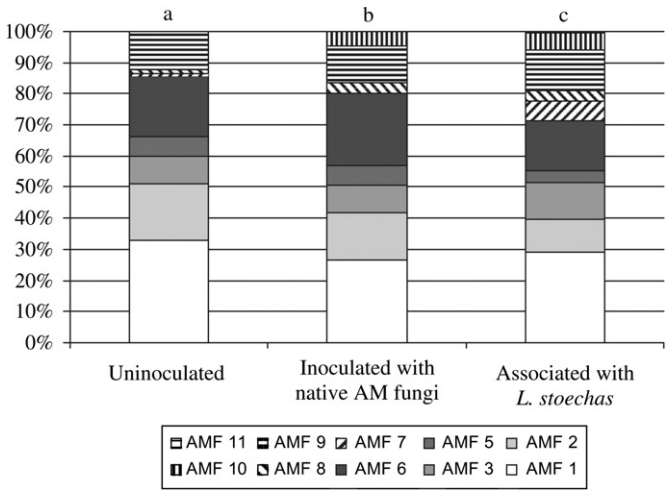

Fig. 5. Distribution of AM fungus species in soils collected under inoculated or uninoculated *C. atlantica* and under *C. atlantica* associated with *L. stoechas* plants. The distributions of AM fungus species in column indexed by different letters are significantly different according to a χ^2 test ($p < 0.05$). AMF 1: *G. fasciculatum*; AMF 2: *G. manihotis*; AMF 3: *Glomus* sp. 1; AMF 5: *Glomus* sp. 2; AMF 6: *Glomus* sp. 3; AMF 7: *Glomus* sp. 4; AMF 8: *Acaulospora* sp.; AMF 9: *G. aggregatum*; AMF 10: *Glomus* sp. 5; AMF 11: *Glomus* sp. 6.

important consequence of the mycorrhizal networks is nutrient transfer between plants and more particularly on carbon transfer (Simard and Durall, 2004). The C transfer is bidirectional in some studies (Simard et al., 1997) and the magnitude and direction of carbon transfer depends on plant–fungus combinations and on the plant environment (Simard et al., 1997). Since during the first year of plantation, shoot biomass of *C. atlantica* was lower than that of *L. stoechas* (Ouahmane, personal communication) and mycorrhizal propagules were less abundant in the *C. atlantica* rhizosphere soil (Ouahmane, personal communication). Hence it could be assumed that there is a net C gain by *L. stoechas* over its connected plant partner that affects *C. atlantica* performance. After two year plantation, opposite effect of *L. stoechas* on the *C. atlantica* growth have been recorded. At this step, mycorrhizal network could equalize the distribution of soil resources among both species. It results to a higher growth of *C. atlantica* and a higher nutrient uptake (P, N). In addition, growth increment of *C. atlantica* at the end of the first dry season was significantly higher than those measured in the other treatments (inoculated or uninoculated *Cupressus* plants). It is likely that the external mycelium facilitated direct water uptake and transport of water by mycorrhizal roots (Faber et al., 1991) and assist AM plants to exploit available soil moisture (Subramanian et al., 1997). The higher development of external mycelium in the dual cultivation treatment has enhanced soil microbial activities (total and dehydrogenase activities) at the same levels of those recorded in the mycorrhizal inoculation treatment. In addition to the quantitative external mycelium effect on fluorescent pseudomonad population, the introduction of *L. stoechas* has also modified the functional activities of this bacterial group. Present results showed that most of fluorescent pseudomonad strains isolated from the hyphosphere soil compartment are able to solubilize tricalcium orthophosphate compared to those isolated from the soils collected in the other treatments. It has been previously demonstrated that some phosphate-solubilizing bacteria can interact synergistically with AM fungi for translocation of soluble phosphorus to the host plant (Kim et al., 1998). It suggests that the selective effect of external mycelium on soil microbiota can improve the phosphorus soil content around the hyphae and, consequently enhance the phosphorus uptake by the external mycelium that further transfer this nutrient to the host plant. This proposal is in accordance with the trophic complex described by Frey-Klett et al. (2005) where multitrophic interactions take place between mycorrhizal fungi, mycorrhizosphere microbiota and host plants. These authors suggested that the mycorrhizal symbiosis had a direct positive effect on the plant growth (nutrient uptake facilitations, etc) but also an indirect effect via its selective pressure on bacterial communities.

The *L. stoechas*/*C. atlantica* association has also modified the structure of AM fungus communities by enhancing their species evenness and the abundance of AM spores. van der Heijden et al. (1998) proposed that the species composition and diversity of AM fungus communities have the potential to determine plant biodiversity in natural ecosystems. Hence, the introduction of *L. stoechas* in afforestation programs could be a beneficial tool to rehabilitate sustainably degraded soils in Mediterranean ecosystems.

From a practical point of view and despite the fact that the dual cultivation did not lead to a higher height growth as that recorded in the controlled mycorrhization treatment, the results of the present study show that the cultural process involving two mycotrophic plant species could be of great relevance to rehabilitate degraded areas as (i) it improves *C. atlantica* growth in field conditions without mass production of mycorrhizal inocula and (ii) it enhances soil microbial characteristics, more particular AM fungus communities known to be a key component in soil biofunctioning. Our results show that this pioneer shrub facilitates the

establishment of Cypress seedlings and, since the facilitative effect generally increases with abiotic stress, this reforestation technique might be relevant under stressful environment (dryness, rainfall variability, soil nutrient deficiencies, etc) generally encountered in Mediterranean degraded areas.

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