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Use of Nitrate Reductase Activity for Assessing Effectiveness of Mycorrhizal Symbiosis in *Dorycnium pentaphyllum* Under Induced Water Deficit

Fuensanta Caravaca,[1,*] María del Mar Alguacil,[1] Gisela Díaz,[2] and Antonio Roldán[1]

[1]CSIC-Centro de Edafología y Biología Aplicada del Segura, Department of Soil and Water Conservation, Murcia, Spain
[2]SACE-University of Murcia, Murcia, Spain

ABSTRACT

The effects of arbuscular-mycorrhizal (AM) fungi, *Glomus intraradices*, *Glomus mosseae* or *Glomus deserticola* on root nitrate reductase (NR) activity, growth parameters, colonization rate and foliar nutrient (NPK) concentrations were assessed in *Dorycnium pentaphyllum* L. seedlings grown under well-watered (−0.03 MPa) or drought conditions (−0.60 MPa). Under water stress, plants colonized by AM fungi exhibited root dry weights and root/shoot ratios, which were lower than

*Correspondence: Fuensanta Caravaca, CSIC-Centro de Edafología y Biología Aplicada del Segura, Department of Soil and Water Conservation, P.O. Box 4195, Campus de Espinardo, Murcia 30100, Spain; Fax: +34-968-396213; E-mail: fcb@cebas.csic.es.

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un-inoculated plants. *G. deserticola* had more effect on N uptake whereas *G. intraradices* and *G. mosseae* had more effect on phosphorus (P) uptake under the water-limited conditions tested. Nodulation was inhibited and AM colonization rate was increased by water stress in *D. pentaphyllum* seedlings inoculated with *G. deserticola*. Root NR activity increased significantly in *G. deserticola*-colonized plants, under both well-watered (by 137%) and drought-stressed conditions (by 140%), with respect to un-inoculated plants. NR activity could be used as an index for assessing the effectiveness of fungus-host plant combinations for mitigation of water-deficit stress.

Key Words: *Glomus intraradices*; *Glomus mosseae*; *Glomus deserticola*; Semiarid areas; Drought conditions.

INTRODUCTION

In semiarid Mediterranean areas the un-productiveness of the soil and the water deficit seriously limit plant growth. Thus, to carry out successful reforestation programs, it is necessary to improve the viability of plants prior to their planting in the field, to resist semiarid environmental conditions. Inoculation with symbiotic microorganisms, especially arbuscular-mycorrhizal (AM) fungi, is an effective method to protect host plants against the detrimental effects of drought.[1] Mycorrhizae help plants to thrive in arid conditions by increasing the supply of nutrients to the plant, particularly nitrogen (N) and P,[2,3] and reducing water stress.[4] Nitrate has been suggested to be the preferential N source for AM fungi associated with plants grown in neutral to alkaline soils.[5] Uptake and transport of nitrate occur through the extraradical mycelium of AM fungi.[6] Differences in nitrate utilization have been described between isolates of a given species as well as between species of the same genus.[7]

The importance of mycorrhizal colonization against drought stress is commonly diagnosed by measuring the N, P, and potassium (K) concentrations in plant tissues and plant growth. Alternatively, assay of plant enzymes involved in N assimilation by plants, such as nitrate reductase (NR) activity, has been proposed.[8] NR catalyzes the rate-limiting step in the nitrate assimilation pathway. It has been shown that NR activity decreases in plants exposed to water limitation because of a lower flux of nitrate from the soil to the root.[6,9] The presence of such an enzymatic activity in AM fungi[7] and the increase of NR activity in the AM symbiosis have also been shown.[10] Under stress conditions, the maintenance of nitrate reductase activity is a factor

involved in the drought tolerance of mycorrhizal plants.[10] However, this effect depends on the associated mycorrhizal fungus. The objective of this study was to compare the effectiveness of inoculation with three AM fungi in increasing nitrate reductase activity in roots, mycorrhizal colonization, plant growth and nutrient uptake in *Dorycnium pentaphyllum* L. seedlings under well-watered and drought stress conditions. The results obtained should help to optimize nursery production of mycorrhizal seedlings for use in soil revegetation programs in a semiarid Mediterranean area.

MATERIALS AND METHODS

Plants and Mycorrhizal Treatments

The plant used, *Dorycnium pentaphyllum*, is a leguminous low-growing shrub, widely distributed in the Mediterranean area. It is also well adapted to water stress conditions and, therefore, frequently used in the revegetation of semiarid disturbed lands.

The mycorrhizal fungi used in the experiment, *Glomus intraradices* Schenck & Smith, *Glomus mosseae* (Nicol & Gerd.) Gerd. & Trappe, and *Glomus deserticola* (Trappe, Bloss. & Menge), were obtained from the collection of the experimental field station of Zaidín, Granada. Arbuscular mycorrhizal inoculum 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) mixed with each of inocula (5%). The same amount of the autoclaved mixture of the inoculum was added to control plants, supplemented with a filtrate ($< 20\,\mu m$) of culture to provide the microbial populations accompanying the mycorrhizal fungi. Inoculated and non-inoculated seedlings were grown for 8 months under nursery conditions without any fertilization treatment.

Water Stress Treatments and Experimental Design

The experiment was conducted as a completely randomized factorial with two factors. The first factor had four levels: non-inoculation and inoculation with three AM fungi (*G. intraradices*, *G. mosseae* or *G. deserticola*), 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 40 seedlings. Plants were watered regularly with deionizated

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°C to 24°C, and the relative humidity was between 40% and 80%. Midday photosynthetically active radiation (PAR) averaged 260 $\mu E\, m^{-2}\, s^{-1}$.

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 −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.[11]

Measurements

After the water stress period, basal stem diameters and heights of plants were measured with calipers and rulers. Plants were harvested, and the roots were washed free from soil under a stream of cold tap water. Fresh and dry (105°C, 5 hr) 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 N was determined by Kjeldahl method, the P concentration was determined by colorimetry[12] and the K uptake was estimated by a flame photometer.[13] The percentage of root length colonized by arbuscular mycorrhizal fungi was calculated by the gridline intersect method[14] after staining with trypan blue.[15]

Nitrate reductase activity was assayed *in vivo* by measuring NO_2^- production in tissue that has been vacuum infiltrated with buffered NO_3^- solutions.[16] The roots from the non-stressed and stressed seedlings were collected in the morning between 8:30 and 11:00 solar time. Roots of *D. pentaphyllum* were cut into 5-mm sections. Approximately 300 mg of root was 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.[17]

Statistical Analysis

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

RESULTS

After the water stress period, mycorrhizal inoculation and water regime had no significant effect on the height, the basal diameter or shoot dry weight of the *D. pentaphyllum* seedlings (Table 1). In the case of plants growing under water-stressed conditions, plants colonized by AM fungi presented root dry weights and root/shoot ratios, which were lower than un-inoculated plants.

Inoculation with *G. intraradices*, *G. mosseae*, or *G. deserticola* significantly enhanced AM colonization in well-watered *D. pentaphyllum* seedlings (Table 1), similar levels of root colonization being reached in all the mycorrhizal inoculation treatments. Under water stress, *G. deserticola* was the most effective fungus for colonizing the roots of *D. pentaphyllum*.

The number of *Rhizobium* nodules was increased significantly in well-watered *D. pentaphyllum* seedlings inoculated with *G. deserticola* with respect to control plants (Table 1). Nodulation in *D. pentaphyllum* seedlings inoculated with *G. mosseae* and *G. deserticola* was sharply inhibited by water stress.

Most of the NR activity was in the roots of *D. pentaphyllum*, since activity was not detected in the shoots of these seedlings. Root NR activity increased significantly in *G. deserticola*-colonized plants, under both well-watered (by 137%) and drought-stressed conditions (by 140%), with respect to un-inoculated plants (Fig. 1). A positive significant correlation between NR activity in roots of *D. pentaphyllum* and mycorrhizal inoculation was demonstrated (Table 2).

Mycorrhizal inoculation treatments positively affected N acquisition of plants grown under drought-stressed conditions, particularly in those colonized by *G. deserticola* (Fig. 1). In contrast, *G. intraradices* and *G. mosseae* fungi had a higher capacity for P acquisition than *G. deserticola*, which induced foliar P concentrations similar to control plants. Neither of the mycorrhizal inoculation treatments affected, generally, K acquisition in leaves of *D. pentaphyllum*, regardless of the water regime. There was a positive

Table 1. Growth parameters, mycorrhizal colonization and number of nodules of *D. pentaphyllum* seedlings as affected by the mycorrhizal inoculation treatments and the water regime.

	Treatment	Height (cm)	Basal diameter (mm)	Shoot (g dw)	Root (g dw)	Root/shoot ratio	Colonization (%)	No. nodules
Well-watered	Control	33a[a]	1.8b	0.47ab	0.27b	0.57b	0a	0a
	G1	34a	1.5a	0.36a	0.18a	0.54b	69b	9ab
	G2	34a	1.7ab	0.43ab	0.26ab	0.52b	64b	23ab
	G3	43a	1.7ab	0.78b	0.28b	0.36a	63b	36b
Drought-stressed	Control	30a	1.6ab	0.54a	0.34c	0.58c	6a	2ab
	G1	26a	1.8b	0.48a	0.15ab	0.32a	60b	0a
	G2	30a	1.9b	0.47a	0.22b	0.47b	67b	3ab
	G3	31a	1.4a	0.37a	0.13a	0.34ab	76c	7b

G1 = plants inoculated with *G. intraradices*; G2 = plants inoculated with *G. mosseae*; G3 = plants inoculated with *G. deserticola*.
[a] Values sharing the same letter are not significantly different ($P < 0.05$) by the LSD test.

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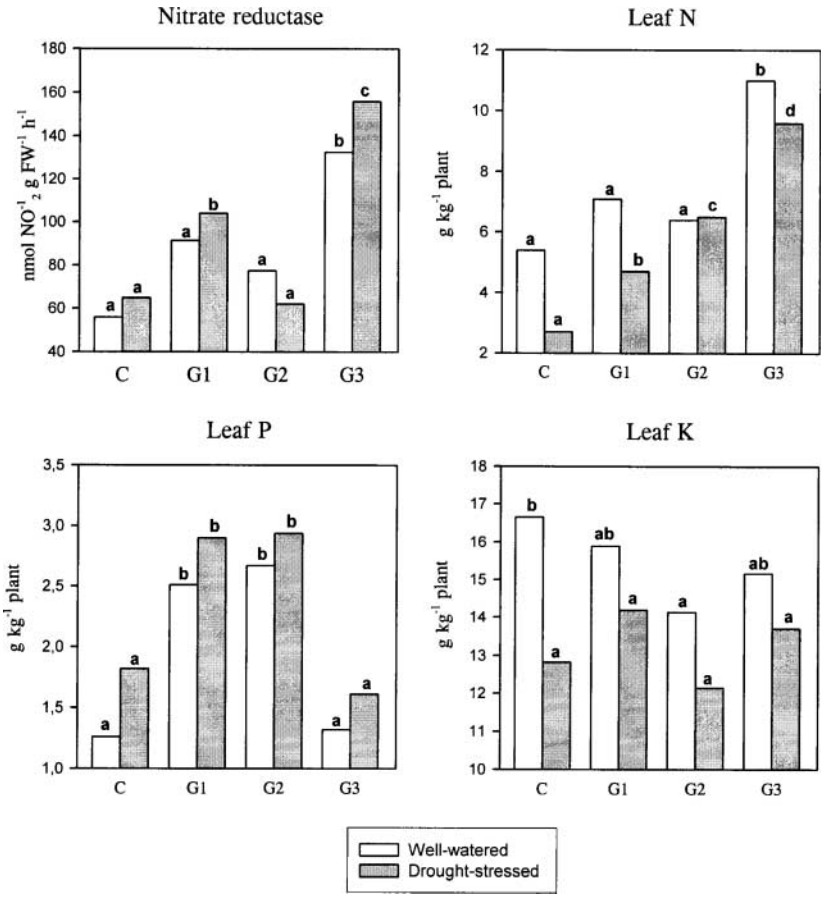

Figure 1. Root nitrate reductase activity and foliar nutrient concentrations of *D. pentaphyllum* seedlings as affected by mycorrhizal inoculation treatments and the water regime. C = uninoculated plants; G1 = plants inoculated with *G. intraradices*; G2 = plants inoculated with *G. mosseae*; G3 = plants inoculated with *G. deserticola*. For each water regime, bars with the same letter are not significantly different at $P < 0.05$, according to LSD test.

Table 2. Pearson's coefficients of correlation between root colonization, foliar nutrient concentrations and nitrate reductase in root of *D. pentaphyllum* seedlings subjected to mycorrhizal inoculation treatments and water deficit ($n = 40$).

Parameters	Correlation coefficient
Foliar N-colonization	0.484**
Foliar P-colonization	0.378*
Foliar K-colonization	ns
Root NR-colonization	0.487**
Foliar N-nodules	0.485**
Foliar P-nodules	ns
Foliar K-nodules	ns
Root NR-nodules	ns
Foliar N-NR	0.649***

*, **, *** significant at $P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively. Ns = not significant.
NR = nitrate reductase.

significant correlation between mycorrhizal inoculation and foliar N and P concentrations in *D. pentaphyllum* seedlings (Table 2).

DISCUSSION

NR activity is responsible for nitrate assimilation and is inducible by its substrate.[18] Thus, NR activity is highly sensitive to the metabolic and physiological plant status. The increased NR activity found in AM plants is an indication of the mycorrhizal ability to promote plant adaptation to drought resistance.[19] In this study, *G. deserticola* was the most effective fungus in increasing root NR activity and foliar N concentration of *D. pentaphyllum* when grown under either well-watered or water-stressed conditions. The mycorrhizal effect on N-nitrate uptake was positively correlated with an increase of N-nitrate assimilation activity.

Some authors have indicated that the increase in NR activity of mycorrhizal plants with respect to non-mycorrhizal ones can be related to the phosphate requirements of this enzyme.[20] This relationship was observed for *G. intraradices- and G. mosseae*-colonized plants, which showed the highest foliar P concentrations. However, *G. deserticola*-colonized plants had the highest NR

activity and showed similar P concentration to un-inoculated plants. This means that NR activity was regulated not only according to the P content in the host plant, but by also the colonizing AM fungus, indicating specific physiological behaviors of the different AM fungi. The present results are also consistent with the finding by Ruíz-Lozano and Azcón[10] that the NR activity differs in various AM fungal species colonizing roots of lettuce plants. Likewise, the different effects of AM fungi on this enzymatic activity could be a consequence of fungal NR activity. In fact, Kaldorf et al.[21] described assimilatory NR activity in mycorrhizal fungi. On the other hand, the fact that *G. deserticola*-colonized plants assimilated less P than plants colonized by *G. intraradices* or *G. mosseae* may be related to the increase in rhizosphere pH, caused by the highest amount of nitrate being reduced in the roots colonized by *G. deserticola*.[22] In general, the increase in rhizosphere pH would decrease P uptake and at the same time might favor the formation of relatively insoluble P compounds.

Many drought-adapted species from arid environments have a highly developed root system and thus the root/shoot ratio is high, which may be considered a mechanism of drought tolerance. Mycorrhizal symbiosis is known to decrease the root/shoot ratio.[23] The lack of roots is then compensated for the extension of the mycorrhizal fungus extraradical mycelium. The lower root/shoot ratio observed in stressed *D. pentaphyllum* seedlings colonized by three AM fungi, as compared to non-inoculated plants, may indicate partitioning of carbon to the fungus at the expense of root production.[24] In this case, the extraradical mycelium may have contributed to a more effective uptake of nutrients and water by plants submitted to water deficit.[4]

Nodulation and AM colonization are interactive processes in roots of legumes related to the N nutrition of the host plant. In well-watered conditions, both rhizobial and mycorrhizal symbiosis could have contributed to N uptake in mycorrhizal *D. pentaphyllum* plants. Drought stress did not affect the AM colonization rate, except in roots colonized by *G. deserticola*, where it that increased. However, drought stress decreased the number of nodules in roots colonized by *G. mosseae* and *G. deserticola*, therefore decreasing their ability for nitrogen fixation. The decrease in nodule formation under drought could be due to the competition between symbionts for the limited supply of photosynthates, especially considering that a large proportion of C is required by the plant for amino acid and protein synthesis. In the case of *G. deserticola*-colonized roots, the additional C requirements for supporting the increased NR activity, as well as extended AM colonization, could also limit nodule formation in these roots. Under drought-stressed conditions, plant N acquisition through the external AM mycelium may account for the increased plant N content found in the mycorrhizal *D. pentaphyllum* plants when water availability was limited.[8]

Considering the physiological plant responses to mycorrhizal symbiosis tested, we can conclude that NR activity could be used as an index for assessing the effectiveness of fungus-host plant combinations in mitigation of water-deficit stress. This result may be relevant in the selection of particular AM fungi to be used as inoculants in semiarid soil revegetation programs.

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