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RESEARCH ARTICLE

Potential of mycorrhizal inocula to improve growth, nutrition and enzymatic activities in *Retama sphaerocarpa* compared with chemical fertilization under drought conditions

E. Armada[1], O. López-Castillo[1], A. Roldán[2] and R. Azcón[1*]

[1]Departamento de Microbiología del Suelo y Sistemas Simbióticos, Estación Experimental del Zaidín, CSIC, 18008 Granada, Spain. [2]Department of Soil and Water Conservation, Centro de Edafología y Biología Aplicada del Segura, CSIC, Campus de Espinardo, 30100 Murcia, Spain. *******Corresponding author: rosario.azcon@eez.csic.es*

Abstract

The growth of *Retama sphaerocarpa* under drought conditions was similarly increased by arbuscular mycorrhizal (AM) colonization [native AM fungal consortium (M), allochthonous *Rhizophagus intraradices* (RI)] or H_3PO_4 application [25 ppm P (1P) or 50 ppm P (2P)]. However, the antioxidant ascorbate peroxidase (APX) activity was increased by P-fertilization and decreased by AM colonization in plants of similar size, which revealed possible AM protection against drought. RI was most effective in enhancing P and it also reduced glutathione reductase (GR) activity compared with plants of similar biomass from other treatments. In a subsequent study the mixture of autochthonous inocula (AM fungal consortium (M) plus native *Bacillus thuringiensis* (B)) were able to fortify K_2SO_4 fertilization [5 mM K (1K) or 10 mM K (2K)] on *R. sphaerocarpa* under drought. Dual inocula increased nutrient content only in plants fertilized with 1K, while 2K even decreased the abundance of arbuscules. The reduced superoxide dismutase (SOD) and APX and the elimination of catalase (CAT) and GR activities found in co-inoculated K-fertilized plants suggested the lowest oxidative stress and the highest potential to cope with drought irrespective of nutrition. In both experiments inocula enhanced soil enzymatic activities, which also contributed to higher performance of inoculated plants under drought.

Keywords: Drought stress, mycorrhizal-colonization, P or K fertilization, antioxidant activities, *Retama sphaerocarpa* nutrition, enzymatic soil activities

1. Introduction

Drought and water stress are considered the most important environmental factors limiting plant growth in the world (Panozzo and Eagles 1999). High temperatures and dry climate have a strong relationship with soil degradation and desertification. The restoration of degraded areas must be started by reclaiming the vegetation as shrubs species and most of the revegetation programs have been developed by

using autochthonous plant species that are the most appropriate for reclaiming degraded soils.

Restoration of natural vegetation in arid drought zones is quite difficult due to water and nutrient limitation. Application of chemical fertilizers implies the appearance of insoluble mineral complexes, these insoluble forms precipitated cannot be absorbed by plants (Rengel and Marschner 2005). As a consequence, a minimum of the applied fertilizers are acquired by plants. Nevertheless, the establishment of plant cover in these disturbed sites can be facilitated by beneficial soil microorganisms such as arbuscular mycorrhizal (AM) fungi. The interest of using microbial inoculation to recover degraded dry lands has led to evaluation of inocula effectiveness to enhance plant establishment under drought conditions (Armada *et al*. 2014). This biotechnology has been proposed to be ecologically important for the plant development in degraded ecosystems (Jeffries and Barea 2012).

Mycorrhizal fungi colonize the roots of more than 90% of plant species having mutual plant and fungus benefit (Smith and Read 2008) and this symbiosis can improve the nutritional status and growth of plants under both optimal and restricted water levels. AM fungi represent an important biological factor for plants to thrive in water-limited conditions not only by increasing the supply of nutrients, but also by helping plants to support water stress (Medina and Azcón 2012). Selected microorganisms may contribute to plant establishment and growth particularly limited under semiarid conditions (Armada *et al*. 2014).

Drought may reduce AM fungus root colonization, but the inoculation of efficient fungi may help enhancing colonization and consequently population of AM fungi in this environment. The low microbiological activity of arid soils, due to the low density of microbial propagules, may be critical to the successful reestablishment and recovery of desertified ecosystems. Thus, native strains could be, presumably, the most effective in semiarid sites (Armada *et al*. 2014). Among different AM fungal species *Rhizophagus intraradices* have been reported an efficient endophyte in terms of soil water uptake (Marulanda *et al*. 2003) and in increasing plant growth and nutrition under drought conditions (Marulanda *et al*. 2006).

We hypothesised that under drought conditions autochthonous plants will be particularly benefited from inoculation with a whole native AM fungi consortium due to the diversity and functionality of autochthonous mycorrhizal community than from the inoculation with a single drought adapted mycorrhizal isolate as *R. intraradices* (from our collection). Presumably the inoculation with a complex fungal community would have a greater buffer capacity against the water stress than a single fungal inoculum (Caravaca *et al*. 2005).

A general trend in the beneficial effect of mycorrhization is associated with phosphorus acquisition in colonized plants and this is an important mechanism related to plant drought tolerance as has been reported by Augé (2004).

The inoculation with adapted symbiotic microorganism as autochthonous AM fungi has proved their effectiveness under water stress (Marulanda *et al*. 2006) and they been proposed with regards the improvement of the performance of plants in semi-arid and arid areas (Caravaca *et al*. 2005). However, information on the comparative role of autochthonous AM fungal consortium with fertilizers on plant stress tolerance is missing. Revegetation with the native woody legumes *Retama sphaerocarpa* in nutrient-deficient arid soils has proven to be more effective than with exotic plants under such unfertile and water limited conditions (Caravaca *et al*. 2005).

Drought stress can trigger an oxidative burst, induce an array of oxidant enzymes expression (Gururani *et al*. 2013) and as result, plants are able to counteract drought stress by modulating levels of some antioxidant enzymatic systems (Koussevitzky *et al*. 2008).

Plants have this alternative defence strategy as a tool to overcome the stress constraints. There are few studies regarding the changes in the activity of antioxidant enzymes in plants modulated by the microbial inoculations under water stress conditions and results reported are highly variable (Armada *et al*. 2014; Ortiz *et al*. 2015).

To address the mycorrhizal drought tolerance strategies in *R. sphaerocarpa* we selected in a first experiment two types of drought adapted mycorrhizal inocula (autochthonous fungal mixture or the reference *R. intraradices*) and the effect of these biological treatments on drought tolerance were compared with two levels of phosphorus fertilization, 25 and 50 ppm P as H_3PO_4. Drought tolerance of *R. sphaerocarpa* was determined assessing growth, nutrient acquisition, mycorrhizal development and antioxidant enzymatic activities according to the chemical or biological treatments applied using a Mediterranean arid soil under drought conditions. A second experiment was also carried out using the same plant, soil and environmental conditions. This second experiment was planned based on the well-known fact that potassium is the main element related with the alleviation of osmotic stress by being involved in photosynthetic CO_2 fixation and the protection of chloroplasts from photooxidative damage (Romheld and Kirkby 2010). K^+ as inorganic osmolyte is important in water homeostasis under drought and able to regulate osmotic balance, turgor pressure, stomatal opening and transpiration (Loutfy *et al*. 2012). The objective of this second study was to assess to what extent the effect of the single potassium fertilizer [two levels, 5 or 10 mM K (1K or 2K) as K_2SO_4], may be improved by the addition of autochthonous inocula [*Bacillus thuringiensis* (B) plus the mycorrhizal consortium (M)]. Both inocula were isolated from the same rhizosphere samples as *B. thuringiensis*, which had been selected for exerting a significant effect on drought tolerance in native plants such as *Lavandula*, *Trifolium*, *Salvia* and others (Armada *et al*. 2014). Moreover, in a recent study with a plant of agronomic interest as maize the coordinated effect of these autochthonous two microbial groups (*B. thuringiensis* + AM fungi) under drought conditions was evidenced. *B. thuringiensis* increased mainly maize nutrition and AM fungi were more active improving stress tolerance/homeostatic mechanisms (as plant aquaporins and physiological functions) (Armada *et al*. 2015). Thus, we here hypothesised that these combined biological treatments (MB) could interact with chemical fertilizers (1K or 2K) influencing growth, nutrition, mycorrhizal development and drought tolerance in those drought-adapted Mediterranean plants growing under drought conditions.

Soil enzyme activities are an alternative way of monitoring the soil alterations and perturbations (Naseby and Lynch 1997). In arid and degraded soils the microbial populations and their activities are low mainly due to the lack of water and suitable substrates (Medina and Azcón 2012). Thus, soil enzymatic activities play an important role in the mineralization of the organic products and were assessed as index of microbial soil activity.

The efficacy of native strains from different sites lead to different effects on plant growth and nutrients uptake (Ortiz *et al*. 2015). The aims of this study is to ascertain the comparative effect of mycorrhizal inocula (autochthonous or allochthonous) and P fertilizers (two levels), and to verify the relevance of the AM strain origin or P-fertilizer to the ability to enhance plant growth, nutrition, biochemical antioxidant values related to drought tolerance under semiarid conditions. In addition, to test the impact of dual microbial inoculations (autochthonous AM fungi and the bacteria *B. thuringiensis*) and/or the application of fertilizer potassium (two levels) on *R. sphaerocarpa* growth, nutrition and plant antioxidant activities. In both experiments

changes in soil enzymatic activities were measured.

2. Materials and Methods

2.1. Experimental design

Two independent greenhouse pot experiments were carried out in this study. In Experiment I, we determined the effectiveness of a consortium of autochthonous mycorrhizal fungi (M) or *R. intraradices* (RI) compared with two phosphorus levels [25 ppm P (1P) or 50 ppm P (2P) as H_3PO_4] over non-inoculated unfertilized control to increase plant growth, nutrition mycorrhizal development and biochemical activities under drought conditions.

In Experiment II, we examined how two levels of K in the growing medium [5 mM K (1K) and 10 mM K (2K) as K_2SO_4], applied as single fertilizer or when these two K levels were dually inoculated with autochthonous microorganism as AM fungi (consortium) plus *B. thuringiensis* affected plant drought tolerance in terms of plant growth, nutrition, mycorrhizal development and biochemical parameters under drought conditions.

In both experiments soil enzymatic activities, as soil quality biomarkers, were determined. Five replicates of each treatments were included resulting a total of 25 pots in experiment I and 20 pots in experiment II.

2.2. Soil characteristics

The experimental soil used was selected from an area located in the Natural Ecological Park "Vicente Blanes" in Molina de Segura, province of Murcia (southeastern Spain) (coordinates 38º12´ N, 1º13´ W, 393 m altitude). The climate is semiarid Mediterranean, with an average annual rainfall lower than 270 mm and the potential evapotranspiration (ETP) reaches approximately 1000 mm. The mean annual temperature is 19.2 ºC with absence of frost period. The soil in is a Typic Torriorthent (SSS 2006) very little developed with a low organic matter content and a silty clay texture that facilitates the degradation of soil structure. The vegetation in the zone was dominated by *Piptatherum miliaceum* L. Cosson., *Trifolium repens* L., some shrubs of *Thymus vulgaris* L., *Rosmarinus officinalis* L. and *R. sphaerocarpa* growing with patchy distribution.

The main soil characteristics were pH 8.90, P-Olsen 1.36 mg kg^{-1}, organic carbon 0.94%, total N 0.22%, and an electric conductivity of 1.55 dS m^{-1}. Both microcosm experiments were conducted in this soil.

2.3. Isolation, production and identification of drought-tolerant microorganism

The microbial inocula used in these experiments were isolated from the rhizosphere of plants naturally growing in this semiarid soil described above. This rhizosphere soil containing colonized roots, spores and mycelia belonging to the native adapted AM fungi was cultivated for inoculum production (Marulanda *et al*. 2006).

For inocula production the rhizosphere soil was bulked in an open pot culture of *Zea mays* and *Trifolium repens* with sterile soil/sand (1:1 v/v) mixture. After six months of plant growth the shoots were eliminated and the under-grown part (mycorrhizal roots plus soil possessing fungal spores and mycelium) was maintained by storage for three to six months in polyethylene bags at 4 ºC and used as a stock culture. The mycorrhizal fungus *R. intraradices* (EEZ 195) from our collection (Estación Experimental del Zaidín) was also used in the Experiment I as reference.

Plants were inoculated with *R. intraradices* or a consortium of indigenous AM fungi. The fungal spores were isolated by wet-sieving and decanting as de-

scribed by Ruíz-Lozano and Azcón (1995) and all the spores obtained were morphologically similar to *Septoglomus constrictum* (EEZ 198), *Diversispora aunantia* (EEZ 199), *Archaeospora trappei* (EEZ 200), *Glomus versiforme* (EEZ 201) and *Paraglomus ocultum* (EEZ 202) compared to those from our current EEZ collection. We used as autochthonous mycorrhizal (M) inoculum a mixture of each one of these fungal species.

From the corresponding stock culture, 5 g of this fungal AM consortium (M) or *R. intraradices* (RI) from collection was applied as inocula to the corresponding pots, having both inocula similar an average of 80 spores/g of soil and roots with 75% of AM colonization. The M inoculum or the reference *R. intraradices* were applied to each one of the appropriate pots at transplanting time just below the seedlings. Non-mycorrhizal treatments received the same amount of autoclaved inoculum together with a 2 mL aliquot of natural soil filtrate (< 20 mm) containing soil microorganisms with the exception of AM fungi.

In Experiment II an autochthonous bacteria was also used in interaction with autochthonous AM fungi. The bacterium was isolated from the above-mentioned soil (a mixture of rhizospheres from several autochthonous plants species). A homogenate of 1 g soil in 9 mL sterile water was diluted (10^{-2} to 10^{-4}), plated on three different media [Yeast Manitol Agar, Potato Dextrose Agar or Luria-Bertani (LB) Agar] and then incubated at 28 ºC for 48 h, to isolate bacteria from different taxonomic groups.

Identification of isolated autochthonous bacteria was done by sequencing the 16S rDNA gene. Bacterial cells were collected, diluted, lysed and their DNA used as a template in the PCR reactions. All reactions were conducted in 25 µL volume containing PCR buffer 10X, 50 mM $MgCl_2$, 10 µM each primers 27F (AGAGTTTGATCCTGGCTCAG) and 1492R (GGTTACCTTGTTACGACTT), 5 U/µL of *Taq* polymerase (Platinum, Invitrogen). The PCR was performed in a thermal cycle with the following conditions: 5 min at 95 ºC, followed by 30 cycles of 45s at 95 ºC, 45s at 44 ºC and 2 min at 72 ºC, and finally one cycle of 10 min at 72 ºC. The products PCR of were analysed by 1% agarose gel electrophoresis and DNA was extracted and purified with the QIAquick Gel extraction kit (QUIAGEN) for subsequent sequencing in an automated DNA sequencer (PerKin-Elmer ABI Prism 373). Sequence data were compared to gene libraries (NCBI) using BLAST program, unambiguously identified the bacterium as *Bacillus thuringiensis* (Acession NR 043403.1, similarity 98%). The bacteria were grown in 250-mL flasks containing 50 mL of LB medium for 48 h at 28 ºC. In the corresponding pots, plants were inoculated with 1 mL of the bacterial culture (10^8 cfu mL^{-1}) in the planting hole at sowing time. The bacterial inoculum was applied again 15 days later. In control treatments, 1 mL of sterilized bacterial culture was added.

Prior use soil was sieved (mesh diameter = 2mm) and sterilized by steam (100 ºC for 1h on 3 consecutive days). One month old seedlings of *R. sphaerocarpa* plants were transplanted to pots containing 0.750 kg of a 1:1 mixture of soil: sand (v/v) in Experiment I and 1:2 soil: sand mixture (v/v) in Experiment II. At transplanting time plants were inoculated with the appropriate inoculum.

2.4. Plant growth conditions

Plants (one per pot) were grown for seven and half months in a greenhouse under a day/night cycle of 16/8 h, 21/15 °C and 50% relative humidity. The photosynthetic photon flux density (PPFD) was 503 µmol m^{-2} s^{-1}, as measured with a light-meter (LICOR, model LI-188B). Water loss was compensated by watering every day to reach 50% of water-holding capacity (WHC). During the first 2 weeks

of plant growth constant soil water content close to water holding capacity was maintained. After this time, plants were allowed to dry until soil water content was 50% of water holding capacity and maintained under these conditions for additional 30 weeks. To achieve that, the soil moisture in the pots was measured each 24 h and the water was added to reach a maximum of 50% of water holding capacity. However, during the 24-h period between each rewatering the soil water content was progressively decreased to a minimum value of 40% of water holding capacity. Soil moisture was measured with an ML2 X ThetaProbe (AT Delta-T Devices Ltd, Cambridge, UK), which measures volumetric soil moisture content by responding to changes in the apparent dielectric constant of moist soil (White *et al*. 1994). This volumetric soil moisture is considered to be a normal environmental condition in dry Mediterranean areas. During the experimental time, a Hewitt's nutritive solution was applied weekly (10 mL pot^{-1}) modified to have ½ N and ¼ P concentrations. In Experiment I, the two P fertilization treatments were applied twice a week (10 mL) of H_3PO_4 solution along the four weeks after transplanting to reach the desired P concentrations (25 or 50 ppm P) in the appropriate pots. Similarly, in Experiment II the two K fertilization treatments were applied twice a week (10 mL) of K_2SO_4 solution along the four weeks after transplanting to reach the desired K concentrations (5 mM or 10 mM K) in the corresponding pots.

2.5. Parameters measured

2.5.1. Biomass production

At harvest, seven and half months after transplanting in both Experiments, the root system was separated from the shoot and dry weights were measured after drying at 75 ºC for 2 days.

2.5.2. Nutrient content

Shoot content (mg per plant) of P, K, Ca and Mg as well as of Fe, Mn, Zn and Cu (µg per plant) were determined by inductively coupled plasma optical emission spectrometry (ICP-OES). Mineral analyses were carried out by the Instrumentation Service (EEZ-CSIC), Granada, Spain.

2.5.3. Mycorrhizal development

Fungal colonization was assessed after clearing washed roots in 10% KOH and staining with 0.05% trypan blue in lactic acid (v/v), according to Philips and Hayman (1970). The extent of mycorrhizal colonization was calculated according to the gridline intersect method Giovanneti and Mosse (1980) after counting 150 intersections. Mycorrhizal development was evaluated by the method of Trouvelot *et al*. (1986) using MYCOCALC software (http://www.dijon.inra.fr/mychintec/Mycocalc-prg/download.html). The parameters measured according to this method were the frequency of AM colonization in the sample (%F), intensity of AM colonization (%m) in the whole root system (%M), and relative and absolute arbusculum richness (% a and % A) referred to the calculated whole root system respectively.

2.5.4. Antioxidant enzymatic activities

Shoot tissues were homogenized (Aroca *et al*. 2003) in a cold mortar with 4 mL 100 mM phosphate buffer (pH 7.2) containing 60 mM KH_2PO_4, 40 mM K_2HPO_4, 0.1 mM diethylenetriaminepenta acetic acid (DTPA) and 1% (w/v) polyvinylpolypyrrolidone (PVPP). The

homogenate was centrifuged at 18,000 *g* for 10 min at 4 ºC, and the supernatant was used for enzyme activity determination. Total superoxide dismutase (SOD) activity (EC 1.15.1.1) (Burd *et al*. 2000) was measured on the basis of SOD's ability to inhibit the reduction of nitroblue tetrazolium (NBT) by superoxide radicals generated photochemically. One unit of SOD was defined as the amount of enzyme required to inhibit the reduction rate of NBT by 50% at 25 ºC. Catalase (CAT) activity (EC 1.11.1.6) was measured as described by Aebi (1984) conducted in 2 mL reaction volume containing 50 mM potassium phosphate buffer (pH 7.0), 10 mM H_2O_2 and 50 µL of enzyme extract. Consumption of H_2O_2 (extinction coefficient (ε_{240}) of 39.6 mM^{-1} cm^{-1}) at 240 nm for 1 min was monitored. Ascorbate peroxidase (APX) activity (EC 1.11.1.11) was measured in a 1 mL reaction volume containing 80 mM potassium phosphate buffer (pH 7.0), 2.5 mM hydrogen peroxide and 0.5 mM sodium ascorbate. The H_2O_2 was added to start the reaction, and the decrease in absorbance at 290 nm was recorded for 1 min to determine the oxidation rate for ascorbate (Amako *et al*. 1994). Glutathione reductase (GR) activity (EC 1.20.4.2.) was estimated by measuring the decrease of absorbance at 340 nm due to the oxidation of NADPH (Carlberg and Mannervik 1985). The reaction mixture (1 mL) contained 50 mM Tris buffer 3 mM $MgCl_2$ (pH 7.5), 1 mM oxidized glutathione, 50 µL enzyme extract, and 0.3 mM NADPH was added and mixed thoroughly to begin the reaction. The results were expressed in mmol NADPH oxidized mg^{-1} protein, and the activity was calculated from the initial speed of reaction and the molar extinction coefficient of NADPH (e_{340}=6.22 mM^{-1} cm^{-1}). Total soluble protein amount was determined using the Bradford (Bradford 1976) method and bovine serum albumin (BSA) as standard.

2.5.5. Soil enzymatic activities

In rhizosphere soil samples enzymatic activities were determined in both experiments.

Dehydrogenase activity was determined following Skujins' method (Skujins 1976), as modified by 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 ºC in darkness. The INTF (iodo-nitrotetrazolium formazan) formed was extracted with 10 mL of methanol, by shaking vigorously for 1 min and filtering through a Whatman N° 5 filter paper. INTF was measured spectrophotometrically at 490 nm.

Alkaline phosphatase activity was determined using *p*-nitrophenyl phosphate disodium (PNPP) 0.115 M as substrate. Two milliliters of 0.5 M sodium acetate buffer adjusted to 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 in a spectrophotometer at 398 nm (Tabatabai and Bremner 1969). In controls, the substrate was added before the $CaCl_2$ and NaOH addition. β-glucosidase was determined using *p*-nitrophenyl-β-D-glucopyranoside (PNG), 0.05 M (Masciandaro *et al*. 1994) as substrate. This assay is also based on the release and detection of PNP. Two milliliters of 0.1 M maleate buffer (pH 6.5) and 0.5 mL of substrate were added to 0.5 g of sample and incubated at 37 ºC for 90 min. The reaction was stopped with tris-hydroxymethyl aminomethane (THAM) according to Tabatabai (1982). The amount of PNP was determined in a spectrophotometer at 398 nm (Tabatabai and Bremner 1969).

Urease activity was determined by the method of Nannipieri *et al*. (1980), and expressed as µmol $N\text{-}NH_3$ g^{-1} soil $\cdot h^{-1}$.

2.6. Statistical analyses

Data from both experiments were analyzed using the SPSS 21 software package for Windows, employing one-way general linear model ANOVA (analysis of variance) to determine the effect of two mycorrhizal inocula compared with two levels of phosphorus fertilizer [25 ppm P (1P) or 50 ppm P (2P) as H_3PO_4]) (Experiment I), and to evaluate the effect of dual microbial inoculations (autochthonous AM fungi and the bacteria *B. thuringiensis*) and /or the application of potassium fertilizer (two levels [5 mM K (1K) and 10 mM K (2K) as K_2SO_4] (Experiment II). The Duncan's multiple-range test (Duncan 1955) was used for post hoc analysis to determine differences between means. Differences were considered significant at $p \leq 0.05$. Percentage values were arc-sine-transformed before statistical analysis.

3. Results

3.1. Experiment I

Regarding the results of this experiment, all mycorrhiza inocula applied behaved similarly to P fertilization (1P or 2P) in terms of shoot dry mass production. The four treatments applied, similarly enhanced shoot dry biomass compared to control plants, but root development was lower in RI colonized plants (Table 1). In spite of non-significant differences in growth of P treated or mycorrhizal inoculated plants the highest shoot biomass (79% over control) was obtained in plants colonized by the consortium of autochthonous fungi (Table 1).

Shoot and root development of P-fertilized plants as well as some of the analyzed nutrients (P, K, Ca, Fe, Mn, Zn and Cu) reached similar values irrespective of P level applied (Table 1). Thus, the application of 25 ppm P (1P), under these drought environmental conditions, may be considered as the optimum amount of P-fertilization to reach the maximum plant growth and nutrition.

Regarding P and K content no-differences were observed in P fertilized plants, irrespective of P level and M inoculated having similar root development (Table 1). Nevertheless, the uptake of P, K, Ca or Mg was significantly higher in *R. intraradices*-colonized plants than in control plants having similar root biomass (Table 1).

The root development in *R. intraradices* colonized plants was more reduced than in the rest of treated plants (Table 1). As a result of lower root growth, lesser nutrients transport to the above grown parts is expected. However, the opposite effect was observed in *R. intraradices* inoculated plants since the uptake of macronutrients was higher in these plants. Results show that this fungus plays a significant role in the extra acquisition of some nutrients (P, K, Ca and Mg) under drought conditions (Table 1).

Moreover, in the case of P, *R. intraradices*-colonized plants acquired the highest proportion of this nutrient, even more than plants P-fertilized with 50 ppm P (Table 1). Regarding plant content of K, Ca and Mg both mycorrhizal inocula (M or RI) resulted as active as the highest P-fertilization in the uptake of these nutrients (Table 1).

Table 1. Comparative effect of fertilizers [1P (25 ppm P); 2P (50 ppm P)], authochthonous AM fungal consortium (M) or the reference *R. intraradices* (RI), over control (C) on the dry weight of shoot and root (g) and content of P, K, Ca, Mg (mg per plant), Fe, Mn, Zn and Cu (μg per plant) of *R. sphaerocarpa* under drought conditions.

	Shoot dry weight	Root dry weight	P	K	Ca	Mg	Fe	Mn	Zn	Cu
C	1.37 a	1.71 a	1.3 a	7.7 a	10.6 a	2.1 a	158.6 b	156.0 b	140.6 b	8.6 b
1P	2.28 b	2.81 b	2.2 b	16.2 b	8.9 a	3.0 a	152.7 b	142.9 b	160.2 b	8.1 b
2P	2.02 b	2.78 b	2.2 b	14.5 b	15.7 ab	4.1 b	136.9 ab	138.1 b	151.6 b	7.4 b
M	2.45 b	2.70 ab	2.9 b	15.1 b	17.2 b	6.5 b	144.3 b	143.4 b	128.7 b	8.7 b
RI	2.05 b	1.57 a	4.8 c	17.7 b	17.2 b	5.3 b	91.4 a	72.2 a	41.6 a	4.3 a

Within each parameter values having a common letter are not significantly different ($p \leq 0.05$) as determined by Duncan's multiple-range test (n=5).

The content of the micronutrients Fe, Mn, Zn and Cu did not significantly change irrespective of the applied treatments with the exception of *R. intraradices* that highly depressed the content of whatever micronutrient here analyzed (Table 1).

There were no-significant differences in the percentage of mycorrhizal root length produced by both mycorrhiza inocula. The intraradical mycorrhizal development was analyzed in terms of frequency (%F), intensity (%M and %m) and relative and absolute arbusculum richness (%a and %A) in colonized roots (Table 2).

Table 2. Mycorrhizal symbiotic development by the authochthonous AM fungal consortium (M) or the reference *R. intraradices* (RI) on colonization frequency (%F), intensity (%M), intensity of colonization (%m), arbuscule abundance (%a) and richness of arbuscules (%A).

	%F	%M	%m	%a	%A
M	100 a	28.9 a	28.9 a	56.6 a	20.6 a
RI	100 a	30.4 a	30.4 a	64.9 a	21.1a

Within each parameter values having a common letter are not significantly different ($p \leq 0.05$) as determined by Duncan's multiple-range test (n=5).

No nodule production was observed in spite of the application of an extract from non-sterilized natural soil presumably having *Rhizobium*.

Antioxidant activities were here analyzed as an index of the ability of plants to counteract the oxidative damage caused by the drought imposed. Results show that the APX activity was the highest in P-fertilized plants and the lowest in mycorrhizal inoculated plants of similar size (Table 1, Figure 1). For SOD and CAT activities the lowest values were observed in M-colonized plants that decreased both these activities compared to whatever level of P fertilization, while *R. intraradices* reduced GR activity (Figure 1).

The β-glucosidase activity increased by 176% in response to M inoculum application (Figure 2) and dehydrogenase activity was enhanced by both inocula but only 10%. Non-significant differences on these enzymatic activities as result of the P fertilization were observed. Treatments applied did not change phosphatase and urease activities (Figure 2).

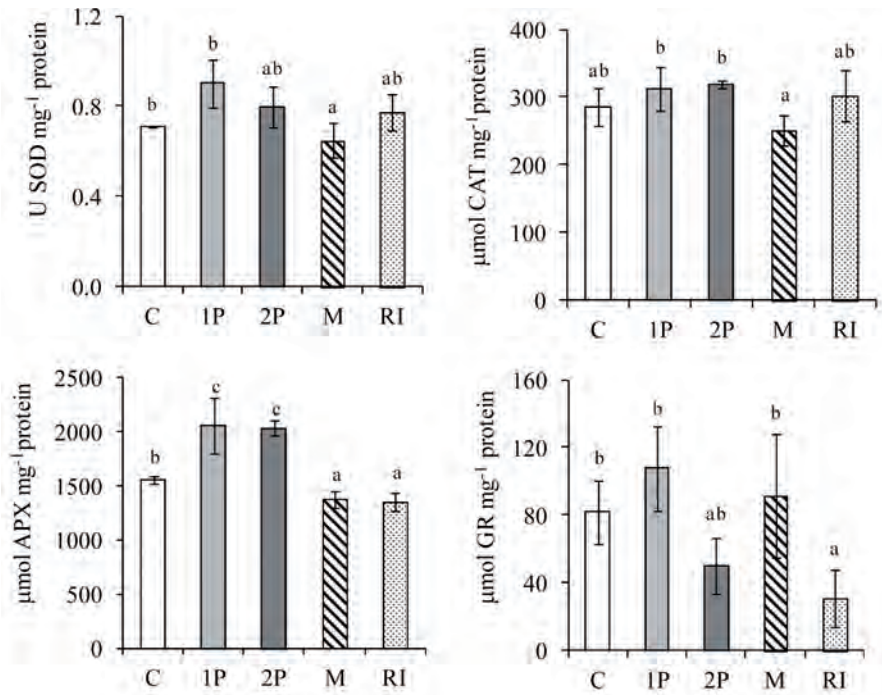

Figure 1. Comparative effect of fertilizers [1P (25 ppm P); 2P (50 ppm P)], authochthonous AM fungal consortium (M) or the reference *R. intraradices* (RI), over control (C) on superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX) and glutathione reductase (GR) antioxidant activities in shoot of *R. sphaerocarpa* under drought conditions. Within each value bars having a common letter are not significantly different ($p \leq 0.05$) as determined by Duncan's multiple-range test (n=3).

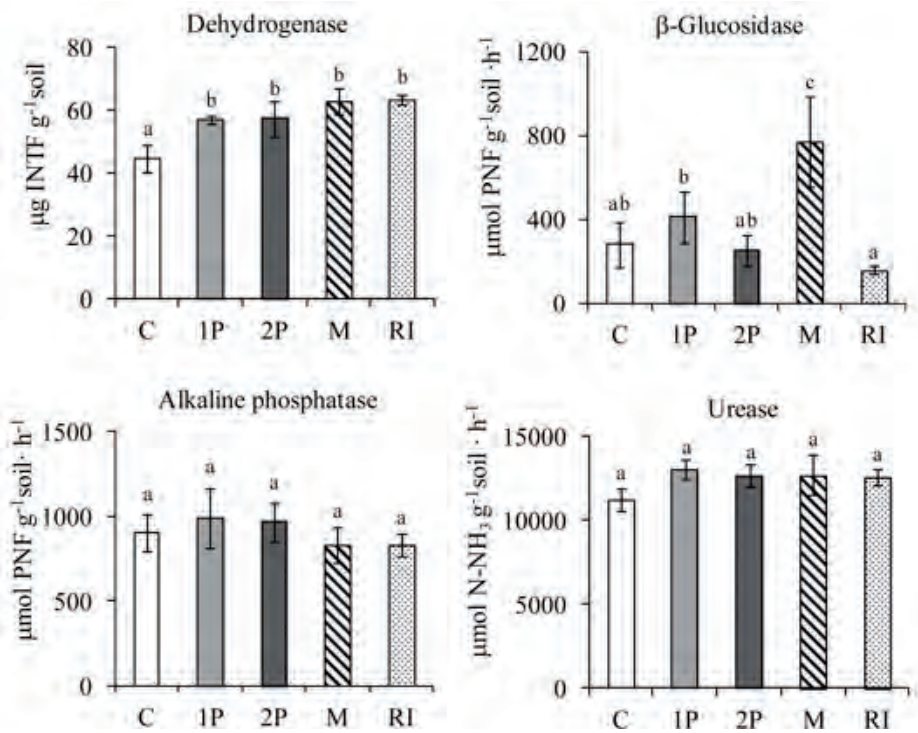

Figure 2. Comparative effect of fertilizers [1P (25 ppm P); 2P (50 ppm P)], authochthonous AM fungal consortium (M) or the reference *R. intraradices* (RI), over control (C) on dehydrogenase, β-glucosidase, alkaline phosphatase and urease enzymatic activities in soil under drought conditions. Within each value bars having a common letter are not significantly different ($p \leq 0.05$) as determined by Duncan's multiple-range test (n=3).

3.2. Experiment II

Growth of untreated control plants was not represented since it resulted negligible in this experiment which is indication on the effectiveness of applied treatments. The shoot biomass of *R. sphaerocarpa* plants was the highest in 1K fertilized plants irrespective of inocula applied (Table 3). The highest K level (2K) has a reducing, but non-significant effect on shoot growth particularly in inoculated plants. The highest level of K-fertilization (2K) increased root growth, but the inocula reduced this value (Table 3). The comparative effect on plant biomass production shows that inoculation was not important for plant growth promotion irrespective of a lower (5 mM K) or a higher (10 mM K) K level in the growing medium. Regarding plant nutrition a significant increase of P (583.3%), K (78.6%), Zn (90.8%) and Cu (219%) content was obtained in inoculated plants compared to the respective 1K fertilized non-inoculated plants (Table 3) under drought conditions. In contrast, the microbial inocula interaction with 2K decreased the plant acquisition of K, Ca, Fe and Mn compared to single 2K fertilized plants. Nevertheless, 2K fertilization enhanced P, K and Mn contents compared to the lowest K level (1K) (Table 3).

Table 3. Comparative effect of fertilizers [1K (5 mM K) and 2K (10 mM K)] in interaction or not with autochthonous fungal consortium (M) and *B. thuringiensis* (B) on the dry weight of shoot and root (g) and content of P, K, Ca, Mg (mg per plant), Fe, Mn, Zn and Cu (µg per plant) of *R. sphaerocarpa* under drought conditions.

	Shoot dry weight	Root dry weight	P	K	Ca	Mg	Fe	Mn	Zn	Cu
1K	2.46 ab	1.88 a	0.6 a	15.4 a	16.9 b	6.0 b	121.8 b	104.8 a	46.9 a	3.7 a
2K	2.14 a	2.58 b	1.8 b	24.7 b	19.3 b	5.5 ab	127.3 b	157.6 b	48.4 a	2.5 a
1K + MB	2.92 b	2.09 ab	4.1 c	27.5 b	11.1 a	3.7 a	104.5 b	67.5 a	89.5 c	11.8 c
2K + MB	1.73 a	1.87 a	2.0 b	13.0 a	10.1 a	4.7 ab	75.2 a	75.6 a	68.3 b	5.4 b

Within each parameter values having a common letter are not significantly different ($p \leq 0.05$) as determined by Duncan's multiple-range test (n=5).

Table 4. Mycorrhizal symbiotic development by the authochthonous AM fungal consortium (M) plus *B. thuringiensis* (B) with whatever of K fertilizers [1K (5 mM) and 2K (10 mM)] on colonization frequency (%F), intensity (%M), intensity of colonization (%m), arbuscule abundance (%a) and richness of arbuscules (%A).

	%F	%M	%m	%a	%A
1K + MB	74.7 a	8.4 b	10.5 b	46.2 b	4.7 b
2K + MB	83.3 a	1.4 a	1.6 a	34.8 a	0.5 a

Within each parameter values having a common letter are not significantly different ($p \leq 0.05$) as determined by Duncan's multiple-range test (n=5)

The impact of each K level on the development of the mycorrhizal colonization was only relevant regarding values as intensity (%M and %m) and richness of arbuscules (%a and %A). Both mycorrhizal parameters were highly depressed by 2K fertilization (Table 4).

All antioxidant activities determined were enhanced by the highest K (2K) level applied (Figure 3). The strongest depressing effect in the antioxidant activities was observed as result of K-fertilization and inocula interaction. The lowest values of whatever antioxidant activity was measured in inoculated plants irrespective of K level being CAT and GR totally depressed. The SOD and APX activities also decreased by the microbial inoculation applied being SOD 1.8 (1K) and 2.4 (2K) times lower while APX was reduced by 2.33 (1K) and 5.92 (2K) times (Figure 3).

The treatments applied did not change dehydrogenase and urease enzymatic activities. Regarding β-glucosidase, this activity increased with the higher level of K and also as result of microbial inoculation. In fact, inoculated plants with 2K resulted most effective in increasing β-glucosidase activity in rhizosphere soil (Figure 4). Nevertheless, the inocula behaved in a different way regarding the alkaline phosphatase activity since the inocula highly enhanced this activity associated to 1K. Non-significant differences between 1K and 2K and between 2K with and without inocula were found (Figure 4).

As in the Experiment I, no nodules were formed by native rhizobial population in roots of *R. sphaerocarpa* plants despite an extract of natural soil being added, after transplanting, in all the pots.

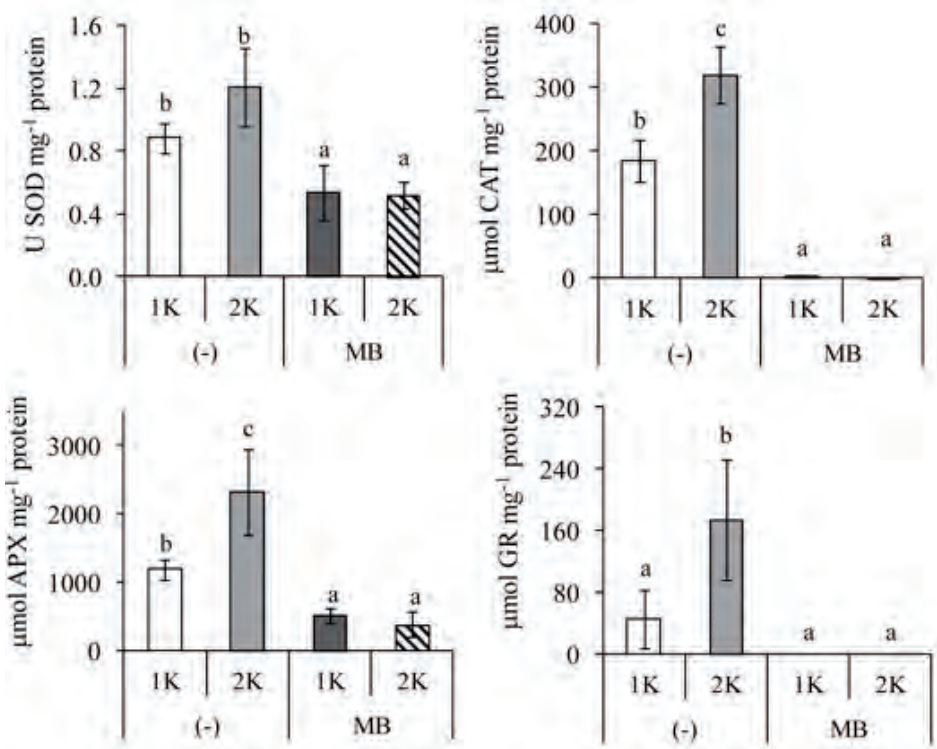

Figure 3. Comparative effect of fertilizers [1K (5 mM K) and 2K (10 mM K)] in interaction or not with authochthonous mycorrhizal fungal consortium (M) and *B. thuringiensis* (B) on superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX) and glutathione reductase (GR) antioxidant activities in shoot of *R. sphaerocarpa* under drought conditions. Within each value bars having a common letter are not significantly different ($p \leq 0.05$) as determined by Duncan's multiple-range test (n=3).

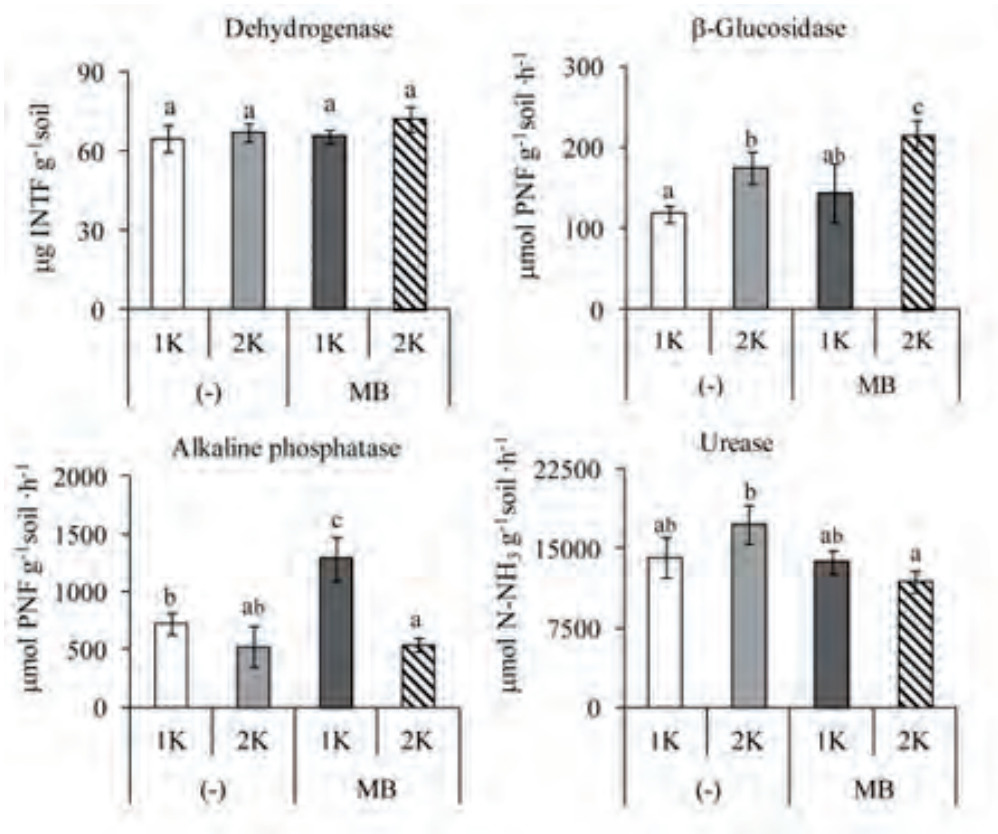

Figure 4. Comparative effect of fertilizers [1K (5 mM K) and 2K (10 mM K)] in interaction or not with authochthonous mycorrhizal fungal consortium (M) and *B. thuringiensis* (B) on dehydrogenase, β-glucosidase, alkaline phosphatase and urease enzymatic activities in soil under drought conditions. Within each value bars having a common letter are not significantly different ($p \leq 0.05$) as determined by Duncan's multiple-range test (n=3).

4. Discussion

Here we addressed the comparative role of P fertilization rate (25 or 50 ppm) and of mycorrhizal symbiosis (from allochthonous and autochthonous strains) in the drought tolerance of *R. sphaerocarpa* plants growing in an arid Mediterranean soil.

The comparative effect of single *R. intraradices* from collection vs. the whole autochthonous AM fungal community evidenced non-significant differences in terms of mycorrhizal colonization, growth and nutritional content (K, Ca and Mg). Moreover, the fungus *R. intraradices* resulted to be more efficient on P acquisition and less on Fe, Mn, Zn and Cu. Our initial hypothesis was that the autochthonous fungal

community ought to be the most efficient inocula under these experimental conditions as suggested by Caravaca *et al*. (2005) in promoting P uptake, more than the autochthonous AM consortium or whatever level of P fertilizer applied (25 or 50 ppm). Marulanda *et al*., (2003) reported that the different impact of mycorrhizal inocula to improve plant growth under drought stress may be attributed to the differences in intra and/or extraradical colonization and proportion of active structures as arbuscules, but in this study the mycorrhizal inoculated plants did not vary in their mycorrhizal development.

Results show that the mycorrhizal inoculation irrespective of fungal origin resulted as positive as phosphorus fertilization in improving *R. sphaerocarpa* growth. In fact, both mycorrhizal inocula behaved differently regarding *R. sphaerocarpa* nutrient acquisition in spite of similar frequency/intensity and arbuscular development of these mycorrhizal colonizations. No correlation between mycorrhizal root colonization and P nutrition was found since no differences in terms of intraradical mycorrhizal development were observed between the two mycorrhizal inocula used.

In general, AM symbiosis can increase plant biomass due to the highest acquisition of nutrients specially those having low mobility. The content of P was maximized in *R. sphaerocarpa* plants colonized by the reference *R. intraradices* and this nutritional effect could be associated with a greater ability for P transfer from soil to the plant for the colonizing RI fungus. The allochthonous RI maintained its positive effect under water stress when inoculated in soil different from their isolation source.

The nutritional results were not related to the growth effect when comparing chemical (1P or 2P) and biological (M or RI) fertilization. The increased K and P content in inoculated plants could have enhanced some physiological and biochemical plant parameters, which may be even more relevant than the nutrition in the tolerance under drought associated with the mycorrhizal activity. It is well known that K has an important role as inorganic osmolyte, which in turn increases the osmotic potential within the cell, while Ca is important in membrane protection and Mg modulates ionic currents across the chloroplasts and vacuole membranes (regulating stomatal opening and ion balance in cells) under dry conditions (Parida and Jha 2013). The enhancement of Mg content in mycorrhizal plants suggests that the functioning of photosynthetic apparatus was not affected by drought in mycorrhizal colonized *R. sphaerocarpa*, though drought lends to severe damage to membrane integrity in many plants (Silva *et al*. 2010).

Antioxidant processes reflect the modified redox status of the stressed cells in plants (Gururani *et al*. 2013). Thus, specific antioxidant activities, particularly APX, were highest in P fertilized plants and lowest in mycorrhizal plants. These results suggest that water seems to be less limited in mycorrhizal plants and thus, these plants did not require to increase APX and CAT activities to counteract ROS production by drought stress (Armada *et al*. 2014). Results indicate that P-fertilized plants suffer a stronger drought stress than mycorrhizal plants having similar growth and nutrition. Antioxidant activities have been proposed as stress indexes since they are highly sensitive to the metabolic and physiological status of plants (Ortiz *et al*. 2015). The efficient destruction of O^{2-} and H_2O_2 generated under water stress requires the action of antioxidant enzymes acting in synchrony to minimize these toxic radicals. Lowest activities of antioxidant enzymes were found in mycorrhizal plants, which preclude a direct role of these enzymes in this process. Particularly APX activity is the main antioxidant related with the maintenance of osmotic balance and also it may facilitate the nutrient uptake in colonized plants. Different roles of this symbiosis in drought

alleviation in relation to changes in particular antioxidant activities have been reported ranging from increase (Garg and Kaur 2013) to decrease (Yang *et al*. 2009). Alternatively, different changes on such activities in plants colonized by different fungi have been found (Marulanda *et al*. 2007). The lowest APX activity in mycorrhizal plants than in P-fertilized plants of similar development supports the view of the crucial role of this symbiosis in alleviating drought stress. The lowest CAT and SOD activities (in AM-colonized plants) and GR activity (in *R. intraradices* inoculated plants) also indicates drought-tolerance in mycorrhizal plants. These results may be regarded as greater protective capacity and as a proof of maintenance of water uptake under low water availability. The autochthonous fungi (M) and also RI resulted an essential component to protect plants against stress conditions. However, the combinations of nutritional, physiological and biochemical mechanisms seem to play a crucial role in drought tolerance. These results suggest that to successfully establish plant in drought arid areas it is important to use efficient and adapted mycorrhizal fungi.

The comparisons of autochthonous mycorrhizal (M) and P-fertilized plants having similar P nutrition and root dry weight, but different antioxidant activities and elements such as Fe, Mn, Zn and Cu have relevance and important impact in plant physiological and biochemical processes related to the drought tolerance (Ruíz-Lozano and Azcón 1995). These results allow to conclude that independent and additional mechanisms other than nutritional are involved in the drought tolerance of mycorrhizal plants (Augé 2004). Nevertheless, the differences in these parameters between both mycorrhizal inocula preclude any broader generalization.

From experiment II we showed that the combination of autochthonous microorganisms applied (AM fungi consortium plus *B. thuringiensis*) resulted highly effective in improving P, K, Zn and Cu content only at the lowest K level applied (5 mM K), but not at the highest K level (10 mM) in the growing medium. According to these results the potential of inocula to alleviate drought stress was limited beyond a certain level of K.

The 2K level (10 mM K) increased P, K and Mn over 1K (5 mM K) in *R. sphaerocarpa* shoots, but at this highest level of K the AM colonization was highly depressed (particularly intensity and arbuscule abundance).

The inocula did not affect *R. sphaerocarpa* growth, but in contrast, pronounced differences in nutrient assimilation was shown according to the inocula/K level interaction. Here, the highest P and K plant content in 1K inoculated plants correlated with the highest intraradical mycorrhizal colonization determined. High differences in these values were observed among plants inoculated under each one of these two K levels. Differences in the amount of active fungal structures as arbuscules is an explanation for the better fungal performance and functioning in arid environments as have suggested by Marulanda *et al*., (2003).

In the experiment I, whatever chemical or biological treatment applied increased dehydrogenase activity particularly each one of the mycorrhizal inocula. This enzymatic activity reflected soil microbial community. The reactivation of the rhizosphere microbial populations by the inocula is an indication of rehabilitation of degraded soils. β-glucosidase activity was only increased in soil inoculated with the autochthonous mycorrhizal consortium (M) that was increased by 2.76 times, which indicates carbohydrates transformation that is important as energy source for microorganism. Consequently, mycorrhizal inoculation not only increased plant characteristics, but also the microbial properties and quality of arid soils. In a previous study (Azcón *et al*. 2013) reported that autochthonous mycorrhizal fungi not only affected the bacterial

microbial structure, but also increased the microbial diversity (by 233%) compared to P fertilization.

Similarly, in Experiment II, the inocula significantly increased the phosphatase activity (in 1K fertilized soil) and β-glucosidase activity (in 2K fertilized soil). Measurement of these soil hydrolases are indicators of changes in soil fertility since they are involved in the mineralization of compounds that provide nutrients as N, P and C. The effectiveness of inocula in this experiment was based on a direct improvement of nutrient status particularly P, K, Zn and Cu. The highest P shoot content in these plants could be explained by the PGPR (plant growth promoting rhizobacteria) abilities of inocula applied and also by the highest value of phosphatase activity in the rhizosphere of these plants. The main role of this phosphatase is to catalyze the hydrolysis of organic phosphates increasing the P available to plants and thus improving plant P uptake. The enhancement of soil enzyme activities, particularly β-glucosidase, by the inocula may be related to the reactivation of the rhizosphere microbial population by increasing water soluble C. Carbohydrates are also involved in aggregate stabilization and soil water retention. Thus, these values indicated that the applied inocula may enhance rehabilitation of arid degraded soils contributing to soil fertility and quality (Medina and Azcón 2012).

As the results show, the higher performance of inoculated plants than those fertilized reaffirm the important role of inocula applied in sustaining the plant cover under drought in these nutrient deficient arid soils.

5. Conclusions

The mycorrhizal effect in enhancing shoot biomass and growth was similar to this produced by P fertilization however a drop in particular antioxidant activities in mycorrhizal colonized plants as APX (by M and RI inocula) CAT (by M inoculum) and GR (by RI inoculum) may indicate the highest potential of mycorrhizal colonization to alleviate drought stress in these plants.

Inocula (M+B) positively interacted on nutrient acquisition with the lowest K fertilization (5 mM K) and negatively with the highest (10 mM K). Lower SOD and APX activities and the suppression of CAT and GR in inoculated K-fertilized plants may indicate the highest ability of inoculated plants to cope with drought independently of nutritional status.

Mycorrhizal inoculants may be more important than chemical fertilization orchestrating antioxidant activities along the process of drought tolerance.

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