Available online at www.sciencedirect.com
SCIENCE DIRECT®
Applied Soil Ecology 25 (2004) 169–180
Applied Soil Ecology
www.elsevier.com/locate/apsoil

Comparing the effectiveness of mycorrhizal inoculation and amendment with sugar beet, rock phosphate and *Aspergillus niger* to enhance field performance of the leguminous shrub *Dorycnium pentaphyllum* L.

F. Caravaca [a,*], M.M. Alguacil [a], R. Azcón [b], G. Díaz [c], A. Roldán [a]

[a] *Department of Soil and Water Conservation, CSIC-Centro de Edafología y Biología Aplicada del Segura, P.O. Box 164, Campus de Espinardo, Murcia 30100, Spain*
[b] *Microbiology Department, CSIC-Estación Experimental del Zaidín, Profesor Albareda, 1, Granada 18008, Spain*
[c] *Campus de Espinardo, SACE-University of Murcia, Murcia 30100, Spain*

Received 12 May 2003; received in revised form 6 August 2003; accepted 8 August 2003

Abstract

A field experiment was undertaken to evaluate the effectiveness of mycorrhizal inoculation with *Glomus intraradices* (Schenck & Smith) or *Glomus deserticola* (Trappe, Bloss. & Menge) and the addition of sugar beet (SB), rock phosphate and *Aspergillus niger* with respect to the establishment of *Dorycnium pentaphyllum* L. seedlings, in a semiarid Mediterranean area, and the improvement of the physical-chemical, biochemical and biological properties of the soil. Eighteen months after planting, amended soil had higher available phosphorus and aggregate stability levels than control soil (165 and 157%, respectively). All assayed treatments had higher soil total carbohydrates and water soluble C values and enzyme activities (dehydrogenase, urease, acid phosphatase and β-glucosidase), and lower soil pH and bulk density than control soil. Biomass C and dehydrogenase, urease and β-glucosidase activities of the rhizosphere of *G. deserticola*-inoculated plants were higher than those of the rhizosphere of *G. intraradices*-inoculated plants. Mycorrhizal inoculation treatments stimulated significantly the production of shoot biomass, to a higher extent than the addition of the amendment alone to soil or the combined treatment. The highest levels of mycorrhizal colonisation and of foliar N and K were recorded in the seedlings inoculated with *G. deserticola*. The shoot biomass of *G. deserticola*-inoculated *D. pentaphyllum* plants was greater (about 219% with respect to control plants) than that of *G. intraradices* (about 116% with respect to control plants).

Keywords: Aggregate stability; Arbuscular mycorrhizal fungi; Enzyme activities; *Glomus intraradices*; *Glomus deserticola*; Microbial biomass C

1. Introduction

The revegetation of desertification-threatened areas is particularly difficult in semiarid Mediterranean ecosystems due to severe water stress, scarcity of plant available nutrients, particularly P and N, weak structural stability, and low microbiological activity

* Corresponding author. Tel.: +34-968-396-337; fax: +34-968-396-213.
E-mail address: fcb@cebas.csic.es (F. Caravaca).

0929-1393/$ – see front matter © 2003 Elsevier B.V. All rights reserved.
doi:10.1016/j.apsoil.2003.08.002

of the soils (Caravaca et al., 2002). Drought-tolerant, native shrub species have been recommended for re-establishment of functional shrublands and recovery of desertified Mediterranean ecosystems (Francis and Thornes, 1990). Previous studies have demonstrated that woody legumes are useful for such nutrient-deficient, arid ecosystems because of their ability to fix N_2 in association with rhizobial species, and to form mycorrhizas which improve nutrient acquisition and help plants to become established and cope with stress (Herrera et al., 1993; Requena et al., 2001). In particular, shrub legumes are important because they constitute a reservoir of arbuscular mycorrhizal (AM) fungi for the ecosystem and a source of N input for the non-N-fixing vegetation (Caravaca et al., 2003b). The P supplied by arbuscular mycorrhizal activities is thought to be critical for both plant growth and N_2-fixation.

The successful reestablishment of native plants in degraded soils such as those of semiarid ecosystems may be limited by the low density of mycorrhizal propagules, which represent a significant factor for soil fertility, by governing the cycles of major plant nutrients (Requena et al., 2001; Palenzuela et al., 2002; Jeffries et al., 2003). It is well known that mycorrhizas help plants to thrive in arid conditions by increasing the supply of nutrients to the plant, particularly N and P (Toro et al., 1997), reducing water stress (Augé, 2001) and increasing disease resistance (Pozo et al., 1999). Thus, inoculation with suitable symbiotic microorganisms, especially AM fungi, has been proposed as a promising tool for improving restoration success in semiarid degraded areas (Caravaca et al., 2003c). However, there are no studies regarding field testing of the effects of mycorrhizal inoculation on the performance of leguminous shrub seedlings planted in these areas.

The use of rock phosphate as a fertiliser for P-deficient soils has received significant interest in recent years. Rock phosphates are natural, inexpensive and available fertilisers, but their solubility is low in non-acidic soils. The solubilisation of rock phosphate by microorganisms able to excrete organic acids has been tested under fermentation conditions (Rodríguez et al., 1999). The addition of microbiologically-solubilised phosphate and treated agrowastes (such as sugar beet (SB)) to agricultural soils has been used for improving plant growth and nutrition (Vassilev et al., 1996, 1998). Rodríguez et al. (1999) demonstrated that the effectiveness of these biosystems for increasing the growth and nutrient uptake of *Medicago sativa* L. plants decreased with the time of preincubation of the lignocellulosic substrate with *Aspergillus niger*. However, the effectiveness of rock phosphate solubilisation on agrowastes by microorganisms inoculated directly into soil under field conditions is unclear because of the possible re-fixation of phosphate ions before they reach the root surface. In addition, no information is available on the use of such materials in revegetation programmes.

Revegetation with native shrub legumes has proved to be more effective than with exotic shrub legumes under semiarid conditions (Herrera et al., 1993). For this study, we chose as the target species *Dorycnium pentaphyllum* L., which is a leguminous shrub suitable for use in the reafforestation of semiarid disturbed lands. However, knowledge of revegetation strategies involving *D. pentaphyllum* is still very limited. The objectives of this study were: (1) to compare the effectiveness of inoculation with two AM fungi in increasing mycorrhizal colonisation, plant growth and nutrient uptake in *D. pentaphyllum* seedlings afforested in a degraded Mediterranean semiarid soil; and (2) to evaluate the potential application of SB, rock phosphate and *A. niger* directly into the planting hole for improving plant performance and soil properties.

2. Materials and methods

2.1. Study sites

The experimental area is located in Los Cuadros in the Province of Murcia (southeast Spain) (1°05′W and 38°10′N). The climate is semiarid Mediterranean with an average annual rainfall of 300 mm and a mean annual temperature of 19.2 °C; the potential evapo-transpiration reaches 1000 mm per year. The soil used was a Typic Haplocalcid (Soil Survey Staff, 1999) developed from quaternary sediments with a loam texture. The analytical characteristics of the soil are shown in Table 1.

2.2. Materials

The SB residue was dried in a 60 °C oven and then ground to 2 mm fragments. The SB is a lignocellulosic

Table 1
Chemical, biochemical, microbiological and physical characteristics of the soil used in the experiment

pH (H_2O)	8.5 ± 0.0[a]
EC (1:5, $\mu S\,cm^{-1}$)	225 ± 2
Texture	loam
Total organic C ($g\,kg^{-1}$)	10.3 ± 0.3
Total carbohydrates ($\mu g\,g^{-1}$)	552 ± 20
Water soluble C ($\mu g\,g^{-1}$)	100 ± 1
Water soluble carbohydrates ($\mu g\,g^{-1}$)	8 ± 0
Total N ($g\,kg^{-1}$)	0.95 ± 0.02
Available P ($\mu g\,g^{-1}$)	7 ± 0
Extractable K ($\mu g\,g^{-1}$)	222 ± 4
Microbial biomass C ($\mu g\,g^{-1}$)	396 ± 11
Dehydrogenase (μg INTF g^{-1})	51 ± 1
Urease ($\mu mol\ NH_3\,g^{-1}\,h^{-1}$)	0.31 ± 0.03
Protease-BAA ($\mu mol\ NH_3\,g^{-1}\,h^{-1}$)	0.60 ± 0.04
Phosphatase (μmol PNP $g^{-1}\,h^{-1}$)	0.28 ± 0.02
β-Glucosidase (μmol PNP $g^{-1}\,h^{-1}$)	0.46 ± 0.01
Aggregate stability (%)	11.5 ± 0.4
Bulk density ($g\,cm^{-3}$)	1.10 ± 0.02

[a] Mean ± standard error ($N = 6$).

material characterised by: cellulose, 29%; hemicellulose, 23%; lignin, 5%, total C, 55% and total N, 1.7%. The strain of *A. niger* NB2 used in this study was selected because it produces citric acid on complex substrates (Vassilev et al., 1995). Rock phosphate (RP), i.e. flourapatite from Morocco with 12.8% P (1 mm mesh) was used in this study.

D. pentaphyllum is a leguminous low-growing shrub, widely distributed in the Mediterranean area. It is well adapted to water stress conditions and, therefore, frequently used in the revegetation of semiarid disturbed lands (Wills et al., 1989).

2.3. Mycorrhizal inoculation of seedlings

The mycorrhizal fungi used in the experiment, *Glomus intraradices* (Schenck & Smith) and *Glomus deserticola* (Trappe, Bloss. & Menge), were obtained from the collection of the experimental field station of Zaidín, Granada. Both mycorrhizal fungi were isolated from the experimental area.

AM fungal inoculum consisted of a mixture of rhizospheric soil from trap cultures (*Sorghum* sp.) containing spores, hyphae and mycorrhizal root fragments. Once germinated, seedlings were transplanted into the growth substrate, consisting of peat and cocopeat (1:1 (v/v)). The corresponding arbuscular mycorrhizal inoculum was applied at a rate of 5% (v/v). The same amount of an autoclaved mixture of the inocula was added to control plants, supplemented with a filtrate (<20 μm) of culture to provide the microbial populations accompanying the mycorrhizal fungi. Inoculated and non-inoculated seedlings were grown for 8 months under nursery conditions without any fertiliser treatment.

Table 2
Nomenclature used for non-mycorrhizal (−M) and mycorrhizal (+M) plants grown in the soil without (−A) and with (+A) addition of SB + *A. niger* + RP (SB: sugar beet, RP: rock phosphate)

		−A	+A
	−M	C	A
+M	*G. intraradices*	G1	AG1
+M	*G. deserticola*	G2	AG2

2.4. Experimental design and layout

The experiments were arranged in a randomised block design, with two factors and five replication blocks. The first factor was the addition or not of SB + *A.niger* + RP to the soil (soil amendment), and the second was the inoculation or not of *D. pentaphyllum* plants with two AM fungi (*G. intraradices* or *G. deserticola*) in the nursery. The treatments established are named specifically in Table 2. An experimental plot of 1000 m^2 was established on a flat surface. Planting holes 40 cm wide, 40 cm long and 30 cm deep were manually dug. In early November 2001, a mixture of SB and RP in the proportion of 1:0.05 (w/w) and a spore suspension of *A. niger* (1.2×10^7) were added to half of the holes (0–20 cm depth) at a rate of 3%. The seedlings were planted at least 1 m apart, with 3 m between blocks. At least 48 seedlings per replication block were planted (eight plants × six treatments in each block).

2.5. Sampling procedures

Eighteen months after planting soil samples were collected ($N = 30$). Each sample consisted of eight bulked subsamples (200 cm^3 soil cores), collected randomly at 0–20 cm in the rhizospheres of eight individual plants. The sampling was carried out in early April (before the dry season) when the highest microbial

activity would be expected (Lax et al., 1997). Five plants of each treatment (one per block) were harvested every 6 months after planting.

2.6. Plant analyses

Basal stem diameters and heights of plants were measured, fresh and dry (105 °C, 5 h) mass of shoots and roots were recorded. Plant tissues were ground prior to chemical analysis. Foliar concentrations of N, P and K were determined after digestion in nitric-perchloric acid (5:3) for 6 h (Plank, 1992). Foliar P was determined by colorimetry (Murphy and Riley, 1962), foliar N was colorimetrically measured after Kjeldhal digestion (Page et al., 1982) and foliar K was estimated by flame photometry (Schollemberger and Simon, 1954). The percentage of root length colonised by AM fungi was calculated by the gridline intersect method (Giovannetti and Mosse, 1980) after staining with trypan blue (Phillips and Hayman, 1970).

2.7. Soil physical-chemical, chemical, biological and biochemical analyses

Soil pH and electrical conductivity were measured in a 1:5 (w/v) aqueous solution. In soil aqueous extracts, water soluble carbon was determined by wet oxidation with $K_2Cr_2O_7$ and measurement of the absorbance at 590 nm (Sims and Haby, 1971). Water soluble carbohydrates and total carbohydrates were determined by the method of Brink et al. (1960). Total N, available P, extracted with 0.5 M $NaHCO_3$, and extractable (with NH_4OAC) K were determined following the methods described above for plant tissues.

Microbial biomass C was determined using a fumigation-extraction method (Vance et al., 1987). Ten gram of soil at 60% of field capacity were fumigated in a 125 ml Erlenmeyer flask with purified $CHCl_3$ for 48 h. After removal of residual $CHCl_3$, 40 ml of 0.5 M K_2SO_4 solution was added and the sample shaken for 1 h before filtration. The K_2SO_4-extracted C was measured as indicated for water soluble carbon and microbial biomass C was calculated as the difference between fumigated and non-fumigated samples.

Dehydrogenase activity was determined according to García et al. (1997). For this, 1 g of soil at 60% of its field capacity was exposed to 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 the dark. The INTF (iodo-nitrotetrazolium formazan) formed was extracted with 10 ml of methanol by shaking vigorously for 1 min and filtering through Whatman No. 5 filter paper. INTF was measured spectrophotometrically at 490 nm.

Urease and *N*-α-benzoyl-L-argininamide (BAA) hydrolyzing protease activities were determined in 0.1 M phosphate buffer at pH 7; 1 M urea and 0.03 M BAA were used as substrates, respectively. Two millilitre of buffer and 0.5 ml of substrate were added to 0.5 g of sample, which was incubated at 30 °C (for urease) or 39 °C (for protease) for 90 min. Both activities were determined as the NH_4^+ released in the hydrolysis reaction (Nannipieri et al., 1980).

Acid phosphatase activity was determined using *p*-nitrophenyl phosphate disodium (PNPP, 0.115 M) as substrate. Two millilitre of 0.5 M sodium acetate buffer at pH 5.5 using acetic acid (Naseby and Lynch, 1997) and 0.5 ml of substrate were added to 0.5 g of soil and incubated at 37 °C for 90 min. The reaction was stopped by cooling at 2 °C for 15 min. Then, 0.5 ml of 0.5 M $CaCl_2$ and 2 ml of 0.5 M NaOH were added, and the mixture was centrifuged at 4000 rpm for 5 min. The *p*-nitrophenol (PNP) formed was determined by spectrophotometry at 398 nm (Tabatabai and Bremner, 1969). Controls were made in the same way, although the substrate was added before the $CaCl_2$ and NaOH.

β-glucosidase was determined using *p*-nitrophenyl-β-D-glucopyranoside (PNG, 0.05 M; Masciandaro et al., 1994) as substrate. This assay is based on the release and detection of PNP. Two millilitre of 0.1 M maleate buffer pH 6.5 and 0.5 ml of substrate was added to 0.5 g of sample and incubated at 37 °C for 90 min. The reaction was stopped with tris-hydroxymethyl aminomethane (THAM) according to Tabatabai (1982). The amount of PNP was determined by spectrophotometry at 398 nm (Tabatabai and Bremner, 1969).

2.8. Physical analysis

The percentage of stable aggregates was determined by the method described by Lax et al. (1994). A 4 g aliquot of sieved (0.2–4 mm) soil was placed on a small 0.250 mm sieve and wetted by spray. After 15 min, the soil was subjected to an artificial rainfall of 150 ml

with energy of 270 J m^{-2}. The remaining soil on the sieve was placed in a previously weighed capsule (T), dried at 105 °C and weighed ($P1$). Then, the soil was soaked in distilled water and, after 2 h, passed through the same 0.250 mm sieve with the assistance of a small stick to break the remaining aggregates. The residue remaining on the sieve, which was made up of plant debris and sand particles, was dried at 105 °C and weighed ($P2$). The percentage of stable aggregates with regard to the total aggregates was calculated by

$$\frac{(P1 - P2) \times 100}{4 - P2 + T}$$

Soil bulk density was determined as described by Barahona and Santos (1981). Oven-dried soil clods were coated with paraffin and weighed in water (P_w) and in air (P_a). Bulk density was calculated by $P_a/(P_a - P_w)$.

2.9. Statistical analysis

Aggregate stability and percentage colonisation were arcsin-transformed, and the other parameters were log-transformed to compensate for variance heterogeneity before analysis of variance. SB + *A. niger* + RP addition, mycorrhizal inoculation and their interactions effects on measured variables were tested by a two-way analysis of variance and comparisons among means were made using the Least Significant Difference (LSD) test, calculated at $P < 0.05$. Statistical procedures were carried out with the software package SPSS 10.0 for Windows.

3. Results

3.1. Physical-chemical, chemical and physical parameters

Eighteen months after planting, soils from the (SB + *A. niger* + RP) amendment and mycorrhizal inoculation treatments had significantly lower pH than the control soil, the greatest differences being due to the amendment treatments (Table 3). Only electrical conductivity from amended soil was higher than that of the control soil (Tables 3 and 4).

Available P, extractable K, total N and aggregate stability were higher in the amended soil than in the soils from the mycorrhizal inoculation treatments and control soil (Tables 3 and 4). All assayed treatments had lower bulk density of the *D. pentaphyllum* rhizosphere soil than control soil, particularly the amendment treatments. There were no significant differences between the mycorrhizal inoculation treatments with respect to the soil bulk density (Table 4).

3.2. Biological and biochemical parameters

Rhizosphere soil from the amendment and mycorrhizal inoculation treatments had significantly higher total carbohydrate and water soluble C values and enzyme activities (dehydrogenase, urease, acid phosphatase and β-glucosidase) than the control soil (Tables 4 and 5). All the microbiological and biochemical parameters were higher in amended soil than in the soils from the mycorrhizal inoculation treatments. Rhizosphere soil of *G. deserticola*-inoculated plants

Table 3
Physical-chemical, chemical and physical properties of rhizosphere soil of *D. pentaphyllum* in response to mycorrhizal inoculation treatments and SB + *A. niger* + RP addition 18 months after planting ($n = 5$)[a]

	pH (H_2O)	EC (1:5, μS cm^{-1})	Total N (g kg^{-1})	Available P (μg g^{-1})	Extractable K (μg g^{-1})	Aggregate stability (%)	Bulk density (g cm^{-3})
C	8.43 ± 0.00	253 ± 6	0.87 ± 0.02	1.7 ± 0.1	117 ± 2	20.5 ± 1.0	1.17 ± 0.02
A	8.06 ± 0.01	400 ± 9	1.67 ± 0.06	4.5 ± 0.4	156 ± 4	52.7 ± 2.6	1.01 ± 0.01
G1	8.27 ± 0.02	292 ± 5	1.17 ± 0.04	1.8 ± 0.3	111 ± 4	20.7 ± 1.0	1.07 ± 0.01
AG1	8.12 ± 0.01	377 ± 9	1.29 ± 0.01	3.7 ± 0.4	150 ± 3	51.5 ± 0.7	1.02 ± 0.01
G2	8.22 ± 0.04	298 ± 4	1.06 ± 0.01	2.0 ± 0.1	118 ± 4	25.9 ± 0.4	1.06 ± 0.01
AG2	8.05 ± 0.00	359 ± 9	1.49 ± 0.01	4.6 ± 0.3	165 ± 4	49.6 ± 2.6	1.01 ± 0.00

C: control; A: SB + *A. niger* + RP addition; G1: inoculation with *G. intraradices*; AG1: SB + *A. niger* + RP addition and inoculation with *G. intraradices*; G2: inoculation with *G. deserticola*; AG2: SB + *A. niger* + RP addition and inoculation with *G. deserticola*; EC: electrical conductivity.

[a] Means ± standard errors.

Table 4
Results of two factor ANOVA (*P*-values) for all parameters studied in the rhizosphere soil of *D. pentaphyllum* seedlings 18 months after planting

	Amendment (A)	Mycorrhiza (M)	Interaction (A × M)	Comparison between means		
				C vs. G1	C vs. G2	G2 vs. G1
pH	<0.001	0.002	0.001	0.090	0.001	0.037
Electrical conductivity	<0.001	0.408	0.001	0.192	0.386	0.650
Total N	<0.001	0.424	<0.001	0.565	0.196	0.462
Available P	<0.001	0.367	0.910	0.388	0.585	0.165
Extractable K	<0.001	0.183	0.799	0.239	0.506	0.072
Aggregate stability	<0.001	0.848	0.281	0.809	0.745	0.572
Bulk density	<0.001	0.010	0.002	0.016	0.004	0.580
Total carbohydrates	<0.001	<0.001	<0.001	0.519	<0.001	0.001
Water soluble C	<0.001	<0.001	<0.001	<0.001	<0.001	0.102
Water soluble carbohydrates	<0.001	0.132	0.033	0.126	0.058	0.692
Biomass C	<0.001	0.001	0.006	0.001	0.966	0.001
Dehydrogenase	<0.001	0.006	<0.001	0.640	0.003	0.009
Urease	<0.001	<0.001	0.054	0.607	<0.001	<0.001
Protease	<0.001	0.999	0.100	0.997	0.967	0.970
Phosphatase	<0.001	<0.001	<0.001	<0.001	<0.001	0.400
β-Glucosidase	<0.001	0.002	0.002	0.089	<0.001	0.028

C: control with and without SB + *A. niger* + RP addition; G1: inoculation with *G. intraradices* with and without SB + *A. niger* + RP addition; G2: inoculation with *G. deserticola* with and without SB + *A. niger* + RP addition.

had higher total carbohydrate and biomass C values and dehydrogenase, urease and β-glucosidase activities than rhizosphere soil of *G. intraradices*-inoculated plants (Table 4). In general, the combined treatments, involving mycorrhizal inoculation of seedlings with *G. intraradices* or *G. deserticola* and addition of SB + *A. niger* + RP to soil, changed the microbiological and biochemical parameters of the rhizosphere soil equally to or to a lesser extent than each treatment applied separately.

3.3. Foliar nutrients, growth parameters of *D. pentaphyllum* and mycorrhizal infection

At the time of planting, foliar N and K contents of non-inoculated plants were greater than for plants inoculated with *G. intraradices* and equal to those of plants inoculated with *G. deserticola* (Table 6). The plants inoculated with *G. deserticola* had the highest contents of foliar P. At the time of planting, the inoculated plants exhibited averages of 70 and 63% of roots colonised by *G. intraradices* and *G. deserticola*, respectively (Table 6). The roots of the non-inoculated plants were not colonised by mycorrhizal fungi.

Eighteen months after planting, the N, P and K contents in shoot tissues of plants inoculated with both AM fungi were significantly higher than those in control plants, the N and K contents being significantly higher in shoot tissues of plants inoculated with *G. deserticola* than for plants inoculated with *G. intraradices* and non-inoculated plants (Tables 6 and 7). Only the foliar N content of plants grown in the amended soil was higher than that of the control plants (Table 7). The highest levels of mycorrhizal colonisation were recorded in the seedlings inoculated with *G. deserticola*, followed by the plants inoculated with *G. intraradices* and non-inoculated plants, all grown in the non-amended soil (Table 6). Application of the amendment had a negative effect on the levels of mycorrhizal colonisation of plants inoculated with *G. intraradices* or *G. deserticola*.

Eighteen months after planting, the percentages of plant survival were 69% for non-inoculated plants and 80% for inoculated plants, although there were no statistically significant differences between treatments (LSD test). At the time of planting, shoot and root dry weight of non-inoculated plants were slightly greater than for inoculated plants, although differences in growth were not statistically significant (Fig. 1). Six months after planting, the amendment and mycorrhizal inoculation treatments had stimulated the production of shoot biomass, although

Table 5
Carbon fractions, enzyme activities of rhizosphere soil of *D. pentaphyllum* in response to mycorrhizal inoculation treatments and SB + *A. niger* + RP addition 18 months after planting ($n = 5$)[a]

	Total CH ($\mu g\,g^{-1}$)	Water soluble C ($\mu g\,g^{-1}$)	Water soluble CH ($\mu g\,g^{-1}$)	Biomass C ($\mu g\,g^{-1}$)	Dhase (μg INTF g^{-1} soil)	Urease (μmol NH_3 $g^{-1}\,h^{-1}$)	Protease (μmol NH_3 $g^{-1}\,h^{-1}$)	Phosphatase (μmol PNP $g^{-1}\,h^{-1}$)	β-glucosidase (μmol PNP $g^{-1}\,h^{-1}$)
C	389 ± 22	93 ± 1	5 ± 1	642 ± 15	66.4 ± 2.6	0.39 ± 0.02	0.33 ± 0.03	0.30 ± 0.02	0.41 ± 0.02
A	979 ± 37	371 ± 16	36 ± 1	860 ± 33	131.9 ± 2.0	0.98 ± 0.07	0.82 ± 0.06	0.77 ± 0.01	1.44 ± 0.08
G1	616 ± 40	136 ± 2	8 ± 0	626 ± 21	82.8 ± 2.2	0.47 ± 0.02	0.41 ± 0.02	0.64 ± 0.01	0.68 ± 0.05
AG1	687 ± 26	376 ± 12	35 ± 4	629 ± 16	109.2 ± 1.9	0.71 ± 0.02	0.65 ± 0.05	0.69 ± 0.02	1.18 ± 0.05
G2	744 ± 20	181 ± 6	9 ± 1	651 ± 19	94.9 ± 2.3	0.73 ± 0.08	0.36 ± 0.00	0.50 ± 0.01	0.74 ± 0.05
AG2	981 ± 49	326 ± 4	34 ± 2	848 ± 7	118.3 ± 2.1	1.35 ± 0.03	0.72 ± 0.04	0.96 ± 0.02	1.65 ± 0.07

C: control; A: SB + *A. niger* + RP addition; G1: inoculation with *G. intraradices*; AG1: SB + *A. niger* + RP addition and inoculation with *G. intraradices*; G2: inoculation with *G. deserticola*; AG2: SB + *A. niger* + RP addition and inoculation with *G. deserticola*. CH: carbohydrates; Dhase: dehydrogenase.

[a] Means ± standard errors.

Table 6
Foliar nutrients and root infection of *D. pentaphyllum* seedlings in response to mycorrhizal inoculation treatments and SB + *A. niger* + RP addition at the time of planting and 18 months after planting ($n = 5$)[a]

	Months	
	0	18
Nitrogen (mg per plant)		
C	3.2 ± 0.1	19.5 ± 0.4
A	3.2 ± 0.1	24.4 ± 0.7
G1	2.4 ± 0.1	48.3 ± 1.8
AG1	2.4 ± 0.1	43.6 ± 0.5
G2	3.3 ± 0.1	68.4 ± 0.6
AG2	3.3 ± 0.1	45.1 ± 1.5
Phosphorus (mg per plant)		
C	0.87 ± 0.04	0.55 ± 0.01
A	0.87 ± 0.04	0.71 ± 0.02
G1	0.81 ± 0.02	1.79 ± 0.15
AG1	0.81 ± 0.02	1.68 ± 0.12
G2	0.97 ± 0.04	1.85 ± 0.11
AG2	0.97 ± 0.04	1.53 ± 0.12
Potassium (mg per plant)		
C	6.8 ± 0.1	13.0 ± 0.6
A	6.8 ± 0.1	18.3 ± 0.2
G1	5.1 ± 0.1	30.2 ± 0.7
AG1	5.1 ± 0.1	23.3 ± 0.9
G2	6.2 ± 0.2	41.1 ± 2.0
AG2	6.2 ± 0.2	30.2 ± 0.8
Colonisation (%)		
C	0.0 ± 0.0	20.3 ± 2.8
A	0.0 ± 0.0	19.1 ± 2.5
G1	70.3 ± 1.4	49.3 ± 5.1
AG1	70.3 ± 1.4	36.9 ± 4.2
G2	63.4 ± 1.8	60.7 ± 9.5
AG2	63.4 ± 1.8	35.7 ± 5.9

C: control; A: SB + *A. niger* + RP addition; G1: inoculation with *G. intraradices*; AG1: SB + *A. niger* + RP addition and inoculation with *G. intraradices*; G2: inoculation with *G. deserticola*; AG2: SB + *A. niger* + RP addition and inoculation with *G. deserticola*.

[a] Means ± standard errors.

the amendment × mycorrhiza interaction produced even higher values (on average, about 115% greater with respect to the control plants). The differences in seedling growth produced by the amendment, with respect to control plants, tended to decrease with time. Inoculation with *G. deserticola* had the strongest effect on the growth of seedlings from 12 months after planting onwards, producing about 219% more shoot dry matter than the control plants at the end of the growth period. Eighteen months after planting, in the combined treatment there was no additive effect, for the addition of the amendment together with either of the mycorrhizal inoculation treatments, on the growth of *D. pentaphyllum* seedlings.

4. Discussion

4.1. Effectiveness of soil amendment

The results of this study have demonstrated the viability of applying SB, rock phosphate and *A. niger* directly into soil in order to improve the growth and (NPK) nutrient status of the target shrub legume species, *D. pentaphyllum*, selected for revegetation of a semiarid Mediterranean area. This effectiveness relies on the improvement of soil fertility. In this regard, the addition of SB + *A. niger* + RP increased the total N, available P and extractable K contents of the soil, the greatest increase being observed for available P. These results confirm the effectiveness of *A. niger*, inoculated directly into soil, for solubilising rock phosphate, and support the use of such an approach to improve P bioavailability in P-deficient soils. Such microorganisms are able to excrete organic acids, which increase the concentration of phosphorus in solution by mechanisms involving chelation and exchange reactions (Vassilev et al., 1996). In fact, we have also recorded that the soil amendment caused a decrease in soil pH.

The benefits of the amendment were also due to improvement of the physical characteristics of the soil, such as aggregate stability and bulk density, which in turn favours the establishment and viability of a stable plant cover (Cox et al., 2001; Caravaca et al., 2002). Roldán et al. (1996) found that the restoration of soil structure may depend on the amount and nature of the organic matter added. The biological transformations that the SB underwent under soil conditions increased the quantity of chemical aggregate-stabilising agents, such as polysaccharides and water-soluble organic matter. Likewise, these C fractions can be used as carbon and energy sources by soil microorganisms, such as bacteria and fungi, which are principally responsible for the formation of aggregates larger than 0.2 mm (Roldán et al., 1994; Caravaca et al., 2002).

Table 7
Results of two factor ANOVA (*P*-values) for growth parameters, foliar nutrients and mycorrhizal colonisation of *D. pentaphyllum* seedlings 18 months after planting

	Amendment (A)	Mycorrhiza (M)	Interaction (A × M)	Comparison between means		
				C vs. G1	C vs. G2	G2 vs. G1
Shoot	0.358	<0.001	0.128	0.001	<0.001	0.026
Root	0.624	<0.001	0.030	0.058	<0.001	<0.001
Foliar N	0.004	<0.001	<0.001	<0.001	<0.001	<0.001
Foliar P	0.988	<0.001	0.064	<0.001	<0.001	0.851
Foliar K	0.107	<0.001	<0.001	<0.001	<0.001	<0.001
Mycorrhizal roots	<0.001	<0.001	0.005	<0.001	<0.001	0.107

C: control with and without SB + *A. niger* + RP addition; G1: inoculation with *G. intraradices* with and without SB + *A. niger* + RP addition; G2: inoculation with *G. deserticola* with and without SB + *A. niger* + RP addition.

It is worth noting that this type of residue is, in the short-term, more effective than other residues such as sewage sludge and urban waste widely used for improving soil structure under semiarid Mediterranean conditions (Roldán et al., 1996).

4.2. Effectiveness of the mycorrhizal inoculation treatments

The role of AM in the stimulation of growth and nutrient uptake of many host plants is well

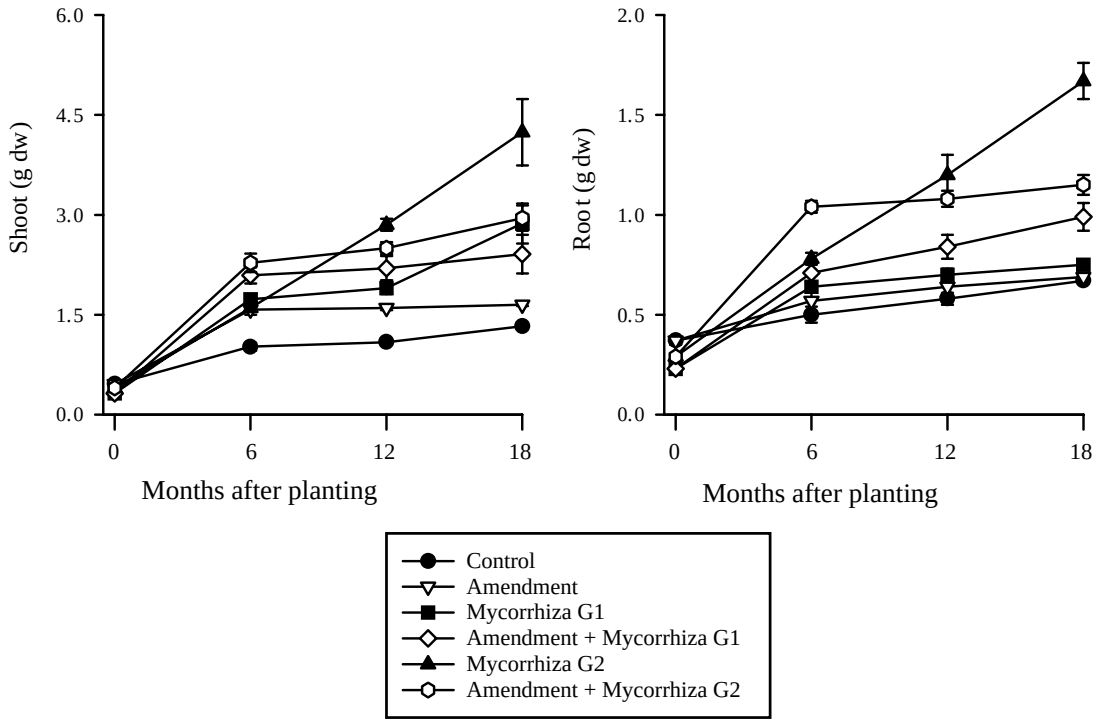

Fig. 1. Changes in shoot and root dry weights of *D. pentaphyllum* seedlings in response to mycorrhizal inoculation treatments (G1: inoculation with *G. intraradices*; G2: inoculation with *G. deserticola*) and SB + *A. niger* + RP addition during an 18 months growth period ($n = 5$). Bars represent standard errors.

documented (Smith and Read, 1997; Jeffries et al., 2003). In this study, the inoculation of seedlings with *G. intraradices* or *G. deserticola* stimulated significantly the production of shoot biomass, to a greater extent than the addition of the SB + *A. niger* + RP alone to soil. The mycorrhizal inoculation treatments showed different levels of effectiveness with respect to improving the performance of *D. pentaphyllum* seedlings. *G. deserticola* was the more effective for increasing plant growth. This increase was significantly above that of *G. intraradices*-colonised seedlings from 12 months after planting onwards, which is the most critical period for revegetation, particularly in Mediterranean semiarid areas. Recently, we have reported the effectiveness of *G. deserticola* with respect to increasing the root nitrate reductase activity and N uptake in shoot tissues of *D. pentaphyllum* seedlings under water-stressed conditions (Caravaca et al., 2003a). Ruíz-Lozano et al. (1995) also found that *G. deserticola* was the most effective fungus, compared to six other *Glomus* species, for enhancement of the water-deficit tolerance of lettuce plants. Our results support the hypothesis that there are differences in the symbiotic physiology of different host-endophyte associations (Ruíz-Lozano et al., 1995).

Total plant nutrient content can be taken as a representative indicator of mycorrhizal effectiveness, because it takes into account the well-balanced effects of nutrient acquisition and biomass production (Jeffries et al., 2003). Both mycorrhizal inoculation treatments appeared effective in improving nutrient content. The highest contents of N and K in shoot tissue were observed in the seedlings inoculated with *G. deserticola*, which might explain why the growth of *D. pentaphyllum* was greatest in this treatment. The fact that the foliar P contents of inoculated plants were higher than those of non-inoculated plants grown in the amended soil reaffirms the key role of mycorrhizas in sustaining the plant cover in P-deficient soils, as well as showing the necessity of including mycorrhizal inoculation in revegetation programmes, to guarantee plant performance.

The extent of mycorrhizal infection is of importance when studying the influence of AM fungi on the host plant. High infection rates may not be a prerequisite for growth responses in all plants inoculated with AM fungi. Thus, Requena et al. (1996) observed that native fungi were ineffective for promoting growth of *Anthyllis cytisoides*, despite colonising a relatively large percentage of the roots. In the present revegetation experiment, the effect of AM inoculation on shoot biomass was related positively to the colonisation level of each AM fungus. At the end of the growth period, mycorrhizal inoculation with *G. deserticola* had increased the shoot biomass of *D. pentaphyllum* to a greater extent (about 219% with respect to control plants) than with *G. intraradices* (about 116% with respect to control plants).

4.3. Effectiveness of the combination of the soil amendment and mycorrhizal inoculation treatments

The revegetation experiment with *D. pentaphyllum* showed that the combination of soil amendment with each mycorrhizal inoculation treatment can stimulate considerably the shoot biomass in semiarid conditions. The combined treatment increased the growth of the seedlings to a lesser extent than either mycorrhizal inoculation treatment alone but it was more effective than soil amendment alone. This result agrees with the widely accepted idea that mycorrhizas present little advantage to seedlings grown in amended soils (Yanai et al., 1995). Perhaps, the release of lignin, as a consequence of mineralisation of SB, could have had adverse effects on the functional activity of the ectomycorrhizal fungi (Rodríguez et al., 1999).

In conclusion, mycorrhizal inoculation of seedlings with *G. deserticola* was the most effective treatment for stimulating the growth of *D. pentaphyllum* plants in a semiarid Mediterranean area. The addition of sugar beet, rock phosphate and *A. niger* directly into the soil was the most effective treatment for improving the physical, chemical, microbiological and biochemical quality of the rhizosphere soil of the seedlings, with a subsequently enhanced plant growth. Likewise, the P demand of plants can be satisfied by phosphate-solubilising microorganisms, such as *A. niger*, inoculated directly into soil.

Acknowledgements

This research was supported by the EC + CICYT co-financed FEDER programme (REN 2000-1724-CO3-01).

References

Augé, R., 2001. Water relations, drought and vesicular-arbuscular mycorrhizal symbiosis. Mycorrhiza 11, 3–42.

Barahona, E., Santos, F., 1981. Un nuevo método para la determinación de densidades aparentes y del coeficiente de extensividad lineal (COLE) por método de parafina. Anal. Edaf. Agrobiol. 40, 721–725.

Brink, R.H., Dubar, P., Lynch, D.L., 1960. Measurement of carbohydrates in soil hidrolysates with anthrone. Soil Sci. 89, 157–166.

Caravaca, F., Barea, J.M., Roldán, A., 2002. Synergistic influence of an arbuscular mycorrhizal fungus and organic amendment on *Pistacia lentiscus* L. seedlings afforested in a degraded semiarid soil. Soil Biol. Biochem. 34, 1139–1145.

Caravaca, F., Alguacil, M.M., Díaz, G., Roldán, A., 2003a. Use of nitrate reductase activity for assessing the effectiveness of mycorrhizal symbiosis in *Dorycnium pentaphyllum* under induced water deficit. Commun. Soil Sci. Plant Anal. 34, 2291–2302.

Caravaca, F., Alguacil, M.M., Figueroa, D., Barea, J.M., Roldán, A., 2003b. Re-establishment of *Retama sphaerocarpa* as a target species for reclamation of soil physical and biological properties in a semiarid Mediterranean land. For. Ecol. Manage. 182, 49–58.

Caravaca, F., Barea, J.M., Palenzuela, J., Figueroa, D., Alguacil, M.M., Roldán, A., 2003c. Establishment of shrub species in a degraded semiarid site after inoculation with native or allochthonous arbuscular mycorrhizal fungi. Appl. Soil Ecol. 22, 103–111.

Cox, D., Bezdicek, D., Fauci, M., 2001. Effects of compost, coal ash, and straw amendments on restoring the quality of eroded Palouse soil. Biol. Fert. Soils 33, 365–372.

Francis, D.F., Thornes, J.B., 1990. Matorral: erosion and reclamation. In: Albaladejo, J., Stocking, M.A., Díaz, E. (Eds.), Soil Degradation and Rehabilitation in Mediterranean Environmental Conditions. CSIC, Murcia, Spain, pp. 87–115.

García, C., Hernández, M.T., Costa, F., 1997. Potential use of dehydrogenase activity as an index of microbial activity in degraded soils. Commun. Soil Sci. Plant Anal. 28, 123–134.

Giovannetti, M., Mosse, B., 1980. An evaluation of techniques for measuring vesicular-arbuscular mycorrhizal infection in roots. New Phytol. 84, 489–499.

Herrera, M.A., Salamanca, C.P., Barea, J.M., 1993. Inocualtion of woody legumes with selected arbuscular mycorrhizal fungi and rhizobia to recover desertified Mediterranean ecosystems. Appl. Environ. Microb. 59, 129–133.

Jeffries, P., Gianinazzi, S., Perotto, S., Turnau, K., Barea, J.M., 2003. The contribution of arbuscular mycorrhizal fungi in sustainable maintenance of plant health and soil fertility. Biol. Fert. Soils 37, 1–16.

Lax, A., Díaz, E., Castillo, V., Albaladejo, J., 1994. Reclamation of physical and chemical properties of a salinized soil by organic amendment. Arid Soil Res. Rehab. 8, 9–17.

Lax, A., Roldán, A., Caravaca, F., García-Orenes, F., 1997. Relationships between aggregate improvement, microbiological activity and organo-mineral complex formation in soils from semiarid areas. In: Pandalai, S.G. (Ed.), Recent Research Developments in Soil Biology and Biochemistry. Research Signpost, Trivandrum, India, pp. 77–92.

Masciandaro, G., Ceccanti, B., García, C., 1994. Anaerobic digestion of straw and piggery wastewater: Part II. Optimization of the process. Agrochimica 3, 195–203.

Murphy, J., Riley, J.P., 1962. A modified single solution method for determination of phosphate in natural waters. Anal. Chim. Acta 27, 31–36.

Nannipieri, P., Ceccanti, B., Cervelli, S., Matarese, E., 1980. Extraction of phosphatase, urease, protease, organic carbon and nitrogen from soil. Soil Sci. Soc. Am. J. 44, 1011–1016.

Naseby, D.C., Lynch, J.M., 1997. Rhizosphere soil enzymes as indicators of perturbation caused by a genetically modified strain of *Pseudomonas fluorescens* on wheat seed. Soil Biol. Biochem. 29, 1353–1362.

Page, A.L., Miller, R.H., Keeney, D.R., 1982. Methods of soil analysis. Part II. American Society of Agronomy Madison.

Palenzuela, J., Azcón-Aguilar, C., Figueroa, D., Caravaca, F., Roldán, A., Barea, J.M., 2002. Interactive effects of mycorrhizal inoculation of representative shrub species from Mediterranean ecosystems and composted residue application on transplant performance and mycorrhizal developments in a desertified soil. Biol. Fert. Soils 36, 170–175.

Phillips, J.M., Hayman, D.S., 1970. Improved procedures for clearing roots and staining parasitic and vesicular-arbuscular mycorrhizal fungi for rapid assessment of infection. Trans. Br. Mycol. Soc. 55, 158–161.

Plank, C.O., 1992. Reference Plant Analysis Procedures for the Southern Region of the United States. Southern Co-operative Series Bulletin No. 368, pp. 71.

Pozo, M.J., Azcón-Aguilar, C., Dumas-Gaudot, E., Barea, J.M., 1999. β-1,3-glucanase activities in tomato roots inoculated with arbuscular mycorrhizal fungi and/or *Phytophthora parasitica*: time course analysis and possible involvement in bioprotection. Plant Sci. 141, 149–157.

Requena, N., Jeffries, P., Barea, J.M., 1996. Assessment of natural mycorrhizal potential in a desertified semi-arid ecosystem. Appl. Environ. Microb. 62, 842–847.

Requena, N., Pérez-Solis, E., Azcón-Aguilar, C., Jeffries, P., Barea, J.M., 2001. Management of indigenous plant-microbe symbioses aids restoration of desertified ecosystems. Appl. Environ. Microb. 67, 495–498.

Rodríguez, R., Vassilev, N., Azcón, R., 1999. Increases in growth and nutrient uptake of alfalfa grown in soil amended with microbially-treated sugar beet waste. Appl. Soil Ecol. 11, 9–15.

Roldán, A., Albaladejo, J., Thornes, J.B., 1996. Aggregate stability changes in a semiarid soil after treatment with different organic amendments. Arid Soil Res. Rehab. 10, 139–148.

Roldán, A., García-Orenes, F., Lax, A., 1994. An incubation experiment to determine factors involving aggregation changes in an arid soil receiving urban refuse. Soil Biol. Biochem. 26, 1699–1707.

Ruíz-Lozano, J.M., Azcón, R., Gómez, M., 1995. Effects of arbuscular-mycorrhizal *Glomus* species on drought tolerance:

physiological and nutritional plant responses. Appl. Environ. Microb. 61, 456–460.
Schollemberger, C.J., Simon, R.H., 1954. Determination of exchange capacity and exchangeable bases in soils. Soil Sci. 59, 13–24.
Sims, J., Haby, V., 1971. Simplified colorimetric determination of soil organic matter. Soil Sci. 112, 137–141.
Smith, S.E., Read, D.J., 1997. Mycorrhizal Symbiosis. Academic Press, San Diego, pp. 605.
Soil Survey Staff, 1999. Soil Taxonomy: A Basic System of Soil Classification for Making and Interpreting Soil Surveys. USDA Natural Resources Conservation Service. Agric. Hdbk. 436. US Government Printing Office Washington DC.
Tabatabai, M.A., 1982. Soil enzymes. In: Page, A.L., Miller, E.M., Keeney, D.R. (Eds.), Methods of Soil Analysis. Part II. ASA and SSSA Madison Wisconsin, pp. 501–538.
Tabatabai, M.A., Bremner, J.M., 1969. Use of *p*-nitrophenol phosphate in assay of soil phosphatase activity. Soil Biol. Biochem. 1, 301–307.
Toro, M., Azcón, R., Barea, J.M., 1997. Improvement of arbuscular mycorrhiza development by inoculaltion of soil phosphate-solubilizing rhizobacteria to improve rock phosphate bioavailability (^{32}P) and nutrient cycling. Appl. Environ. Microb. 63, 4408–4412.
Vance, E.D., Brookes, P.C., Jenkinson, D., 1987. An extraction method for measuring microbial biomass carbon. Soil Biol. Biochem. 19, 703–707.
Vassilev, N., Baca, M.T., Vassileva, M., Franco, I., Azcón, R., 1995. Rock phosphate solubilization by *Aspergillus niger* grown on sugar-beet waste medium. Appl. Microbiol. Biot. 44, 546–549.
Vassilev, N., Franco, I., Vassileva, M., Azcón, R., 1996. Improved plant growth with rock phosphate solubilized by *Aspergillus niger* grown on sugar beet waste. Bioresource Technol. 55, 237–241.
Vassilev, N., Baca, M.T., Vassileva, M., Franco, I., De Nobili, M., 1998. Mineralization of three agro-industrial wastes by an acid-producing strain of *Aspergillus niger*. In: De Bertoldi, M., Sequi, P., Lenunes, B., Papi, T. (Eds.), The Science of Composting. Chapman and Hall London, pp. 1375–1379.
Wills, B.J., Begg, J.S.C., Foote, A.G., 1989. *Dorycnium* species—Two new legumes with potential for dryland pasture rejuvenation and resource conservation in New Zealand. Proc. New Zealand Grassland Assoc. 50, 169–174.
Yanai, R.D., Fahey, T.J., Miller, S.L., 1995. Efficiency of nutrient acquisition by fine roots and mycorrhizae. In: Smith, W.K., Hinckley, T.M. (Eds.), Resource Physiology of Conifers: Acquisition, Allocation and Utilization. Academic Press London, pp. 75–103.