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Antioxidant enzyme activities in shoots from three mycorrhizal shrub species afforested in a degraded semi-arid soil

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Mycorrhizae may help plants to thrive in Mediterranean semi-arid ecosystems by altering antioxidant enzyme activities. Our objective was to determine the influence of mycorrhizal inoculation with an allochthonous arbuscular mycorrhizal (AM) fungus, *Glomus claroideum*, Schenck & Smith, or with a mixture of native AM fungi, on the activity of antioxidant enzymes from shoots of *Olea europaea* L. ssp. *sylvestris*, *Retama sphaerocarpa* (L.) Boissier and *Rhamnus lycioides* L. seedlings afforested in a degraded Mediterranean semi-arid soil. One year after planting, shoot biomass of inoculated *O. europaea* seedlings was about 630%, of non-inoculated plants. Shoot biomass of G. *claroideum*-colonized *R. sphaerocarpa* was greater than that of seedlings inoculated with the mixed native AM fungi after 12 months. Inoculation with a mix of native AM fungi was the most effective treatment for increasing shoot biomass and N, P and K contents in shoot tissues of *R. lycioides*. Both mycorrhizal inoculation treatments increased the nutrient contents in shoots of *O. europaea* and *R. lycioides*. In *O. europaea* plants, the inoculation treatments increased catalase, ascorbate peroxidase and dehydroascorbate reductase activities, but not monodehydroascorbate reductase and glutathione reductase activities. Inoculation with G. *claroideum* increased the activities of all antioxidant enzymes in *R. sphaerocarpa*. Monodehydroascorbate reductase, glutathione reductase and superoxide dismutase activities in *R. lycioides* leaves were preferentially increased by inoculation with the mixture of native AM fungi. This work support the view that increased antioxidant enzyme activities could be involved, at least in part, in the beneficial effects of mycorrhizal colonization on the performance of shrub species grown under semi-arid Mediterranean conditions.

Introduction

Mycorrhizae are widespread under natural conditions and occur in nearly all soils, from mine spoil (Jasper et al. 1989) to agricultural soils (Abbot and Robson 1982). Arbuscular mycorrhizal (AM) fungi can colonize the roots of most vascular plants and can develop a complex system of extraradical hyphae under natural conditions. Mycorrhizae may help plants to thrive in arid conditions (Requena et al. 2001) by increasing the supply of nutrients to the plant (particularly P) (Toro et al. 1997), improving soil aggregation in eroded soils (Caravaca et al. 2002) and reducing water stress (Augé 2001). Therefore, re-vegetation programmes that are being developed in semi-arid Mediterranean areas of south-east Spain include the use of mycorrhizal inoculation technologies to increase re-vegetation success (Caravaca et al. 2002).

Activated oxygen species (AOS), such as superoxide ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2) and hydroxyl radicals ($^{\bullet}OH$), are formed as by-products of normal metabolism in different cellular organelles (Scandalios 1993). In the absence of protective mechanisms, they can damage cell structure and function. Environmental stresses, such as drought and salt stress, may cause damage to cells either directly or indirectly, through the formation of AOS (Menconi et al. 1995, Tambussi et al. 2000, Hernández et al. 2001). To mitigate and repair damage initiated by

Abbreviations – AM, arbuscular mycorrhizal; AOS, activated oxygen species; ASC–GSH cycle, ascorbate–glutathione cycle; APX, ascorbate peroxidase; CAT, catalase; DHAR, dehydroascorbate reductase; GSH, glutathione reduced form; GR; glutathione reductase; MDHAR, monodehydroascorbate reductase; SOD superoxide dismutase.

AOS, plants have developed a complex antioxidant system. The primary components of this system include carotenoids, ascorbate, glutathione, tocopherols and enzymes such as superoxide dismutase (SOD, EC 1.15.1.1), catalase (CAT, EC 1.11.1.6), glutathione peroxidase (GPX, EC 1.11.1.9), peroxidases and the enzymes involved in the ascorbate–glutathione cycle (ASC–GSH cycle; Foyer and Halliwell 1976): ascorbate peroxidase (APX, EC 1.11.1.1), dehydroascorbate reductase (DHAR, EC 1.8.5.1), monodehydroascorbate reductase (MDHAR, EC 1.6.5.4) and glutathione reductase (GR, EC 1.6.4.2) (Noctor and Foyer 1998). Many components of this antioxidant defence system can be found in different subcellular compartments (Jiménez et al. 1997, Gómez et al. 1999, Hernández et al. 2000, 2001). We hypothesized that mycorrhizae may help plants to thrive in Mediterranean semi-arid ecosystems, where the water deficit seriously limits plant growth, by altering antioxidant enzyme activities.

The effects of mycorrhizal inoculation on SOD isozymes in mycorrhizal roots of red clover (Palma et al. 1993) and *Pisum sativum* L. (Arines et al. 1994) plants and on the SOD activity in shoots of mycorrhizal *Lactuca sativa* L. plants (Ruiz-Lozano et al. 1996) have been reported. Recently, the activity levels of some antioxidant enzymes have been investigated in roots and nodules of mycorrhizal soybean plants (Porcel et al. 2003). However, there are no reports about the role of mycorrhizal inoculation on the activities of catalase and ASC–GSH cycle enzymes in shoots from mycorrhizal plants.

The objective of our study was to determine the influence of mycorrhizal inoculation with an allochthonous AM fungus, *Glomus claroideum*, Schenck & Smith or with a mixture of native AM fungi on the antioxidant enzyme activities (SOD, CAT, APX, DHAR, MDHAR and GR) from shoots of *Olea europaea* L. ssp. *sylvestris*, *Retama sphaerocarpa* (L.) Boissier and *Rhamnus lycioides* L. seedlings afforested in a degraded Mediterranean semi-arid soil. We also investigated whether changes in the antioxidant enzymes were related to the effects on growth of the shrub species induced by each mycorrhizal inoculation treatment.

Materials and methods

Study sites

The experimental area is located on the El Picarcho range in the Province of Murcia (south-east Spain) (co-ordinates: 1°10′ W and 38°23′ N). The climate is semi-arid Mediterranean, with a total rainfall of 315 mm and a mean temperature of 20°C during the experiment. The topography of the area is mainly flat and slopes do not exceed 6%. The climax vegetation is dominated by shrubs of *Olea europaea* ssp. *sylvestris*, *Retama sphaerocarpa* and *Rhamnus lycioides*, which were selected as target species. The plant cover is sparse (less than 20% canopy cover) and degraded due to ancient grazing and logging. In this area, dwarf shrubs (< 1 m high) such as *Rosmarinus officinalis* and *Stipa tenacissima* grass are very common, constituting more than 98% of plant cover. Bare soil surfaces are abundant between the patches of plants. The soil is a Petrocalcic Xerosol (FAO 1988), developed from limestones, with a silt loam texture. Some characteristics of the soil are shown in Table 1.

Plants and mycorrhizal treatments

The plants used, *O. europaea* ssp. *sylvestris*, *R. sphaerocarpa* and *R. lycioides*, are three representative shrub species from semi-arid scrublands in south-east Spain. They are also well-adapted to water stress conditions and therefore frequently used in the re-vegetation of semi-arid disturbed lands.

The mycorrhizal fungi used were either *Glomus claroideum* Schenck & Smith (EEZ 24) or a mixture of endophytes isolated from the El Picarcho range, a semi-arid area where the target plants naturally grow, consisting of *G. geosporum* (Nicol. & Gerd.) Walker (EEZ 31), *G. albidum* Walker & Rhodes (EEZ 39), *G. microaggregatum* Koske, Gemma & Olexia (EEZ 40), *G. constrictum* Trappe (EEZ 42), *G. mosseae* (Nicol. & Gerd.) Gerd. & Trappe (EEZ 43), *G. coronatum* Giovannetti (EEZ 44), *G. intraradices* Schenck & Smith (EEZ 45) and a *Glomus* sp. (EEZ 46). The acronym EEZ refers to Estación Experimental Zaidín, Granada, Spain.

The 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 AM inoculum was applied at a rate of 5% (v : v). The same amount of autoclaved mixture of the inocula was added to control plants, supplemented with a filtrate (< 20 μm) of culture to provide some of the microbial populations accompanying the mycorrhizal fungi. Inoculated and non-inoculated seedlings were grown for 8 months under nursery conditions without any fertilizer treatment by Paisajes del Sur Ltd. (Granada, Spain).

Experimental design and layout

The experiment was conducted as three independent one-factor factorials (one per plant species) with five replica-

Table 1. Some characteristics of the soil used for the re-vegetation experiment.

pH (H_2O)	8.54* (0.01)
Electrical conductivity (1 : 5) ($\mu S\,cm^{-1}$)	237 (4)
Total organic carbon ($g\,kg^{-1}$)	22.2 (0.7)
Water soluble carbon ($\mu g\,g^{-1}$)	134 (13)
Total nitrogen ($g\,kg^{-1}$)	0.7 (0.1)
Available P ($\mu g\,g^{-1}$)	3 (0)
Extractable K ($\mu g\,g^{-1}$)	702 (47)

*Each value is the mean of five soil samples (SE).

tion blocks. The factor had three levels: non-inoculation, inoculation with *G. claroideum* and inoculation with the mixture of native AM fungi. In early January 2000, an area of 1200 m^2 at the El Picarcho site was mechanically prepared with a subsoiler. Three rows (1 m wide, 25 m long, 3 m apart) were established. Seedlings of the three selected shrub species (inoculated and non-inoculated) were planted in individual holes, at least 1 m apart in a single row and with 3 m between blocks. At least 15 seedlings per factor level per replication block of each shrub species were planted (225 plants per shrub species).

The experiment was carried out under natural conditions, without any watering or fertilizer treatments.

Sampling and laboratory procedures

One year after planting, five plants (one per block) of each treatment were harvested, excavating manually a hole 40 cm wide, 40 cm long and 40 cm deep. For mycorrhizal assays three subsamples from the upper, middle and lower root system were taken. Sampling was based on root colour and morphology to get a mixed age sample and avoiding woody roots. Basal stem diameters and heights of plants were measured with callipers and rulers. Fresh and dry (105°C, 5 h) weights of shoots and roots were recorded. The plant tissues were ground before chemical analysis. The foliar concentrations of nitrogen, phosphorus, potassium, iron, copper, manganese, zinc, calcium and magnesium were determined after digestion in nitric-perchloric acid (5 : 3) for 6 h at 210°C. P was determined colorimetrically according to Murphy and Riley (1962), N was determined by the Kjeldhal method and K, Fe, Cu, Mn, Zn, Ca and Mg were estimated by atomic absorption.

The percentage of root length colonized by AM fungi was calculated by the gridline intersect method (Giovannetti and Mosse 1980) after staining with trypan blue (Phillips and Hayman 1970).

Leaf enzyme extraction

All operations were performed at 4°C. Shoots (2 g) were homogenized with a mortar and pestle in 4 ml of ice-cold 50 m*M* K-phosphate buffer (pH 7.8), 0.1 m*M* EDTA containing 5 m*M* cysteine, 2% (w/v) polyvinylpolypyrrolidone (PVPP), 0.1 m*M* phenylmethylsulfonyl fluoride and 0.2% (v/v) Triton X-100. For APX activity, 20 m*M* sodium ascorbate was added. The homogenate was centrifuged at 14 000 *g* for 20 min and the supernatant fraction was filtered through Sephadex G-25 NAP columns (Amersham Pharmacia Biotech AB, Uppsala, Sweden), equilibrated with the same buffer used for the homogenization, with or without 5 m*M* sodium ascorbate.

Enzymatic activities of the ascorbate–glutathione cycle and assays

APX, DHAR, MDHAR and GR activities were assayed according to previously published protocols, as described by Jiménez et al. (1997). Enzyme activities were corrected for non-enzymatic rates and for interfering oxidations (Jiménez et al. 1997). APX was measured in the presence and absence of the specific inhibitor *p*-chloromercuryphenyl sulphonic acid (*p*CMPS) (0.5 m*M*). The *p*CMPS-sensitive ascorbate peroxidase activity was considered as being due to class I ascorbate peroxidase (EC 1.11.1.11).

CAT was measured according to Aebi (1984) and total SOD activity was assayed by the ferricytochrome *c* method using xanthine/xanthine oxidase as the source of $O_2^{\bullet -}$ radicals (McCord and Fridovich 1969).

Protein was estimated according to Bradford (1976).

Statistical analysis

Data were log-transformed to compensate for variance heterogeneity before analysis of variance. 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 (SPSS Inc., Chicago, IL, USA).

Results

Changes in plant growth, mycorrhizal colonization and nutrient acquisition

One year after planting, the survival rates of non-inoculated plants were highest for *R. sphaerocarpa* followed by *R. lycioides* and *O. europaea* ssp. *sylvestris*. The mixture of native AM fungi increased the percentages of plant survival of *O. europaea* ssp. *sylvestris*, *R. sphaerocarpa*, whereas the inoculation with *G. claroideum* was only effective for *O. europaea* ssp. *sylvestris*. Shoot biomass of the *O. europaea* ssp. *sylvestris*, *R. sphaerocarpa* and *R. lycioides* plants inoculated with the mixture of native AM fungi or *G. claroideum* was greater than that of non-inoculated plants (Table 2), particularly for inoculated *O. europaea* ssp. *sylvestris* plants (on average, about 630%). The mycorrhizal inoculation treatments showed different levels of effectiveness in improving the growth of the three shrub species. The mixture of native AM fungi was equally as (in *O. europaea*) or even more (in *R. lycioides*) effective than the allochthonous AM fungus G. *claroideum* in increasing in plant growth. However, shoot dry weight of *R. sphaerocarpa* inoculated with themixture of native AM fungi was lower than for *G. claroideum*-colonized seedlings, 12 months after planting.

The inoculation of plants with *G. claroideum* or with the mixture of native AM fungi resulted in shoot/root ratio of 4.3- and 1.1-fold for *O. europaea* and *R. lycioides,* respectively, in comparison with control plants (Table 2). However, the shoot/root ratio of inoculated *R. sphaerocarpa* seedlings was lower than that of non-inoculated plants. With the exception of *G. claroideum*-colonized *R. lycioides*, the FW/DW ratio was increased 1.1-fold by the mycorrhizal inoculation treatments compared with non-inoculated plants (Table 2). The mixture of native AM fungi was more effective than *G. claroideum*

Table 2. Effect of inoculation with the allochthonous AM fungus *G. claroideum* and with a mixture of native AM fungi on survival rates, growth parameters, root colonization and total nutrient uptake in *O. europaea* ssp. *sylvestris*, *R. sphaerocarpa* and *R. lycioides* plants. C, non-inoculated plants; M, plants inoculated with a mixture of native arbuscular mycorrhizal fungi; G, plants inoculated with *G. claroideum*. FW, fresh weight; DW, dry weight. For plant species, values in rows sharing the same letter are not significantly different ($P < 0.05$) by the LSD test.

	O. europaea ssp. *sylvestris*			*R. sphaerocarpa*			*R. lycioides*		
	C	M	G	C	M	G	C	M	G
Survival (%)	40a	100b	100b	80a	100b	80a	60a	60a	60a
Shoot (g DW)	0.64a	4.92b	4.42b	3.30a	4.47b	5.30c	0.56a	2.22c	0.81b
Shoot/root	0.55a	2.56c	2.13b	1.79b	1.56a	1.63a	1.56ab	1.71b	1.76b
FW/DW	1.71a	1.97b	1.84b	1.92a	2.10b	2.04b	1.56a	1.72b	1.58a
Mycorrhizal root (%)	9a	72c	48b	9a	73b	72b	6a	68c	31b
Nitrogen (mg plant^{-1})	4.80a	68.00b	62.78b	52.69a	91.53b	78.71ab	5.05a	33.49b	7.27a
Phosphorus (mg plant^{-1})	0.36a	4.00c	2.76b	0.97a	2.42b	2.75b	0.29a	1.38b	0.44a
Potassium (mg plant^{-1})	3.52a	34.90b	27.35b	15.59a	26.71b	16.13b	2.09a	6.48b	2.39a
Iron (mg plant^{-1})	0.15a	0.86b	1.81c	0.17a	1.74b	0.41a	0.56a	1.11b	1.25b
Copper (mg plant^{-1})	0.03a	0.09b	0.07b	0.02a	0.02a	0.03a	0.03a	0.03a	0.07b
Manganese (mg plant^{-1})	0.07a	0.28b	0.33b	0.09a	0.26b	0.15ab	0.21a	0.20a	0.43b
Zinc (mg plant^{-1})	0.01a	0.09b	0.10b	0.05a	0.08b	0.10b	0.06a	0.13a	0.15a
Calcium (mg plant^{-1})	9.36a	67.34b	61.10b	8.09a	48.77b	13.57a	27.34a	33.28a	47.89a
Magnesium (mg plant^{-1})	1.44a	12.60b	10.68b	1.11a	6.84b	1.70a	6.02a	10.71ab	12.32b

with respect to colonization of the roots of *O. europaea* and *R. lycioides* after 1 year (Table 2). In contrast, both inoculation treatments produced a similar level (72%) of root colonization in *R. sphaerocarpa*. Naturally colonized seedlings showed $< 10\%$ colonization of the root length in all shrub species.

Inoculation with *G. claroideum* or the mixture of native AM fungi stimulated N, P, K, Mg and Ca accumulation in shoot tissues of *O. europaea* and *R. sphaerocarpa* (Table 2), being higher in *O. europaea*. Macronutrient contents between seedlings inoculated with different fungal treatments were similar, except that the *O. europaea* seedlings colonized by native AM fungi accumulated more P in shoots than did seedlings colonized by *G. claroideum*. As observed for the growth parameters, the highest N, P and K contents were in the *R. lycioides* plants inoculated with the mixture of native AM fungi. However, inoculation with *G. claroideum* did not affect N, P or K contents in shoots of *R. lycioides*.

Both mycorrhizal inoculation treatments increased the micronutrient (Fe, Cu, Mn, Zn) contents in shoots of *O. europaea* and generally were similar (Table 2). The highest micronutrient contents were recorded in shoots of *R. sphaerocarpa* inoculated with the mixture of native AM fungi. With th exception of Zn, inoculation with *G. claroideum* did not affect micronutrient contents of *R. sphaerocarpa* shoots. Inoculation with *G. claroideum* was generally the more effective mycorrhizal treatment for increasing the micronutrient contents of shoots of *R. lycioides*.

Changes in antioxidant enzymes of the shoot

We used three different shrub species that showed different constitutive antioxidant enzyme levels. The non-inoculated *R. lycioides* shoots had the highest antioxidant enzymes activities (Figs 1–3). Activities of CAT, MDHAR and SOD were very low in *R. sphaerocarpa* and especially in *O. europaea*, in which APX and GR, were also low (Figs 1–3).

Mycorrhizal inoculation increased antioxidant enzymes activities in the shoots of the three target shrub species, especially in *R. lycioides*. The effects of the mycorrhizal inoculation treatments on antioxidant enzymes activities varied, depending on the shrub species. Both inoculation treatments increased the activity of CAT in *O. europaea* and *R. sphaerocarpa* shoots (Fig. 1). Only the inoculation of plants with *G. claroideum* increased CAT activity in shoots of *R. lycioides*.

The activity of APX increased in the shoots of *O. europaea* plants inoculated with *G. claroideum* or with the mixture of native AM fungi, and in the shoots of *R. sphaerocarpa* (1.2-fold) and *R. lycioides* (13-fold) plants inoculated with *G. claroideum* (Fig. 1). It is worth noteworthy that the shoots of *G. claroideum*-colonized *R. lycioides* plants had values of APX activity higher than those of *G. claroideum*-colonized *R. sphaerocarpa* (6.4-fold) and than those of *O. europaea* (29-fold).

The activity of MDHAR was not changed by either of the mycorrhizal inoculation treatments in shoots of *O. europaea* (Fig. 2). MDHAR activity increased in *R. sphaerocarpa* plants inoculated with *G. claroideum* or with the mixture of native AM fungi (on average, by about 185%) and in the shoots of *R. lycioides* plants inoculated with the mixture of native AM fungi (by about 204%). DHAR activity showed an increase in shoots of *O. europaea* and *R. sphaerocarpa* plants inoculated with the mixture of native AM fungi or with *G. claroideum* (Fig. 2). In *R. lycioides*, only the inoculation with *G. claroideum* produced a strong induction in the activity of DHAR (nearly 10-fold).

Inoculation with *G. claroideum* increased the activity of GR in shoots of *R. sphaerocarpa* and *R. lycioides* plants, whereas inoculation with the mixture of native AM fungi only had an effect on this antioxidant activity in *R. lycioides* shoots, producing even higher increases than *G. claroideum* (about 9-fold with respect to non-inoculated plants) (Fig. 3). However, the mycorrhizal inoculation treatments had no effect on GR activity

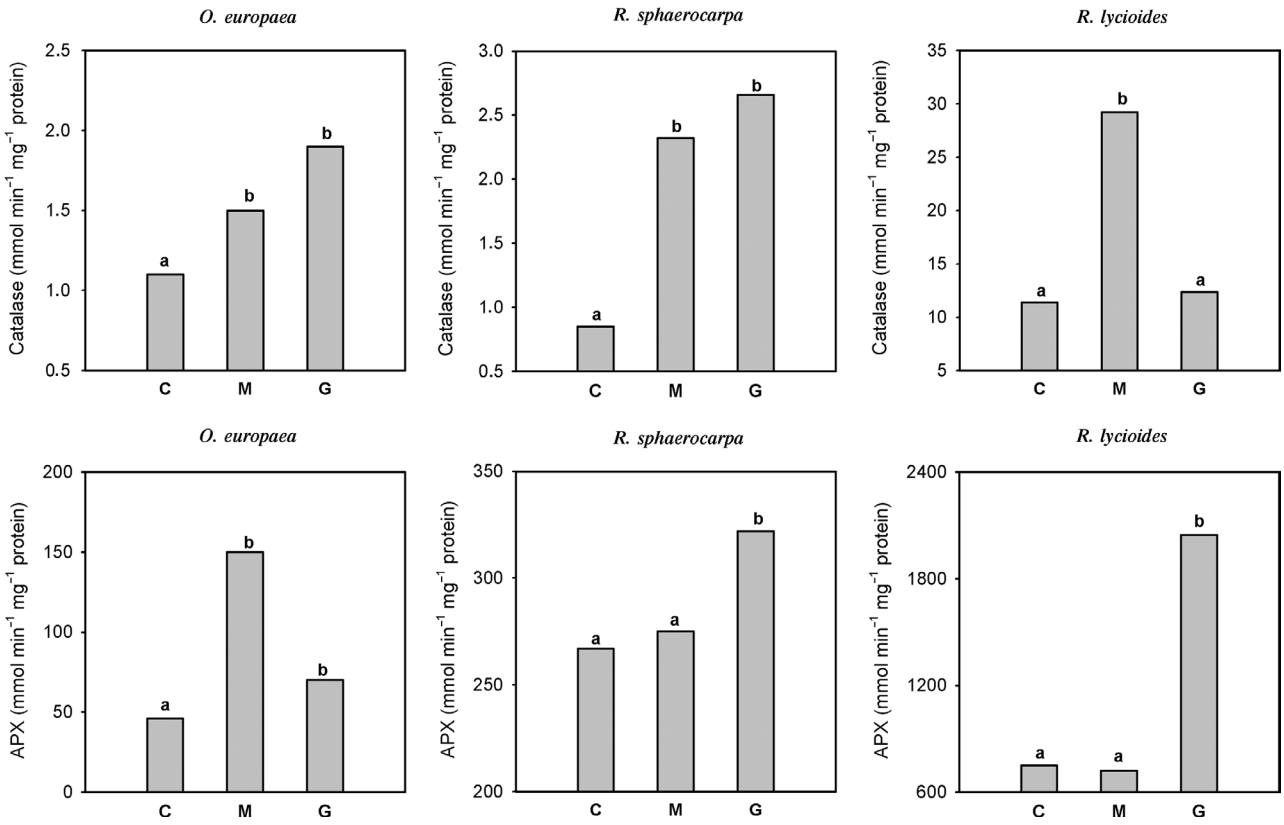

Fig. 1. Effect of inoculation with the allochthonous AM fungus *G. claroideum* and with a mixture of native AM fungi on catalase and APX activities in shoots of *O. europaea* ssp. *sylvestris*, *R. sphaerocarpa* and *R. lycioides* (C: non-inoculated plants; M: plants inoculated with a mixture of native arbuscular mycorrhizal fungi; G: plants inoculated with *G. claroideum*). Values with the same letter are not significantly different at $P < 0.05$, according to the LSD test.

from *O. europaea* shoots. There were marked differences in GR activity between inoculated plants of *O. europaea* and *R. lycioides*, especially with the inoculation of the mixture of native AM fungi (about 112-fold greater in *R. lycioides* than in *O. europaea*).

The activity of SOD increased in shoots of *O. europaea* and *R. lycioides* plants inoculated with the mixture of native AM fungi (Fig. 3). However, both inoculation treatments brought about an increase in SOD activity in shoots from *R. sphaerocarpa* plants.

Discussion

The inoculation of seedlings with an allochthonous AM fungus or a mixture of native AM fungi stimulated shoot biomass production of the three shrub species, especially *O. europaea*. The shoot/root ratio is of importance when studying the degree of AM fungi effectiveness on the host plant (Tobar et al. 1994). In this respect, *O. europaea* was the plant with the greatest increase in shoot/root ratio in response to AM followed by *R. lycioides*. Decreased shoot/root ratio in inoculated *R. sphaerocarpa* plants, compared with non-inoculated plants, indicates low mycorrhizal activity in relation to plant biomass production.

Mycorrhizal symbiosis causes important changes in plant metabolism and growth (Arines et al. 1994), although very little is known of the biochemical mechanisms involved in this mycorrhizal association. Increased growth associated with AM infection in nutrient deficient soils such as Mediterranean semi-arid soils has been attributed to enhanced nutrient uptake, especially of N *and* P (Toro et al. 1998, Requena et al. 2001). In all three shrub species, mycorrhizal inoculation appeared effective in improving macronutrient (NPK) contents, particularly in inoculated *O. europaea* plants. The major efficiency of nutrient acquisition in inoculated plants could explain their large growth differences with respect to non-inoculated plants. In Mediterranean areas, drought stress is one of the most common abiotic factors affecting plant production. Mycorrhizal fungi are known to enhance water absorption by plants grown under water-deficit conditions, owing to altered root morphology or contributions by soil hyphae (Augé 2001). Thus, the increased shoot biomass of inoculated seedlings could be partly related to the increase in water uptake that a high level of root mycorrhizal infection provides under these conditions. In fact, all inoculated plants had higher water contents than non-inoculated plants.

On the other hand, the results obtained under field conditions showed that antioxidant enzyme activities usually increased as a result of mycorrhizal inoculation treatments. In an early study carried out under drought

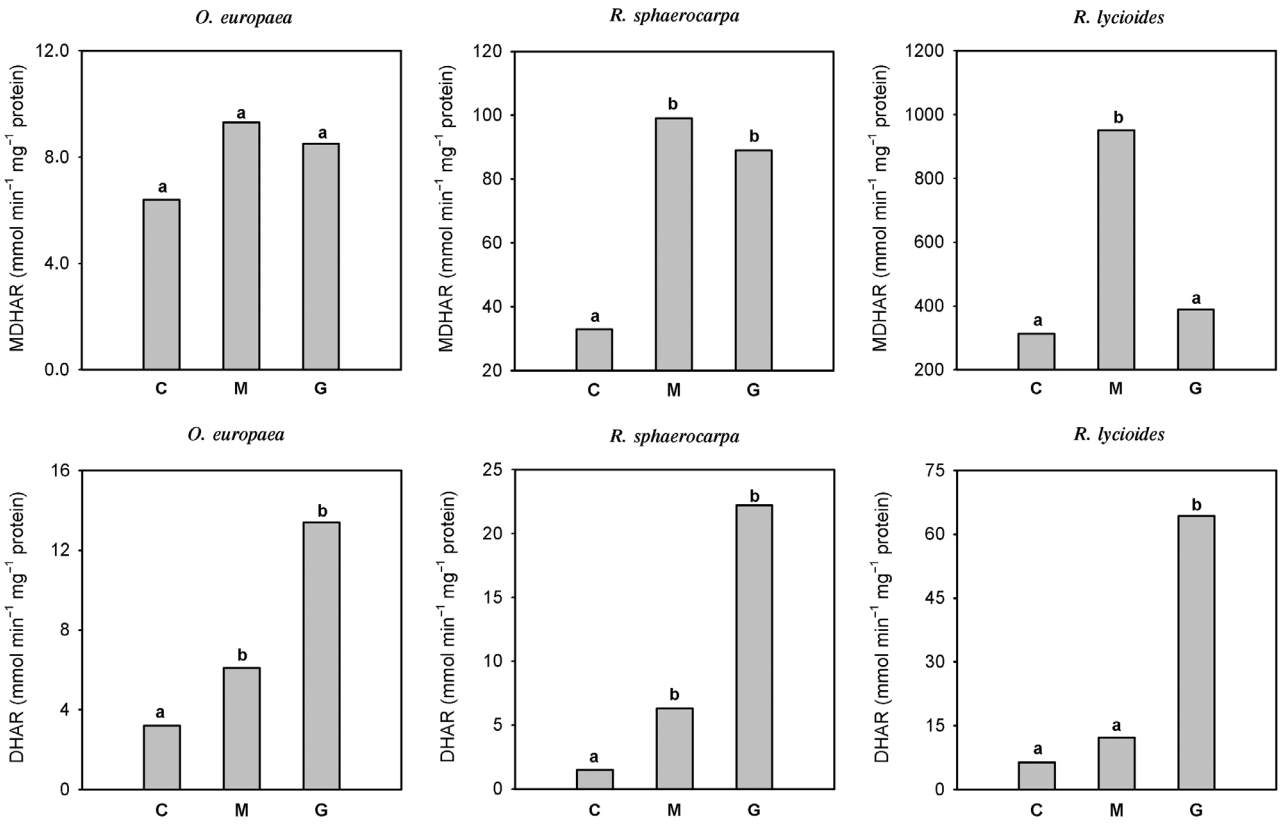

Fig. 2. Effect of inoculation with the allochthonous AM fungus *G. claroideum* and with a mixture of native AM fungi on MDHAR and DHAR activities in shoots of *O. europaea* ssp. *sylvestris*, *R. sphaerocarpa* and *R. lycioides* (C: non-inoculated plants; M: plants inoculated with a mixture of native arbuscular mycorrhizal fungi; G: plants inoculated with *G. claroideum*). Values with the same letter are not significantly different at $P < 0.05$, according to the LSD test.

stress conditions, SOD-specific activity was higher in shoots and roots of inoculated than those of P-fertilized non-inoculated *Lactuca sativa* plants, showing increases from 99 to 150% (Ruiz-Lozano et al. 1996). The increases in antioxidant enzyme activities in shoots of inoculated *O. europaea* were small in spite that such shrub species showed the highest increases in plant growth and N *and* P accumulation. In this shrub species other defence mechanisms against adverse environmental conditions differing to that of antioxidant enzymes activities could be also involved. For example, a higher Ca^{2+} and K^+ uptake were observed in inoculated *O. europaea* plants in relation to the others plant species. Calcium has been found to be involved in the regulation of various responses of plants to environmental conditions, such as salt and drought stress (Knight et al. 1997). Potassium also plays a key role in plant water stress and has been found to be the cationic solute which is responsible for stomatal movement in response to changes in bulk leaf water status (Ruiz-Lozano et al. 1995). On the other hand, mycorrhizal inoculation was less effective in increasing shoot biomass of *R. sphaerocarpa* and *R. lycioides* plants but more effective in inducing their antioxidant enzymes activities, especially in shoots of *R. lycioides*. This agrees with previous results in which *L. sativa* plants colonized with *G. deserticola* grew slowest, but showed the greatest development and stimulation of defence mechanisms against drought stress (Ruiz-Lozano et al. 1996). However, *R. lycioides* showed the lower percentage of survival. Probably, this plant species is more sensitive to the environmental stress under our experimental conditions, and suffered the highest oxidative stress. This fact could be related to both the higher constitutive levels and induction of antioxidant enzymes observed in this plant specie. However, it is also true, that in general, inoculation resulted in an increase in both growth and antioxidant enzyme levels. Since our re-vegetation experiment was carried out in semi-arid conditions, increased antioxidant enzyme activities could be a strategy adopted by such shrub species to cope with the excess of AOS that might be generated under water stress conditions (Menconi et al. 1995, Tambussi et al. 2000). Hence, the increases in antioxidant enzymes of plants inoculated with AM fungi may have important ecological consequences for the adaptation of plants to limiting conditions.

Rhamnus lycioides inoculated with the mixture AM fungi, had higher GR activity than *R. sphaerocarpa* (about 10-fold) and *O. europaea* plants (about 100-fold). The elevated levels of GR may serve to ensure the avail-

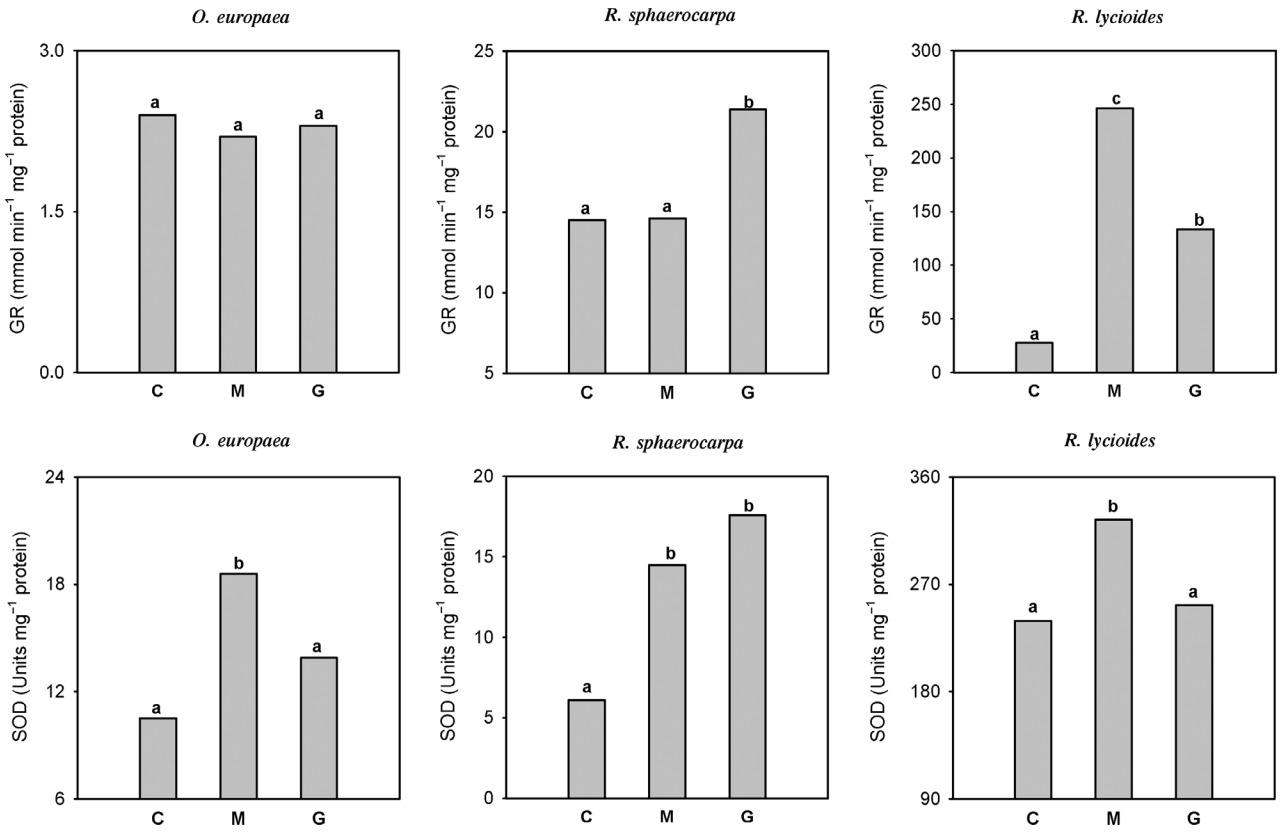

Fig. 3. Effect of inoculation with the allochthonous AM fungus *G. claroideum* and with a mixture of native AM fungi on GR and SOD activities in shoots of *O. europaea* ssp. *sylvestris*, *R. sphaerocarpa* and *R. lycioides* (C: non-inoculated plants; M: plants inoculated with a mixture of native arbuscular mycorrhizal fungi; G: plants inoculated with *G. claroideum*). Values with the same letter are not significantly different at $P < 0.05$, according to the LSD test.

ability of $NADP^+$ to accept electrons derived from photosynthetic electron transport, thereby directing electrons away from oxygen and minimizing the production of $O_2^{\bullet -}$ (Gamble and Burke 1984). Increased GR activity has been also reported in wheat plants in response to water stress (Gamble and Burke 1984, Menconi et al. 1995), suggesting that increased activity may constitute an adaptative response of wheat plants to low water potentials (Gamble and Burke 1984). However, the higher constitutive levels and induction of GR activity observed in inoculated *R. lycioides* seems not be enough to increase the percentage of survival under our experimental conditions.

CAT, APX and SODs are metalloenzymes, and micronutrients can determine the expression of these enzymes. Both excess and deficiency of micronutrients can modulate the expression of metalloenzymes. In leaves of pea plants grown under limiting Mn levels, a significant inhibition of Mn-SOD was found (del Río et al. 1991). On the other hand, it has been shown that APX gene expression can be controlled by iron at the level of mRNA accumulation (Vansuyt et al. 1997). In addition, iron loading of *Nicotiana plumbaginifolia* leaves led to an increase in CAT and APX activities (Kamfenkel et al. 1995). In the present work, the higher contents of Fe in shoots could explain, at least in part, the co-ordinated induction of APX and CAT in inoculated *O. europaea* plants and in *R. sphaerocarpa* plants inoculated with G. *claroideum*. It has been demonstrated that AM fungi increase the foliar concentrations of Zn and Cu, which are of low mobility and are present at low concentrations in the soil solution (Rodríguez et al. 1999). Arines et al. (1994) showed that three SOD isoenzymes were present in pea leaves, Cu, Zn-SOD being the most abundant. Although no isoenzyme studies have been carried out in the present work, the increases in Fe, Cu, Zn and Mn in shoots of inoculated plants could be involved in the increase in total SOD activity observed in mycorrhizal plants. However, in *O. europaea* plants inoculated with *G. claroideum* the rise in total SOD activity was not significant, although it increased by 32%.

The data indicate that mycorrhiza-induced increases in the activity of several antioxidant enzymes were often associated with mycorrhiza-induced increases in shoot biomass and P or N contents. In *O. europarea* both mycorrhizal treatments dramatically increased shoot biomass, N *and* P contents, and APX and DHAR activities. In *R. sphaerocarpa*, as well, increases in shoots dry weight and P contents in both treatments were also

associated with increases in CAT, MDHAR, DHAR and SOD activities. In *R. lycioides*, inoculated with the mixture of native AM fungi, an increase in shoot dry weight, N *and* P contents as well as CAT, MDHAR, GR and SOD activities were observed. On the other hand, to our knowledge, no work concerning the effect on leaf P on the expression and/or the function of antioxidant enzymes has been reported. In this regard, Ruiz-Lozano et al. (1996) showed that under well-watered conditions mycorrhizal *Lactuca sativa* plants had a greater P content than P-fertilized non-inoculated plants, but the two treatments had similar SOD specific activity.

The mycorrhizal inoculation treatments showed different levels of effectiveness in improving the performance of the three shrub species. The mixture of native AM fungi was equally as (in *O. europaea*), more (in *R. lycioides*) or less (*R. sphaerocarpa*) effective than the allochthonous AM fungus G. *claroideum* regarding increases in plant growth.

In conclusion, this work suggests that increased antioxidant enzyme activities could be involved, at least in part, in the beneficial effects of mycorrhizal colonization on the performance of shrub species grown under semi-arid Mediterranean conditions, although no differences were observed regarding the type of inoculated AM fungi.

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References

Abbott LK, Robson AD (1982) The role of vesicular-arbuscular mycorrhizal fungi in agriculture and the selection of fungi for inoculation. Aust J Agric Res 33: 389–408

Aebi H (1984) Catalase in vitro. Meth Enzymol 105: 121–126

Arines J, Quintela M, Vilariño A, Palma JM (1994) Protein pattern and superoxide dismutase activity in non-mycorrhizal and arbuscular mycorrhizal *Pisum sativum* L. Plants Plant Soil 166: 37–45

Augé RM (2001) Water relations, drought and vesicular-arbuscular mycorrhizal symbiosis. Mycorrhiza 11: 3–42

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72: 248–254

Caravaca F, Barea JM, Figueroa D, Roldán A (2002) Assessing the effectiveness of mycorrhizal inoculation and soil compost addition for reafforestation with *Olea europaea* subsp. *sylvestris* through changes in soil biological and physical parameters. Appl Soil Ecol 20: 107–118

FAO (1988) Soil Map of the World: Revised Legend. Food and Agriculture Organization of the Unites Nation, Rome, p. 119

Foyer CH, Halliwell B (1976) Presence of glutathione and glutathione reductase in chloroplasts: a proposed role in ascorbic acid metabolism. Planta 133: 21–25

Gamble P, Burke JJ (1984) Effect of water stress on the chloroplast antioxidant system. I. Alteration in glutathione reductase activity. Plant Physiol 76: 615–621

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

Gómez JM, Hernández JA, Jiménez A, del Río LA, Sevilla F (1999) Differential response of antioxidative enzymes of chloroplasts and mitochondria to long-term NaCl stress of pea plants. Free Rad Res 31: S11–S18

Hernández JA, Ferrer MA, Jiménez A, Ros-Barceló A, Sevilla F (2001) Antioxidant systems and $O_2^{\cdot-}/H_2O_2$ production in the apoplast of *Pisum sativum* L. leaves: its relation with NaCl-induced necrotic lesions in minor veins. Plant Physiol 127: 817–831

Hernández JA, Jiménez A, Mullineaux PM, Sevilla F (2000) Tolerance of pea (*Pisum sativum* L.) to long-term salt stress is associated with induction of antioxidant defences. Plant Cell Environ 23: 853–862

Jasper DA, Abbott LK, Robson AD (1989) Acacias respond to additions of phosphorous and to inoculation with VA mycorrhizal fungi in soils stockpiled during mineral sand mining. Plant Soil 115: 99–108

Jiménez A, Hernández JA, del Río LA, Sevilla F (1997) Evidence for the presence of the ascorbate-glutathione cycle in mitochondria and peroxisomes of pea (*Pisum sativum* L.) leaves. Plant Physiol 114: 275–284

Kamfenkel K, Van Montagu M, Inzè D (1995) Effects of iron on *Nicotiana plumbaginifolia* plants. Implication to oxidative stress. Plant Physiol 107: 725–735

Knight H, Trewavas AJ, Knight MR (1997) Calcium signalling in Arabidopsis thaliana responding to drought and salinity. Plant J 12: 1067–1078

McCord JM, Fridovich I (1969) Superoxide dismutase. An enzymic function for erythrocuprein (hemocuprein). J Biol Chem 244: 6049–6055

Menconi M, Sgherri CLM, Pinzino C, Navari-izzo F (1995) Activated oxygen species production and detoxfication in wheat plants subjected to a water deficit programme. J Exp Bot 46: 1123–1130

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

Noctor G, Foyer C (1998) Ascorbate and Glutathione: Keeping active oxygen under control. Annu Rev Plant Physiol Plant Mol Biol 49: 249–279

Palma JM, Longa MA, del Río LA, Arines J (1993) Superoxide dismutase in vesicular arbuscular-mycorrhizal red clover plants. Physiol Plant 87: 77–83

Phillips JM, Hayman DS (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

Porcel R, Barea JM, Ruiz-Lozano JM (2003) Antioxidant activities in mycorrhizal soybean plants and their possible relationship to the process of nodule senescence. New Phytol 157: 135–143

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

del Río LA, Sevilla F, Sandalio LM, Palma JM (1991) Nutritional effect and expression of SODs: Induction and gene expression; Diagnostics; Prospective protection against oxygen toxicity. Free Rad Res Comms 12–13: 819–827

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

Ruiz-Lozano JM, Azcón R, Gómez M (1995) Effects of arbuscular-mycorrhizal *Glomus* species on drought tolerance: physiological and nutritional plant responses. Appl Environ Microbiol 61: 456–460

Ruiz-Lozano JM, Azcón R, Palma JM (1996) Superoxide dismutase activity in arbuscular mycorrhizal *Lactuca sativa* plants subjected to drought stress. New Phytol 134: 327–333

Scandalios JG (1993) Oxygen stress and superoxide dismutases. Plant Physiol 101: 7–12

Tambussi EA, Bartoli CG, Beltrano J, Guiamet JJ, Araus JL (2000) Oxidative damage to thylakoid proteins in water-stressed leaves of wheat (*Triticum aestivum*). Physiol Plant 108: 398–404

Tobar RM, Azcón R, Barea JM (1994) The improvement of plant N acquisition from an ammonium-treated, drought-stressed soil by the fungal symbiont in arbuscular mycorrhizae. Mycorrhiza 4: 105–108

Toro M, Azcón R, Barea JM (1997) Improvement of arbuscular mycorrhiza development by inoculaltion of soil phosphate-solubilizing rhizobacteria to improve rock phosphate bioavail-

ability (^{32}P) and nutrient cycling. Appl Environ Microb 63: 4408–4412
Toro M, Azcón R, Barea JM (1998) The use of isotopic dilution techniques to evaluate the interactive effects of *Rhizobium* genotype, mycorrhizal fungi, phosphate-solubilizing rhizobacteria and rock phosphate on nitrogen and phosphorus acquisition by *Medicago sativa*. New Phytol 138: 265–273
Vansuyt G, Lopez F, Inzè D, Briat JF, Fourcroy P (1997) Iron triggers a rapid induction of ascorbate peroxidase gene expression in *Brassica napus*. FEBS Lett 410: 195–200

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