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Effect of Mycorrhizal Inoculation on Nutrient Acquisition, Gas Exchange, and Nitrate Reductase Activity of Two Mediterranean-Autochthonous Shrub Species Under Drought Stress

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ABSTRACT

Plant developments in Mediterranean ecosystems are affected by a characteristic multiple stress situation, which mainly derives from water-deficit in these areas. Drought stress conditions restrict nutrient

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and water acquisition capability of plants thus affecting the sustainability of such ecosystems. Because mycorrhizal fungi are known to enhance the ability of plants to establish and cope with stress situations (nutrient deficiency, drought, etc.), the use of these fungi as plant inoculants, is currently being investigated to help plants to thrive in degraded arid/semi arid Mediterranean areas. To assess the role of arbuscular mycorrhizal (AM) fungi on nutrient acquisition by plant under drought stress, this study used a biochemical marker, i.e., the nitrate reductase (NR) activity, both in roots and shoots, because this is the first enzyme involved in nitrogen (N) assimilation by plants. Measurement of the ''intrinsic water use efficiency'' was used to evaluate the effect of AM fungi on some physiological activities related to water relation in plants under drought stress. Three experimental variables were tested including: (i) the use of two Mediterranean-autochthonous shrub host species to the target ecosystem, i.e., *Olea europaea* subsp. *sylvestris* and *Retama sphaerocarpa* L.; (ii) mycorrhizal inoculation with AM fungi either autochthonous or alochthonous from the target area; and (iii) an imposed drought stress to nursery-produced plants. Plant growth, nutrient acquisition, NR activity, both in shoot and root, photosynthesis rate (A) and stomatal conductance (g_s), were measured before transplanting to the target sites. AM inoculation improved plant growth, NR activity (only autochthonous fungi), nutrient acquisition and the ''intrinsic water use efficiency'' (A/g_s) in drought stressed *O. europaea* plants, while in stressed *R. sphaerocarpa* plants AM inoculation benefited only NR activity (with alochthonous fungi) and N acquisition. The advantages of mycorrhizal inoculation to help plant development under Mediterranean climate conditions were therefore demonstrated. The differential plant species responses are discussed in terms of mycorrhizal dependency/functional compatibility criteria.

Key Words: Nitrate reductase; Photosynthetic rate; AM fungi; Drought stress; Mediterranean ecosystems.

INTRODUCTION

Water-deficit, a characteristic of many Mediterranean ecosystems, imposes a multiple stress situation, which becomes a major threat to the sustainability of areas under Mediterranean climate.[1] Among the diverse consequences of a drought effect on plant developments in these ecosystems, a restricted nutrient and water acquisition are commonly recognized.[2] In this context and because mycorrhizal fungi are known to enhance the ability of plants to establish and cope with stress situations

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(nutrient deficiency, drought, etc.), the use of these fungi, as plant inoculants, is being investigated to help plants to thrive in degraded arid/semi arid areas.[3–5] As it is well-known these fungi form the mycorrhizal symbiosis with most plant species from the natural succession in Mediterranean ecosystems, being ubiquitous in these habitats.[6] The trophic relationships ranging from either ectomycorrizal, ectendomycorrhizal or endomycorrhizal habits.[7]

The role of mycorrhizal fungi in plant acquisition of mineral nutrients, as affected by drought stress has been classically ascertained by measuring the N, phosphorus (P), and potassium (K) concentration in plant tissues. However, the use of biochemical marker, for example the nitrate reductase (NR) activity to assess N assimilation by plants has been proposed.[8] NR (EC 1.6.6.1) is the first enzyme in the nitrate assimilation pathway and represents the rate-limiting step in this process.[9] This enzyme is inducible by its substrate, i.e., the nitrate ions.[10] It has been shown that NR activity decreases in plants exposes to water limitation because of a lower flux of nitrate from the soil to the root.[11,12] NR activity was detected both in pure cultures of ectomycorrhizal fungi[13] and in mycorrhizal pine seedlings.[14] The presence of such an enzymatic activity in arbuscular mycorrhizal (AM) fungi[15] and the increase of NR activity in the AM symbiosis have also been shown.[16,17] The expression of NR genes in the symbiotic AM fungi and their expression during the development of the symbiosis have been evidenced by means of molecular approaches.[18,19]

Drought stress is also known to affect many physiological activities related to water relations in plants. These activities concern diverse interacting properties such as stomatal conductance, transpiration, photosynthesis, leaf and root hydration, etc.[20,21] Particularly, stomatal conductance (g_s) to water vapor and the photosynthetic rate are stimulated by AM fungal inoculation under drought conditions,[21–23] effects which are dependent on the fungus involved in the symbiosis.[24,25]

The information on the interactive effects between mycorrhizal inoculation and drought stress on either the NR activity or on some key ecophysiological properties related to gas exchange, is scarce concerning Mediterranean ecosystems. Therefore, a series of experiments were designed accordingly. The aim was to gain information on the effect of AM inoculation of nursery-produced plants when further submitted to drought stress. This is particularly important because these plants are to be used for revegetation of degraded areas, suffering from water-deficit under Mediterranean climate conditions. Three experimental variables were tested. These include: (i) the use of two shrubs host species autochthonous to the target ecosystem, i.e., *Olea europaea* subsp. *sylvestris*

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and *Retama sphaerocarpa* L.; (ii) mycorrhizal inoculation with AM fungi either autochthonous or alochthonous from the target area; and (iii) an imposed drought stress to nursery-produced plants. Plant growth, nutrient acquisition, NR activity, both in shoot and root, photosynthesis rate (A) and stomatal conductance (g_s), were measured before transplantation to the target sites.

MATERIALS AND METHODS

Plants and Mycorrhizal Treatments

The plants used (*Olea europaea* L. subsp. *sylvestris* and *Retama sphaerocarpa*), are slow-growing shrubs, widely distributed in the Mediterranean area. They are also well adapted to water stress conditions and, therefore, frequently used in the revegetation of semiarid disturbed land.

The mycorrhizal fungi used were either *Glomus claroideum* (EEZ 24), or a mixture of endophytes isolated from Cieza (SE Spain), a semiarid area where the target plants naturally grow, consisting of *Glomus geosporum* (EEZ 31), *Glomus albidum* (EEZ 39), *Glomus microaggregatum* (EEZ 40), *Glomus constrictum* (EEZ 42), *Glomus mosseae* (EEZ 43), *Glomus coronatum* (EEZ 44), *Glomus intraradices* (EEZ 45), and a *Glomus* sp. (EEZ 46). The acronym EEZ refers to Estación Experimental Zaidín, Granada (Spain).

AM fungal inocula consisted of a mixture of rhizospheric soil from pure pot culture containing spores, hyphae, and mycorrhizal root fragments. Once germinated, seedlings were transplanted into the growing substrate, consisting of peat and cocopeat (1:1, v:v). The corresponding arbuscular mycorrhizal inoculum was applied at a rate of 5% (v/v). The same amount of the autoclaved mixture of the inocula was added to control plants, supplemented with a filtrate (<20 μm) of culture to provide the microbial populations accompanying the mycorrhizal fungi. Inoculated and noninoculated seedlings were grown for 8 months under nursery conditions without any fertilization treatment. Nursery procedures were conducted at Paisajes del Sur Ltd. (Granada, Spain). At the end of the nursery period, inoculated seedlings were slightly larger than noninoculated ones, although differences in size were not statistically significant.

Water Stress Treatments

The experiment was conducted as two completely randomized factorial (one per plant specie) with two factors. The first factor had

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three levels: noninoculation, inoculation with *G. claroideum* and inoculation with the mixture of arbuscular mycorrhizal fungi in the nursery, and the second factor had two levels: well watered or drought stressed conditions. Five replicates per treatment were set out, thus making a total of 30 seedlings per plant species. Plants were acclimated to greenhouse conditions for one month prior to being submitted to water regime treatments. Fertilizer was not added to the substrate. Plants were watered regularly with deionized water until the initiation of the drought treatment. Soil water shortage was imposed for six weeks (from 20 December until 31 January). During the experiment, the temperatures ranged from 11–24°C, and the relative humidity was between 40% and 80%. Midday photosynthetically active radiation (PAR) averaged $260\,\mu E\,m^{-2}\,s^{-1}$.

Within each plant species, well-watered plants were maintained at a substrate water potential equivalent to field capacity (−0.03 MPa) and stressed plants were maintained at a substrate water potential close to wilting point (averaging −0.60 MPa). Soil moisture was monitored gravimetrically before each watering. Water content in the substrate, calculated as a percentage of dry weight, corresponding with substrate water potential at field capacity and at permanent wilting was determined according to the method of Richards.[26]

Measurements

After the water stress period, basal stem diameters and heights of plants were measured with calipers and rules. Plants were harvested, and the roots were washed free from soil under a stream of cold tap water. Fresh and dry (105°C, 5 h) weights of shoots and roots were recorded. Plant tissues were ground before chemical analysis. The foliar concentrations of nitrogen, phosphorus, and potassium were calculated after digestion in nitric-perchloric acid (5:3) for 6 h. The P concentration was determined by colorimetry,[27] the N concentration was determined by Kjeldahl method and the K concentration was estimated by a flame photometer.[28]

The percentage of root length colonized by arbuscular mycorrhizal fungi was calculated by the gridline intersect method[29] after staining with trypan blue.[30]

NR activity was assayed in vivo by measuring NO_2^- production in tissue that has been vacuum infiltrated with buffered NO_3^- solutions.[31] The leaves and roots from the nonstressed and stressed seedlings were collected in the morning between 8:30 and 11:00 solar time. Circles 5 mm

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in diameter were cut from the leaves of *O. europaea*. Leaves and roots of *R. sphaerocarpa* and roots of *O. europaea* were cut into 5 mm sections. Approximately 300 mg of leaf punches and 300 mg of roots from each plant were placed into 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 during 1 h. The nitrite released into the medium was determined after incubation by treating 1 mL of the aliquots with 1 mL of 1% sulphanilamide in 1 M HCl and 1 mL of 0.01% N-1-napthyl-ethylenediamine hydrochloride (NNEDA). After 15 min, the optical density was measured at 540 nm with Beckman spectrophotometer.

Instantaneous measurements of the photosynthetic rate (A), and stomatal conductance (g_s) were determined using a portable gas analyzers system (ADC, LCA4 configured with PLC4C chamber, UK) according to the methodology developed by Long et al.[32]

Measurements were made on whole shoots of mature leaves with the chamber oriented directly towards the sun. Measurements were always made in the morning between 10:30 and 12:00 solar time. After the A and g_s measurements had been obtained, the shoots used for gas exchange measurement were taken to the laboratory and their total leaf surface area was calculated according to Johnson.[33] A and g_s were expressed on a total leaf surface area basis.

Statistical Analysis

Data were log transformed to adjust for normality. Comparisons among treatment means were made using Least Significant Difference (LSD) multiple range test calculated at $P < 0.05$. Correlation analysis between all the soil parameters measured was carried out using Pearson's rank correlation coefficients. Statistical procedures were carried out with the software packages Statgraphics for Windows 7.0

RESULTS

Plant Growth and Mycorrhizal Colonization

Both mycorrhizal inoculation treatments increased the height of *O. europaea* plants under either well-watered or drought-stressed conditions (Table 1). In spite of both AM fungal inoculation treatments produced a similar level of root colonization, whatever the water regime,

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Table 1. Growth parameters and mycorrhizal colonization of *O. europaea* and *R. sphaerocarpa* seedlings as affected by the inoculation treatment and the water regime.

	Treatment	Height (cm)	Basal diameter (mm)	Shoot (g dw)	Root (g dw)	Mycorrhizal root length (%)
O. europaea	Control	17 a[a]	2.6 ab	0.13 a	0.36 ab	—
Well-watered	M	24 bc	3.0 bc	0.17 ab	0.41 abc	55 a
	G	37 d	3.7 d	0.23 bc	0.55 c	59 a
	Control	14 a	2.4 a	0.14 a	0.32 a	—
Drought-stressed	M	23 b	3.0 bc	0.16 ab	0.37 abc	43 a
	G	28 c	3.3 c	0.24 c	0.54 bc	54 a
R. sphaerocarpa	Control	41 a	3.3 bc	0.12 a	0.65 c	—
Well-watered	M	49 a	3.4 c	0.13 a	0.50 bc	52 a
	G	44 a	3.3 bc	0.12 a	0.41 ab	46 a
	Control	44 a	2.7 ab	0.11 a	0.42 ab	—
Drought-stressed	M	50 a	2.6 a	0.18 b	0.27 a	52 a
	G	44 a	3.0 abc	0.09 a	0.38 ab	58 a

M = plants inoculated with the mixture of arbuscular mycorrhizal fungi; G = plants inoculated with *G. claroideum*.
[a]For each plant species, values sharing the same letter are not significantly different ($P < 0.05$) by the LSD test.

G. claroideum was more effective than the mixture of native AM fungi in improving plant height, the basal diameter and the shoot and root dry weights of *O. europaea* seedlings under both well-watered and drought-stress conditions.

Mycorrhizal inoculation had not significant effect on the height nor on the basal diameter of *R. sphaerocarpa* plants (Table 1). Shoot dry weight significantly increased in plants inoculated with the mixture of arbuscular mycorrhizal fungi under water stress. The two mycorrhizal inoculation treatments actively colonized the root system of the *R. sphaerocarpa* seedlings although there were no significant differences between them with regard to the percentage of root length colonization.

Nutrient Acquisition

With some exceptions, mycorrhizal inoculation treatments increased nutrient concentration in leaves of *O. europaea* in both nonstressed and

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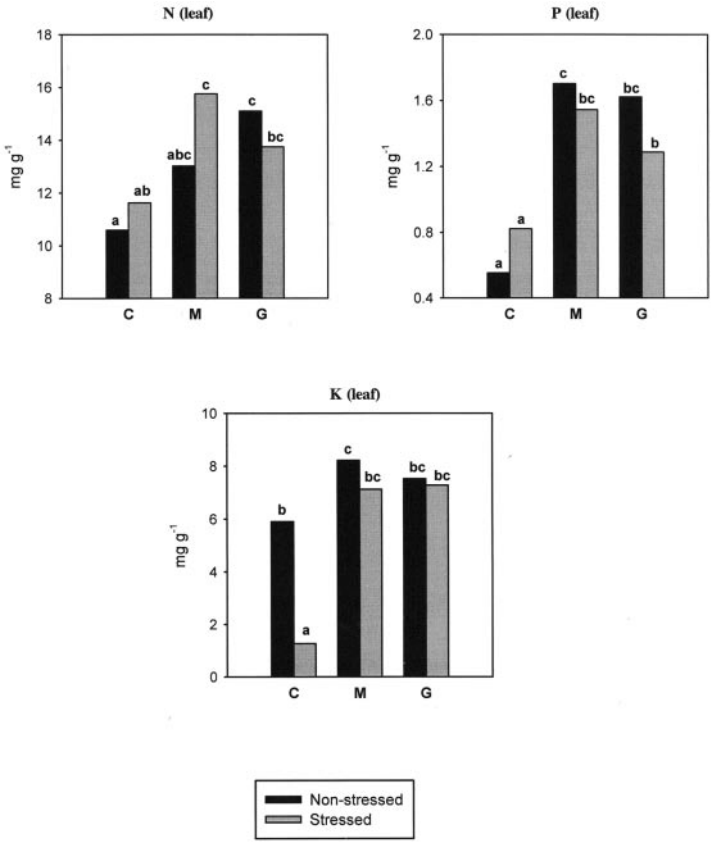

Figure 1. Concentration of nitrogen, phosphorus, and potassium in leaf of *O. europaea* seedlings as affected by the inoculation treatment and the water regime (C = plants control noninoculated; M = plants inoculated with the mixture of arbuscular mycorrhizal fungi; G = plants inoculated with *G. claroideum*). Values with the same letter are not significantly different values at $P < 0.05$, according to LSD test.

stressed plants (Fig. 1). It is particularly noteworthy that mycorrhizal inoculation increased very much K concentration of *O. europaea* seedlings in stress conditions.

In water-stressed *R. sphaerocarpa* plants, only the concentration of N in shoot tissues was significantly increased by both mycorrhizal inoculation treatments (Fig. 2). Well-watered *R. sphaerocarpa* seedlings did not show significant differences in N and P acquisition as a mycorrhizal

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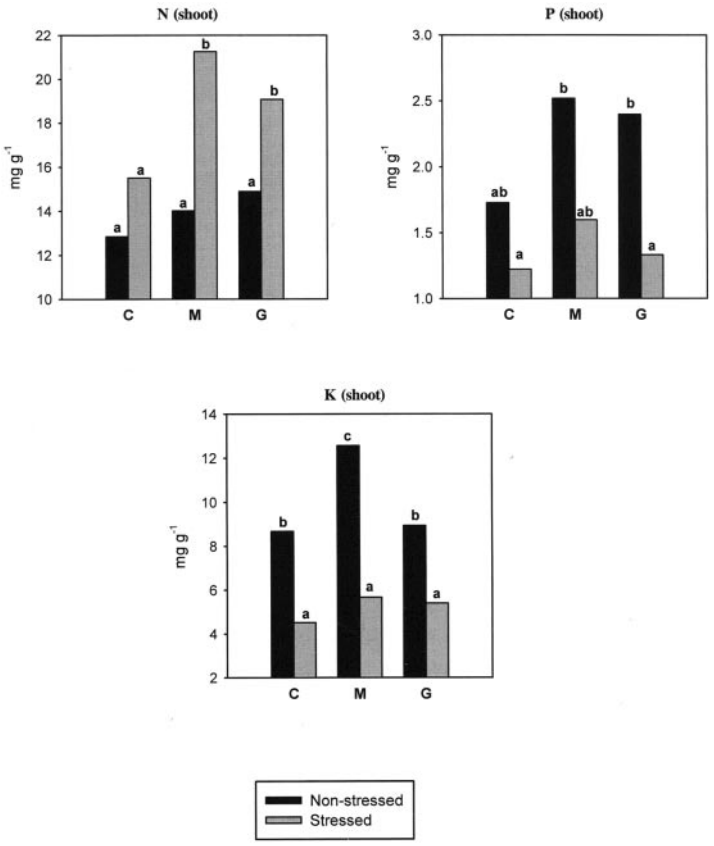

Figure 2. Concentration of nitrogen, phosphorus, and potassium in shoot of *R. sphaerocarpa* seedlings as affected by the inoculation treatment and the water regime (C = plants control noninoculated; M = plants inoculated with the mixture of arbuscular mycorrhizal fungi; G = plants inoculated with *G. claroideum*). Values with the same letter are not significantly different values at $P < 0.05$, according to LSD test.

response, and only the inoculation with the mixture of native AM fungi increased the K concentration in shoot tissues.

Gas Exchange and Intrinsic Water-Use Efficiency

Both mycorrhizal inoculation treatments improved the photosynthetic rate (A) and the A/g_s ratio (intrinsic water-use efficiency) in

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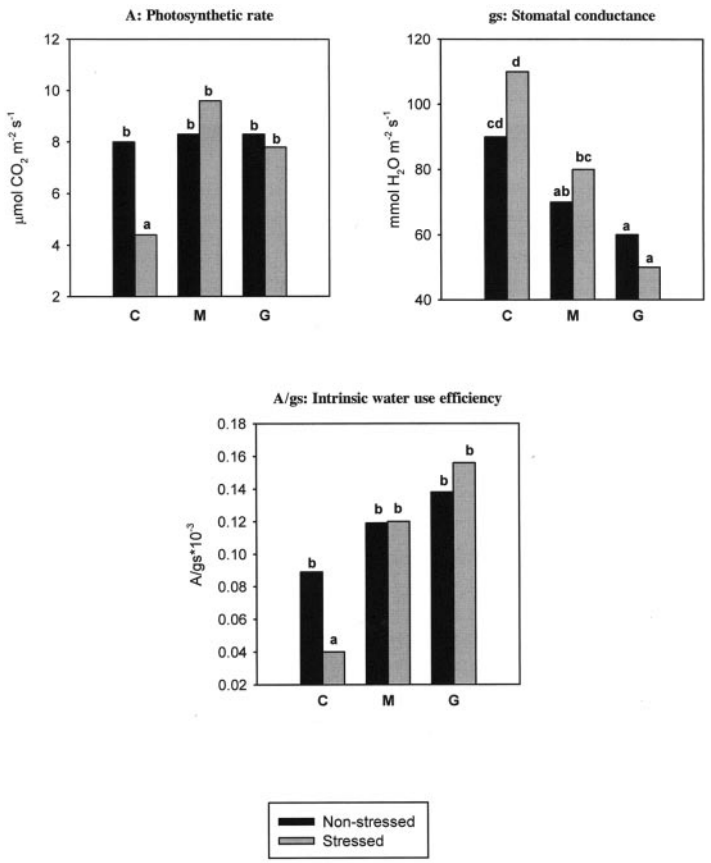

Figure 3. Photosynthetic rate, stomatal conductance, and intrinsic water use efficiency measured in leaf of *O. europaea* seedlings as affected by the inoculation treatment and the water regime (C = plants control noninoculated; M = plants inoculated with the mixture of arbuscular mycorrhizal fungi; G = plants inoculated with *G. claroideum*). Values with the same letter are not significantly different values at $P < 0.05$, according to LSD test.

water-stressed *O. europaea* seedlings (Fig. 3). In contrast, stomatal conductance (g_s) was lower in the inoculated *O. europaea* seedlings than in noninoculated plants, particularly under water stress conditions.

Mycorrhizal inoculation with the mixture of native AM fungi increased the net photosynthesis and A/g_s ratio in well-watered *R. sphaerocarpa* (Fig. 4). Water stress decreased these two physiological parameters in either mycorrhizal and nonmycorrhizal plants.

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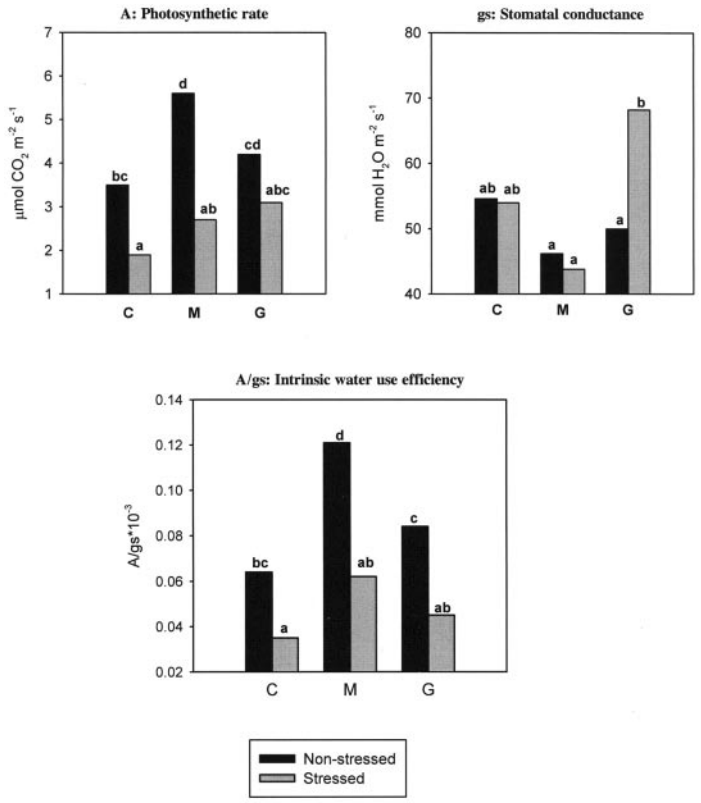

Figure 4. Photosynthetic rate, stomatal conductance, and intrinsic water use efficiency measured in shoot of *R. sphaerocarpa* seedlings as affected by the inoculation treatment and the water regime (C = plants control noninoculated; M = plants inoculated with the mixture of arbuscular mycorrhizal fungi; G = plants inoculated with *G. claroideum*). Values with the same letter are not significantly different values at $P < 0.05$, according to LSD test.

Nitrate Reductase Activity

In general, the mixture of AM fungi was effective increasing the NR activity in the roots of *O. europaea* while *G. claroideum* improved such an activity in *R. sphaerocarpa* plants (Table 2). NR activity in the leaves of *O. europaea* seedlings was higher than in roots and was only significantly increased by the inoculation with the mixture of native AM fungi under drought stress. In contrast, NR activity in roots of *R. sphaerocarpa* was higher than in shoot tissues (Table 2).

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Table 2. Nitrate reductase (NR) in roots and leaves of *O. europaea* and in roots and shoots of *R. sphaerocarpa* seedlings as affected by mycorrhizal inoculation and water regimes.

	Treatment	NR activity (root) (nmol NO_2^- g FW^{-1} h^{-1})	NR activity (leaf or shoot) (nmol NO_2^- g FW^{-1} h^{-1})
O. europaea	Control	58 ab[a]	264 abc
Well-watered	M	70 c	303 bc
	G	58 ab	211 a
	Control	51 a	236 ab
Drought-stressed	M	78 c	320 c
	G	62 ab	234 ab
R. sphaerocarpa	Control	101 ab	12 a
Well-watered	M	125 ab	23 b
	G	181 c	29 b
	Control	93 a	11 a
Drought-stressed	M	82 a	9 a
	G	146 bc	28 b

M = plants inoculated with the mixture of arbuscular mycorrhizal fungi; G = plants inoculated with *G. claroideum*.
[a]For each plant species, values sharing the same letter are not significantly different ($P < 0.05$) by the LSD test.

Correlation Between the Different Parameters

Particularly, mycorrhizal inoculation was positively correlated with some growth parameters such as height ($r^2 = 0.469$, $P < 0.01$), basal diameter ($r^2 = 0.507$, $P < 0.01$) and shoot dry weight ($r^2 = 0.363$, $P < 0.05$) in *O. europaea* plants, while in *R. sphaerocarpa* AM inoculation was only positively correlated with the height of the plants ($r^2 = 0.366$, $P < 0.05$).

There was a positive significant correlation between mycorrhizal inoculation and nutrient (NPK) concentration in *O. europaea* seedlings ($r^2 = 0.491$, $P < 0.01$ for N, $r^2 = 0.796$, $P < 0.001$ for P and $r^2 = 0.624$, $P < 0.001$ for K). The P and K concentration in shoots of *O. europaea* and *R. sphaerocarpa* plants correlated positively with the photosynthetic rate ($r^2 = 0.384$, $P < 0.05$ for P in *O. europaea*, $r^2 = 0.612$, $P < 0.001$ for K in *O. europaea*, $r^2 = 0.532$, $P < 0.01$ for P in *R. sphaerocarpa* and $r^2 = 0.623$, $P < 0.001$ for K in *R. sphaerocarpa*). A positive significant correlation between NR activity in roots of *O. europaea* and mycorrhizal inoculation ($r^2 = 0.525$, $P < 0.01$), and nutrient (NPK) acquisition was

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also demonstrated ($r^2 = 0.495$, $P < 0.01$ for N, $r^2 = 0.437$, $P < 0.05$ for P and $r^2 = 0.385$, $P < 0.05$ for K).

DISCUSSION

It is well known that mycorrhiza formation commonly increase plant nutrient acquisition, particularly P and N.[7] With regard to N, and as it has been well established, the two most important sources of this nutrient for plants, and potentially for AM fungi, are nitrate and ammonium ions, with nitrate as the main N source for plants in neutral to alkaline soils, due to the tendency for a rapid nitrification of ammonium ions.[34] Hence the process of nitrate assimilation, with the participation of the nitrate reductase (NR) enzyme, is of fundamental biological importance in these soils. The contribution of AM symbiosis to nitrate uptake and assimilation has been shown,[35,36] and it has been proposed to use NR activity as a stress index since it is highly sensitive to the metabolic and physiological status of the plant.[17]

In this study, we have found that mycorrhizal inoculation induced an increased NR activity in the roots of both *O. europaea* and *R. sphaerocarpa* plants when grown under either well-watered or water-stressed conditions. Apart from a mycorrhizal effect on the NR activity of the host plants, it has been shown[17] that the AM fungi have the ability to produce such enzymatic activity, per se, as is the case for some fungi.[37,38] The fact that fungal biomass associated with roots possesses NR activity has been further confirmed by Kaldorf et al.[18,19] by using molecular approaches.

Once demonstrated a positive mycorrhizal effect on plant NR activity, a key concern is to realize whether such a biochemical process is actually improving plant N acquisition, particularly under drought conditions. This effect was evident in this study for *O. europaea* where a highly significant correlation ($P < 0.01$) between the NR activity in roots and the N concentration in shoots of mycorrhizal plants was evident. Because NR activity is dependent on the phosphate requirements of this enzyme,[39] it could be argued that an enhanced P acquisition by mycorrhizal *O. europaea* plants can account for the mycorrhizal response on this side.

It is worthy to note that within each plant species the effect of mycorrhizal treatments on NR activity in roots and leaves depended on the mycorrhizal treatment assayed. Thus, the mycorrhizal inoculation with the mixture of AM fungi was most effective for N assimilation in *O. europaea*, while inoculation with *G. claroideum* increased N acquisition

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by *R. sphaerocarpa* plants. These results support that the well-established fact that, although AM fungi are not host specific, they may exhibit host preferences and some fungus-host plant combinations are more effective than others,[40] also applies for this enzymatic activity.

It is also known that in vivo NR activity can be modulated by many metabolic and physiological factors among them the carbohydrate availability,[41] thus by photosynthesis processes functioning. In this context, this study demonstrates that inoculation of *O. europaea* plants with AM fungi enhanced the photosynthetic processes. Thus mycorrhizal colonization may also have affected nitrate reduction via changes in the photosynthesis, which influences a number of activities involved in the regulation of NR.[38] Both the NR activity in roots of *O. europaea* plants and photosynthetic rate were higher in mycorrhizal stressed plants than in the nonmycorrhizal counterparts, in spite of the extra C requirement for fungal development and of competition between nitrate and CO_2 reduction for reductants and ATP.[42] The increased photosynthetic activity recorded in mycorrhizal *O. europaea* plants subjected to water stress is an indication of the mycorrhizal ability to promote plant adaptation to drought resistance. On the other hand, the protection of mycorrhizal *O. europaea* plants against water stress could be also related to the effects that the endophytes had on increasing K uptake. Potassium 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.[24] Accordingly, there was a quite close relationship between intrinsic water use efficiency and leaf K concentrations. The A/g_s ratios were higher in mycorrhizal *O. europaea* plants under water stress, which indicate that these plants are adapted to more arid habitats.[23,43]

In the case of *R. sphaerocarpa*, the photosynthetic rate did not increase in mycorrhizal stressed plants. This could be explained in terms of a competition with the NR activity, which resulted in increased *R. sphaerocarpa* roots. However, a more simple explanation could be argued as based on the fact that AM inoculation did not have a positive effect on plant growth nor on P acquisition, a nutrient fundamental for photosynthesis functioning.[21] This, in turn, may be explained in terms of mycorrhizal dependency/functional compatibility concepts.[40] *Retama sphaerocarpa* is a shrub species known to increase very much the mycorrhizal potential in its rhizosphere, but exhibiting a very slow response to AM inoculation.[44] Thus, in spite of the mycotrophic habit of this species its level of mycorrhizal dependency is low, at least with regard to improvement in shoot P concentration. However, a mycorrhizal P-mediated effect on root-related activities in this legume

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(*R. sphaerocarpa*), such as nodulation,[44] or on NR activity, as shown in the present study, is probably acting to help these plants in drought-stressed conditions.

In conclusion, results from this study show that AM symbiosis increased NR activity and/or gas exchange in *O. europaea* and *R. sphaerocarpa* and that can contribute to the enhanced tolerance of these plants to drought stress.

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