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Advantages of inoculation with immobilized rhizobacteria versus amendment with olive-mill waste in the afforestation of a semiarid area with *Pinus halepensis* Mill

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ABSTRACT

We performed a field assay to assess the influence of the inoculation with a mixture of two immobilized strains of rhizobacteria (*Azospirillum brasilense* and *Pantoea dispersa*) and the addition of organic olive residue (alperujo) on the growth of *Pinus halepensis* Mill. and plant stress parameters, as well as on soil physico-chemical and microbiological properties. Twenty-eight months after planting, the microbial inoculation was the most-effective treatment regarding stimulation of seedling growth (by 48% with respect to the control) and nutrient uptake. The inoculated plants had the lowest proline accumulation, less oxidative damage to lipids and higher shoot water potential. The microbial inoculation and combined treatment enhanced enzyme activities, total carbohydrates and microbial biomass C and nutrients in the soil. The effectiveness of the microbial inoculant with respect to promotion of plant growth and the lower cost of implementation of this restoration biotechnology support its preferential use in re-afforestation tasks with *P. halepensis* in semiarid environments.

1. Introduction

In semiarid Mediterranean areas, the establishment of plant cover is difficult under the severe climate, characterized by low precipitation and frequent drought periods, particularly in soils with low microbial activity. Therefore, it is necessary to use technological restoration methods that can improve both soil quality and the ability of the seedlings to resist semiarid environmental conditions (Caravaca et al., 2005). The establishment of native plant species is a practice widely used for reclaiming degraded lands and constitutes the most-effective strategy in semiarid areas (Alguacil et al., 2003; Mengual et al., 2014; Schoebitz et al., 2014). *Pinus halepensis* Mill. is the prevailing tree species in semiarid areas of central-southern Spain, and it has been used in afforestation programs for degraded soils as it is a pioneer species and one of the few tree species that can thrive in these conditions (Maestre and Cortina, 2004).

Recent studies about the reclamation of semiarid soils have shown the beneficial effects of the application of organic amendments on soil quality, with increase in the proliferation and development of natural populations of soil microflora, since the organic residues can be used by soil microorganisms, as substrates and as carbon and energy sources (Medina and Azcón, 2010), and also improve soil properties. This effect could be extended to the enhancement of soil enzyme activities, which are key factors contributing to soil-borne microorganism activity and soil fertility (Caravaca et al., 2005). The use of organic waste materials not only increases the organic matter and fertility of soils, but also contributes to the palliation of environmental and economic inconveniences related with waste disposal (Rincón et al., 2006).

The Spanish olive-mill industry produces a huge amount of wastes that are difficult to reuse (four million tons per year). The main by-product is alperujo, which can be composted before its application to the soil in order to obtain a high-quality amendment, rich in K and partially-humified organic matter (Alburquerque et al., 2009, 2006). Such characteristics suggest that this residue could be useful for improving soil quality and in the development of afforestation programs in semiarid and degraded areas. The beneficial short-term effects of the addition of alperujo compost in horticultural and revegetation practices have been reported (Alburquerque et al., 2006; Schoebitz et al., 2014).

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However, their effect on the establishment of tree seedlings under semiarid field conditions remains unknown. Additionally, the application of olive-mill waste interacts positively with soil microorganisms (Schoebitz et al., 2014). Among the components of the soil microbiota, rhizobacteria are free-living bacteria, often labeled as plant growth-promoting rhizobacteria (PGPR), which can colonize the rhizosphere and improve root system establishment (Antoun and Kloepper, 2001). In this regard, PGPR have a potential role in the establishment of plant cover in arid environmental conditions (Puente et al., 2004), where they can promote plant growth and improve both water and nutrients uptake (Bashan et al., 2004). Nevertheless, colonization around plant roots following the direct inoculation of free PGPR cells into soil is not easy because this process is highly susceptible to environmental variations (Wu et al., 2012). This unpredictability of the success of PGPR inoculation of plants is due mainly to the quality of the inoculant formulations containing effective rhizobacterial strains, which determines the success or failure of plant growth promotion. Immobilization of microbial inoculants has been used to enhance their effectiveness, by providing nutrients and protection from desiccation (Kim et al., 2012). The success of microbial inoculants introduced into soil requires that an adequate number of bacteria reach suitable habitats where they can survive (Heijnen and Van-Veen, 1991). The aim of the immobilization of rhizobacteria is to protect the microorganisms (Schoebitz et al., 2013) and ensure a gradual and prolonged release into the soil (Wu et al., 2011). In spite of their potential viability, the use of immobilized bacteria has never been tested in the reafforestation with tree species like *P. halepensis* in Mediterranean semiarid conditions. The aim of this work was to study the medium-term effect of olive-mill waste compost and a microbial inoculum constituted by two immobilized strains of rhizobacteria on *P. halepensis* establishment under semiarid field conditions. We hypothesized that the revegetation treatments assayed would confer drought tolerance on the plants and/or enhance soil quality, leading to enhanced plant growth. In this respect, we measured soil physico-chemical, biochemical and microbiological variations as well as the changes in shoot nitrate reductase activity, proline accumulation, oxidative damage to lipids and plant water relations induced by these treatments.

2. Material and methods

2.1. Study site

The experimental area was situated in Vicente Blanes Ecological Park in Molina de Segura (Southeast Spain) (Lat. 38° 12′ N, Long. 1° 13′ W, Elev. 392 m). The climate is semiarid Mediterranean, with a mean annual temperature of 17.5 °C and no frost period. The annual rainfall is around 300 mm and the potential evapotranspiration reaches out to 1000 mm per year. The soil is a Typic Torriorthent (SSS, 2010), with low organic matter content and a silty loam texture (Schoebitz et al., 2014). The vegetation in the study site was dominated by the invasive *Piptatherum miliaceum* (L.) Cosson and some native shrubs of *Thymus vulgaris* L., *Pistacia lentiscus* L., *Cistus clusii* Dunal and *Rosmarinus officinalis* L.

2.2. Plants

The selected plant to carry out this study was *Pinus halepensis* Mill. This is a tree species, belonging to the family *Pinaceae*, which can reach a height of 10 m, widely distributed in the Mediterranean area. It is well adapted to water stress conditions and high temperatures and, therefore, it has been used in revegetation assays of degraded (Díaz and Roldán, 2000; Maestre and Cortina, 2004) and arid soils (Oliveras et al., 2003). Seedlings were grown in Muzalé nursery (Murcia, Spain) with peat as substrate for 1 year prior to experimental procedures. At planting, *P. halepensis* was 20 cm high, with a shoot dry weight of 2.77 g and root dry weight of 1.37 g.

2.3. Microbial inoculant and organic residue

The microbial inoculant was a mixture of two plant growth promoting rhizobacteria (PGPR) *Azospirillum brasilense* Tarrand, Krieg & Döbereiner, 1978 and *Pantoea dispersa* Gavini, Mergaert, Beij, Mielcarek, Izard, Kersters, De Ley 1989 immobilized on clay pellets, being the cells concentrations of both rhizobacteria 10^9 CFU g^{-1}. Bacteria of genus *Azospirillum* that fix nitrogen under microaerobic conditions have a positive effect on plant growth (Flores et al., 2010). *Azospirillum* has been shown to be more successful when it is co-inoculated with other microorganisms such as phosphate-solubilizing bacteria (Bashan et al., 2004). To this end, *A. brasilense* was co-applied with *P. dispersa* whose beneficial effect on plant development arises from its capacity to solubilize phosphorus compounds and help to control pathogenic organism (Son et al., 2006). This microbial inoculant was developed by Probelte, S.A., Murcia. These strains were deposited in the Spanish Type Culture Collection (CECT) with the numbers CECT-5801 (*P. dispersa*) and CECT-5802 (*A. brasilense*).

A composted olive-mill waste was used as organic amendment for soil. Fresh cow bedding was added to the olive-mill waste as bulking agent and composted by using a combination of the Rutgers system and mechanical turning. The analytical characteristics of the organic residue are described on Schoebitz et al., 2014.

2.4. Experimental design

A full-factorial design was established with two factors and five fold replications in a split plot design. The first factor was the inoculation or not of *P. halepensis* seedlings with microbial inoculant and the second was the addition or not of organic olive residue into the soil.

In early February 2011, the seedlings were transported to the experimental field, where planting holes 15×15 cm wide and 15 cm deep were dug manually. There, an amount of 30 g of microbial inoculant pellets was applied per plant. The same quantity of sterilized inoculant was applied to the non-inoculated plants. Olive residue was added at a rate of 2% by weight (186 g of organic olive residue per plant). Microbial inoculant and organic olive residue were manually mixed into 2 kg of soil in plastic bags an introduced in the plantation holes. The seedlings were planted at least 1 m apart between holes, with 3 m between treatment levels. At least 10 seedlings per treatment level were planted.

2.5. Sampling procedures

Twenty-eight months after planting, in early June 2013, samples were collected. Five plants per treatment including root systems and rhizosphere soil were harvested, and introduced in polyethylene bags for transport to the laboratory. Rhizosphere soil samples were separated into two subsamples before physico-chemical and biochemical analyses: one subsample sieved to <2 mm and other subsample sieved between 4 and 0.25 mm.

2.6. Plant analyses

The sampling day, before the harvest, leaf water potential was measured in two fully developed needles per tree of each replicate in a pressure chamber (model 3005, Soil Moisture Equipment Co., Santa Barbara, CA, USA) (Mellisho et al., 2012). Midday (12 h solar time) stem water potential was measured in a similar number and

type of needles as used for leaf water potential, enclosing needles in a small black plastic bag covered with aluminum foil for at least 2 h before measurements in the pressure chamber (Cruz et al., 2012). Leaf osmotic potentials were determined in the same needles used for leaf water potentials. Needles were frozen in liquid nitrogen and the osmotic potential was measured after thawing the samples and expressing sap, using a vapor pressure osmometer (Wescor 5600, Logan, USA). Leaf turgor potential was derived as the difference between osmotic and water potentials (Cruz et al., 2012).

Fresh and dry weights of shoot and root (70 °C, 48 h), basal stem diameter and plant height were recorded. The shoot P and K were determined by ICP/OES spectrometry (Thermo Elemental Co. Iris Intrepid II XDL). Shoot N was determined by dry combustion using a LECO Tru-Spec CN analyzer (Leco Corp., St. Joseph, MI, USA).

Nitrate reductase activity was assayed in vivo by measuring NO_2^- production in tissue that had been vacuum-infiltrated with buffered NO_3^- solutions (Downs et al., 1993). The needles of the trees were collected in the morning at 11:00 h solar time. Needles of *P. halepensis* were cut into 5 mm sections. Approximately 300 mg of needle punches were placed in tubes containing 2 mL of an incubation medium consisting of 0.05 M Tris–HCl, pH 7.8 and 0.25 M KNO_3. The tubes were sealed and kept in the dark at 30 °C for 1 h. The nitrite released into the medium was determined after incubation by treating 1 mL aliquots with 1 mL of 1% sulphanilamide in 1 M HCl and 1 mL of 0.01% *N*-1-napthyl-ethylenediamine hydrochloride. After 15 min, the optical density was measured spectrophotometrically at 540 nm (Alguacil et al., 2006).

Proline accumulation was evaluated using the method developed by Paquin and Lechasseur (1979). This parameter was determined after extraction with sulphosalicylic acid and reaction with ninhydrin of free proline coming from 1 g of fresh needles. The methanolic phase was used for the quantification of proline and a standard curve of L-proline (PRO) was used for calibration. Proline was estimated by spectrophotometric analysis at 515 nm of the ninhydrin reaction, according to Bates et al. (1973).

Lipid peroxidation rates were determined by measuring the malondialdehyde equivalents (MA) according to Hodges et al. (1999). 0.5 g of needles were manually homogenized in a mortar with liquid nitrogen. The homogenates were centrifuged at

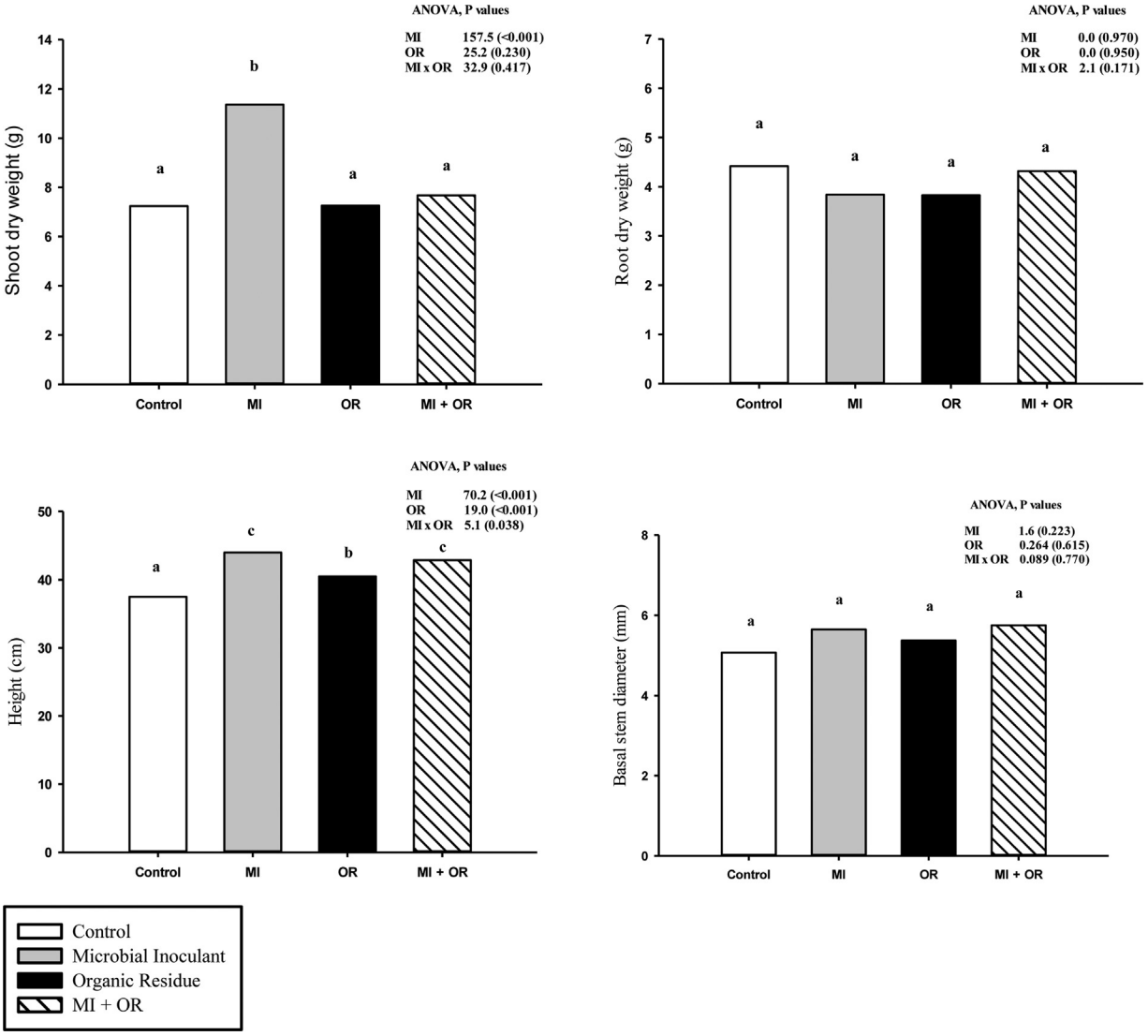

Fig. 1. Plant growth parameters in response to inoculation with immobilized rhizobacteria and addition of organic residue. Values are means of five replicates. Significant differences according to the Duncan test at $P < 0.05$ levels were indicated by different letters. Significance of effects of microbial inoculant, organic amendment and their interaction on the measured variables is also shown.

12,000 × g for 20 min at 4 °C. Samples were then extracted twice with the same solvent. The supernatants were pooled and 1 mL of this sample was added to a test tube with an equal volume of either the solution comprising of 15% trichloroacetic acid (TCA) and 0.01% butylated hydroxy toluene (BHT) or solution of 15% TCA, 0.01% BHT and 0.375% TBA. Samples were heated at 100 °C for 15 min and cooled at 0 °C. Absorbances were read at 535 nm. Lipid peroxidation rate equivalents (nmol MA g fresh weight^{-1}) were calculated by using the formulae given by Hodges et al. (1999).

2.7. Soil physico-chemical analyses

Soil pH and electrical conductivity were measured in a 1:5 (w/v) aqueous solution. Total organic carbon (TOC) and total nitrogen (N) were determined by dry combustion using a LECO Tru-Spec CN analyzer (Leco Corp., St. Joseph, MI, USA). Available P, extracted with 0.5 M $NaHCO_3$, and extractable (with ammonium acetate) K were determined by ICP/OES spectrometry (Thermo Elemental Co. Iris Intrepid II XDL). Total carbohydrates were determined by the method of Brink et al. (1960). In all these analyses soil sieved to <2 mm was used.

2.8. Soil biochemical and microbiological analyses

Alkaline phosphatase activity was determined using, as substrate, *p*-nitrophenyl phosphate disodium (PNPP 0.115 M). 2 mL of 0.5 M sodium acetate buffer at pH 11 using acetic acid (Naseby and Lynch, 1997) and 0.5 mL of substrate were added to 0.5 g of soil sieved to <2 mm and incubated at 37 °C for 90 min. The reaction was stopped by cooling at 0 °C for 10 min. Right after, 0.5 mL of 0.5 M $CaCl_2$ and 2 mL of 0.5 M NaOH were added. The mixture was centrifuged at 4000 rpm for 5 min and the *p*-nitrophenol (PNP) formed was determined by spectrophotometry at 398 nm (Tabatabai and Bremner, 1969). Controls were made in the same way, but the substrate was added before the $CaCl_2$ and NaOH.

β-glucosidase determination is supported on the release and detection of PNP. To carry on this evaluation, 2 mL of 0.1 M maleate buffer at pH 6.5 and a substrate consisting of 0.5 mL of *p*-nitrophenyl-β-D-glucopyranoside (PNG 0.05 M) were added to 0.5 g of soil sieved to <2 mm. Next, sample was incubated at 37 °C for 90 min. The reaction was stopped with tris-hydroxymethyl aminomethano (THAM) according to Tabatabai (1982). The quantity of PNP was determined by spectrophotometry at 398 nm (Tabatabai and Bremner, 1969).

Urease and *N*-α-benzoyl-L-arginine amide (BAA) hydrolyzing protease activities were determined using, respectively, 1 M urea and 0.03 M BAA as substrates and 0.1 M phosphate buffer at pH 7. In order to that, 2 mL of buffer and 0.5 mL of substrate were added to 0.5 g of soil sieved to <2 mm and later it was incubated for 90 min at 30 °C (urease) or 40 °C (protease). Both activities were determined as the NH_4^+ released in the hydrolysis reaction (Nannipieri et al., 1980). Dehydrogenase activity was determined according to García et al. (1997). For that, 1 g of sample sieved to <2 mm 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 during 20 h at 22 °C in darkness. The INTF (iodonitrotetrazolium formazan) formed was extracted with 10 mL of methanol by vortexing for 1 min and filtering through a Whatman No. 5 filter paper. INTF was measured spectrophotometrically at 490 nm.

Microbial biomass C was assayed after glucose was mixed into the soil (at 60% of its field capacity) at a rate of 0.5% (w/w) and monitoring the CO_2 production for 24 h, using the μ-Trac 4200 system (SY-LAB, GmbH P.O. Box 47, A-3002 Neupurkersdorf, Austria). This system is based on the variation of electrical impedance of a KOH 0.2% water solution (Fernández et al., 2004). Respiration rates were calculated in the linear phase of the respiration curves. Basal soil respiration was assessed with the same system described for microbial biomass C but in absence of glucose.

2.9. Statistical analyses

Values were log transformed to achieve normality. The effects of amendment addition, microbial inoculation and their interaction on measured variables were analyzed by a two way ANOVA and post hoc mean separation was performed by Duncan's multiple range test, calculated at $P < 0.05$. All statistical analyses were performed using the software SPSS version 19.0 for windows.

3. Results

3.1. Plant growth parameters

The experimental factors tested, the microbial inoculation (MI) and the addition of organic residue (OR), as well as the MI × OR interaction were significant for *P. halepensis* height, while only MI had a significant effect on shoot biomass. The microbial inoculant produced a significant increase in shoot dry weight by about 48% compared to the control plants (Fig. 1) and by 4 fold with respect to the initial seedlings grown in the nursery. However, the shoot biomass of the plants after the addition of olive residue and the combined treatment did not undergo significant changes respect to the control plants (Fig. 1). Plant height was increased significantly by all the treatments evaluated (Fig. 1). The organic residue and the MI + OR treatment did not increase root dry weight or basal stem diameter, compared with control plants (Fig. 1).

3.2. Nutrients uptake

The ANOVA revealed that the microbial inoculation, organic amendment and MI × OR interaction affected significantly the N, P and K uptake (Table 1). The experimental treatments significantly increased the shoot tissue total N – particularly the microbial inoculant, which yielded an increase of 30%. The P concentration in the plants was improved significantly by the microbial inoculation (57%) and organic amendment (57%), as well as by their combination (69%). Regarding the total K concentration in the shoot, the highest and most-significant increase was recorded with the microbial inoculant (58% greater than control plants); the addition of the organic residue and the combined treatment also gave significant increases (29% and 52%, respectively) (Table 1).

Table 1
Nutrients uptake in plants in response to inoculation with immobilized rhizobacteria and addition of organic residue.

Treatments	N (mg g^{-1})	P (mg g^{-1})	K (mg g^{-1})
Control	9.8 ± 0.1 a	0.54 ± 0.04 a	3.8 ± 0.1 a
Microbial inoculant (MI)	12.7 ± 0.2 c	0.85 ± 0.04 b	6.0 ± 0.1 c
Organic residue (OR)	10.7 ± 0.1 b	0.85 ± 0.05 b	4.9 ± 0.1 b
MI + OR	11 ± 0.2 b	0.91 ± 0.04 b	5.8 ± 0.1 c
ANOVA, *P* values			
MI	157.8 (<0.001)	6.7 (0.020)	358.4 (<0.001)
OR	5.4 (0.034)	24.5 (0.001)	41.2 (<0.001)
MIxOR	107.2 (<0.001)	16.1 (0.001)	70.9 (<0.001)

Values are means of five replicates. Mean ± standard error. Significant differences according to the Duncan test at $P < 0.05$ levels were indicated by different letters. Significance of effects of microbial inoculant, organic amendment and their interaction on the measured variables is also shown.

Table 2
Shoot proline accumulation, oxidative damage to lipids, water and turgor potential and nitrate reductase activity in *P. halepensis* seedlings in response to inoculation with immobilized rhizobacteria and addition of organic residue.

Treatments	Proline (nmol g FW^{-1})	Oxidative stress (nmol MDA g FW^{-1})	Ψstem[a] (MPa)	Ψl[b] (MPa)	Ψp[c] (MPa)	Nitrate reductase (μmol NO_2^{-1} g^{-1} h^{-1})
Control	1.93 ± 0.20 b	4857 ± 495 b	−0.91 ± 0.02 a	−0.94 ± 0.01 a	1.83 ± 0.06 a	0.06 ± 0.001 b
Microbial inoculant (MI)	1.20 ± 0.20 a	2944 ± 447 a	−0.51 ± 0.03 c	−0.56 ± 0.01 c	2.55 ± 0.02 c	0.08 ± 0.002 c
Organic residue (OR)	1.63 ± 0.10 b	4707 ± 496 b	−0.82 ± 0.01 b	−0.86 ± 0.03 bc	2.04 ± 0.05 b	0.05 ± 0.001 a
MI + OR	1.06 ± 0.10 a	4394 ± 305 b	−0.79 ± 0.03 bc	−0.914 ± 0.05 b	1.96 ± 0.09 b	0.10 ± 0.003 d
ANOVA, *P* values						
MI	16.7 (0.001)	5.2 (0.036)	62.8 (<0.001)	128.0 (<0.001)	61.7 (<0.001)	448.6 (0.001)
OR	1.2 (0.293)	2.6 (0.125)	17.6 (<0.001)	108.9 (<0.001)	19.2 (<0.001)	6.2 (0.024)
MIxOR	0.0 (0.914)	3.5 (0.079)	46.9 (<0.001)	201.7 (<0.001)	100.0 (<0.001)	65.1 (0.001)

FW: fresh weight.
Values are means of five replicates. Mean ± standard error. Significant difference according to the Duncan test at $P < 0.05$ levels were indicated by different letters. Significance of effects of microbial inoculant, organic amendment and their interaction on the measured variables is also shown.
[a] Stem water potential.
[b] Leaf water potential.
[c] Leaf turgor potential.

3.3. Plant stress parameters

The MI, organic amendment and MI × OR interaction significantly affected the nitrate reductase activity, stem and leaf water potential and leaf turgor potential, while only MI affected the proline and oxidative stress values (Table 2). The post hoc test showed a significant decrease in proline values after the MI and the combined treatment, and a significant decrease in oxidative stress after MI. Nitrate reductase activity underwent a significant increase after the MI and the application of the combined treatment (33% and 67%, respectively), whereas a significant decrease was recorded with the addition of the organic amendment (Table 2). With regard to the stem water potential, leaf water potential and leaf turgor potential, all the treatments significantly improved the values relative to the control, the highest values occurring after the MI (increases of 56%, 60% and 23%, respectively) (Table 2).

3.4. Soil physico-chemical properties

There were significant effects of the microbial inoculant, organic amendment and MI × OR interaction on the pH, electrical conductivity, available P and extractable K. Total N was significantly affected by MI and the MI × OR interaction, while a significant variation in total organic C was mediated by the organic residue and the MI × OR interaction (Table 3). In many cases, the Duncan test confirmed a significant increase in the values for these treatments compared with the control (Table 3). Only for electrical conductivity was a decrease recorded after the amendment. The pH values increased with all the treatments. With regard to available P, the greatest value was observed after the addition of the organic amendment (a 5-fold increase with respect to the control) (Table 3). The electrical conductivity, total N and extractable K underwent increases after the MI and the combined treatment, the highest values being obtained with the MI for electrical conductivity (14% increase) and with the combined treatment for total N (21%) and extractable K (3-fold higher with respect to the control) (Table 3). The total organic C values were increased by the olive residue and the combined treatment.

3.5. Soil biochemical and microbiological properties

The ANOVA shows that the MI, organic residue and MI × OR interaction significantly affected urease and protease enzyme activities. Microbial inoculation had a significant effect on β-glucosidase, while the addition of organic amendment and the MI × OR interaction affected dehydrogenase activity. Neither of the factors nor the MI × OR interaction had a significant effect on phosphatase (Table 4). Thus, the post hoc test recorded that phosphatase did not undergo any change with respect to the control (Table 4). The activity of β-glucosidase showed an increase with all the treatments, including MI; the highest value was achieved with the combined treatment (a 26% increase, with respect to the control). Urease activity also increased with all the treatments, especially the combined treatment (by 27%, with respect to the control). Protease activity was improved by all the treatments, the MI yielding an increase of 44%. Dehydrogenase activity was improved by the application of the olive residue and the combined treatment, obtaining its maximum value with the latter (a rise of 36% relative to the control) (Table 4).

The MI, the organic residue and the MI × OR interaction significantly affected the total carbohydrates, microbial biomass

Table 3
Soil physico-chemical and chemcial properties in response to inoculation with immobilized rhizobacteria and organic residue addition.

Treatments	pH (H_2O)	EC (1:5, μS cm^{-1})	TOC (g kg^{-1})	Total N (g kg^{-1})	Available P (μg g^{-1})	Extractable K (μg g^{-1})
Control	8.52 ± 0.01 a	149 ± 2 b	18.6 ± 0.1 a	1.40 ± 0.04 a	3 ± 0 a	237 ± 20 a
Microbial inoculant (MI)	8.64 ± 0.01 c	170 ± 1 d	18.8 ± 0.2 a	1.51 ± 0.05 b	7 ± 0 c	437 ± 29 b
Organic residue (OR)	8.64 ± 0.01 c	144 ± 1 a	20.9 ± 0.3 c	1.41 ± 0.06 a	15 ± 0 d	306 ± 16 a
MI + OR	8.56 ± 0.01 b	154 ± 2 c	20.3 ± 0.5 b	1.70 ± 0.05 c	5 ± 0 b	730 ± 10 c
ANOVA, *P* values						
MI	19.1 (<0.001)	102.9 (<0.001)	1.0 (0.340)	29.7 (<0.001)	29.0 (<0.001)	149.0 (<0.001)
OR	7.0 (0.018)	46.8 (<0.001)	97.5 (<0.001)	3.1 (0.096)	706.7 (<0.001)	41.8 (<0.001)
MIxOR	474.0 (<0.001)	13.2 (0.002)	4.9 (0.041)	5.4 (0.033)	1373.1 (<0.001)	4.5 (0.005)

EC: electrical conductivity; TOC: total organic carbon. Values are means of five replicates. Mean ± standard error. Significant difference according to the Duncan test at $P < 0.05$ levels were indicated by different letters. Significance of effects of microbial inoculant, organic amendment and their interaction on the measured variables is also shown.

Table 4
Soil enzymatic activities in response to inoculation with immobilized rhizobacteria and addition of organic residue.

Treatments	Phosphatase (μmol PNF g^{-1} h^{-1})	β-glucosidase (μmol PNF g^{-1} h^{-1})	Urease (μmol NH_3 g^{-1} h^{-1})	Protease (μmol NH_3 g^{-1} h^{-1})	Dehydrogenase ($\mu g g^{-1}$ INTF)
Control	5.2 ± 0.6 a	1.5 ± 0.05 a	1.1 ± 0.02 a	1.8 ± 0.05 a	100 ± 4.2 a
Microbial inoculant (MI)	5.6 ± 0.3 a	1.8 ± 0.09 b	1.3 ± 0.01 b	2.6 ± 0.04 d	94 ± 5.6 a
Organic residue (OR)	5.3 ± 0.4 a	1.6 ± 0.14 a	1.3 ± 0.01 b	2.4 ± 0.01 c	114 ± 5.7 b
MI + OR	5.9 ± 0.2 a	1.9 ± 0.07 c	1.4 ± 0.01 c	2.1 ± 0.04 b	136 ± 4.2 c
ANOVA, *P* values					
MI	1.5 (0.240)	36.0 (<0.001)	127.0 (<0.001)	46.4 (<0.001)	1.4 (0.257)
OR	0.3 (0.567)	3.6 (0.078)	118.6 (<0.001)	5.9 (0.028)	32.4 (<0.001)
MIxOR	0.0 (0.950)	0.3 (0.614)	10.4 (0.03)	216.1 (<0.001)	7.1 (0.017)

Values are means of five replicates. Mean ± standard error. Significant difference according to the Duncan test at $P < 0.05$ levels were indicated by different letters. Significance of effects of microbial inoculant, organic amendment and their interaction on the measured variables is also shown.

and soil respiration (Table 5). The Duncan test confirmed increases in the values of all these parameters for all the treatments. The highest increases in total carbohydrates were recorded after the MI and the addition of the organic amendment (26% and 29%, respectively, compared with the control) (Table 5). Microbial biomass reached its highest value (50% higher than the control) with the MI.

4. Discussion

The results demonstrate that the inoculation with immobilized rhizobacteria was the most-effective treatment for promoting the establishment of *P. halepensis* under semiarid field conditions. Inoculation with rhizobacteria has recently been proved to be a useful strategy for the re-establishment of native shrub species in these degraded environments (Mengual et al., 2014; Schoebitz et al., 2014). Previous afforestation practices with *P. halepensis* in semiarid environments included the use of organic amendment and ectomycorrhizal inoculation (Rincón et al., 2006; Roldán et al., 1996). In this study, we provide the first evidence of the beneficial effect of immobilized PGPR as a microbial inoculant on the growth of this tree species in a degraded semiarid soil.

In our afforestation assay, the microbial inoculant, organic residue and combination of both treatments increased the total N, P and K concentrations in plants. This may be attributable to the nutrients incorporated with the organic residue and their solubilization, mediated by the microbial inoculant (Tejada et al., 2009). The best results were observed after the microbial inoculation. It seems admissible that the inoculated rhizobacteria were rhizosphere-competent bacteria capable of colonizing plant roots (Hozore and Alexander, 1991), which allowed to explore the available ecological niches in the rhizosphere (Kumar et al., 2011; Trivedi et al., 2012) of *P. halepensis*; consequently, they could explore a wider range for mobilization of nutrients. The nitrate reductase values suggest that the N concentration also could have been increased by an improvement in this enzyme activity promoted by these bacterial strains.

It has been demonstrated that the use of rhizobacteria improves plant health and growth performance in degraded soils, as well as enhancing their tolerance of drought and salinity (de-Bashan et al., 2012). The accumulation of proline in plants plays a major role in the process of osmotic adjustment, helping to decrease the cell osmotic potential and thus allow higher water retention during drought periods (Medina et al., 2010). Another mechanism of protection against drought stress is the enzyme nitrate reductase, which catalyses the rate-limiting step in the nitrate assimilation pathway (Alguacil et al., 2006). A decrease in oxidative stress could also be considered a sign of improved plant health. In our study, the control plants and plants treated with organic residue, having high proline levels and low nitrate reductase activities, were the most affected by drought. The treatments that included microbial inoculation gave lower proline and higher nitrate reductase values, while the microbial inoculation produced the lowest value of oxidative stress. This suggests that the inoculated rhizobacteria were highly effective with regard to inducing plant resistance to drought in these semiarid conditions. These results are in accordance with the measurements of the stem water, leaf water and leaf turgor potentials obtained for the needles, where the greatest values were reached after microbial inoculation. Thus, the fact that the highest values of leaf turgor were found in inoculated plants suggests that the rhizobacteria may have contributed to the active osmoregulation and hence to the maintenance of leaf turgor.

The treatments which included the application of organic amendment increased the soil total organic carbon, and all the treatments tested increased the total carbohydrates. These improvements are relevant because these compounds can be

Table 5
Soil microbiological properties in response to inoculation with immobilized rhizobacteria and addition of organic residue.

Treatments	Total CH ($\mu g g^{-1}$)	Microbial biomass C ($\mu g g^{-1}$)	Soil respiration (mg $C–CO_2$ h^{-1} kg^{-1})
Control	1230 ± 35 a	1444 ± 28 a	9.4 ± 0.22 a
Microbial inoculant (MI)	1555 ± 9 c	2185 ± 31 d	12.2 ± 0.32 b
Organic residue (OR)	1591 ± 42 c	1927 ± 97 c	11.6 ± 0.8 b
MI + OR	1436 ± 42 b	1624 ± 18 b	11.5 ± 0.7 b
ANOVA, *P* values			
MI	7.4 (0.015)	144.3 (<0.001)	20.7 (<0.001)
OR	13.1 (0.002)	4.5 (<0.049)	21.9 (<0.001)
MIxOR	48.3 (<0.001)	818.6 (<0.001)	5.6 (0.031)

CH: carbohydrates. Values are means of five replicates. Mean ± standard error. Significant difference according to the Duncan test at $P < 0.05$ levels were indicated by different letters. Significance of effects of microbial inoculant, organic amendment and their interaction on the measured variables is also shown.

used as carbon and energy sources by soil-borne microflora (Mengual et al., 2014). In addition, the carbon compounds released by roots promote the microbial biomass in the rhizosphere soil, since the microbiota produce a cementing effect through the excretion of polysaccharide material (Roldán et al., 1994). This material has the capacity to increase the hydrophobicity of soil particles and stabilize soil aggregates (Caravaca et al., 2002a). Enzyme activities are properties sufficiently sensitive to indicate changes caused by microbial inoculations (Schoebitz et al., 2014). The β-glucosidase results recorded for the treatments involving microbial inoculation are in accordance with the microbial biomass and soil respiration values, which have frequently been used as indicators of soil microbial activity (Caravaca et al., 2002b). The soil microbial activity in semiarid areas is very low due to the low capacity of the soil organic matter for mineralization. Due to this fact, Caravaca et al. (2002b) proposed microbial biomass, soil respiration and enzyme activities as indices of the microbiological activity in semiarid soils.

Although all the treatments increased the total N level in the rhizosphere soil, the highest value was produced by the combined treatment. It can be assumed that the addition of amendment would mediate an input of N and that *A. brasilense* and *P. dispersa* were able to fix N_2. All the treatments evaluated enhanced the protease and urease activities, which are involved in the N cycle. The application of amendments and microorganisms has been used to increase nutrient availability in the soil and improve plant growth and nutrient uptake (Caravaca et al., 2005). In our experiment, available P in the rhizosphere soil was increased by the use of the microbial inoculant, organic residue and combined treatment; presumably, the rhizobacteria inoculated were able to solubilize P from both the soil and the applied organic residue. Soil microflora is able to excrete organic acids and/or enzymes involved in the P cycle (i.e., phosphatases) which increase the concentration of P in rhizosphere soil (Vassilev et al., 2006). However, based on our phosphatase results, it seems that these rhizobacteria were only capable of solubilizing P through their excretion of organic acids (Basak and Biswas, 2010; Mengual et al., 2014).

The microbial inoculation, the addition of organic residue and the combined treatment increased the extractable K content in rhizosphere soil with respect to the control, which may be attributable to the input of K provided by the organic residue and also to the mobilization of K by the soil rhizobacteria. Since the microbial inoculant treatments helped plants to compensate for deficiencies of immobile nutrients, the inoculation with immobilized rhizobacteria can be considered an effective tool for the development of biofertilizers that could substitute partially for chemical fertilization. In this regard, the introduction of a microbial inoculant can improve nutrient availability to plants and thereby increase the efficiency of applied manures (Adesemoye and Kloepper, 2009). It is worth noting that the organic amendment had any effect on plant growth, despite improving soil and plant nutrient status and microbiological quality. In contrast, previous studies have shown the effectiveness of urban residue for improving plants performance during afforestation tasks of a degraded Mediterranean soil with *P. halepensis* (Rincón et al., 2006; Roldán et al., 1996). One possible explanation for this discrepancy is that the composted olive-mill waste used in our experiment could contain phytotoxic compounds after composting (Alburquerque et al., 2006), which would be reducing or eliminating the effectiveness of organic residue for promoting plant growth.

In conclusion, the microbial inoculation of *P. halepensis* seedlings with the mixture of the strains *A. brasilense* and *P. dispersa* improved the plant growth in a degraded soil under semiarid Mediterranean conditions. The lower proline accumulation and lesser oxidative damage to lipids, linked to a higher water potential in the plants inoculated with rhizobacteria, indicate that the plants developed mechanisms to avoid oxidative damage under hydric stress. The capacity of the microbial inoculant to increase plant drought tolerance may have been related to nutrient uptake improvement and an increase in N assimilation through NR activity. Both microbial inoculation and the organic amendment were effective with regard to enhancing soil fertility and microbiological quality. However, the promotion of plant growth by the microbial inoculant was greatest when applied independently, and the lower cost of implementation of this restoration biotechnology supports its preferential use in re-afforestation tasks with *P. halepensis* in semiarid environments.

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