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Mycorrhizal colonization and drought stress as factors affecting nitrate reductase activity in lettuce plants

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Abstract

This study set out to determine the effect of drought stress on nitrate reductase (NR, EC 1.6.6.1.) activity in mycorrhizal plants, and to see if the maintenance of this enzymatic activity under stress conditions is a factor involved in the drought tolerance of mycorrhizal plants. *Lactuca sativa* L. plants were inoculated with three arbuscular mycorrhizal (AM) fungi, *Glomus deserticola* (Trappe. Bloss. and Menge), *G. fasciculatum* (Thax. and Gerd.) Gerd. and Trappe or *G. mosseae* (Nicol. and Gerd.) Gerd and Trappe or remained uninoculated (plus or less P fertilization). The plants were grown under controlled conditions at constant soil water potential (close to −0.04 MPa) or at −0.17 MPa during the last six weeks of plant growth. Results obtained showed that mycorrhizal plants had higher NR activity (NRA) than the uninoculated treatments, particularly under water stress conditions. Control plants had 57% less NRA than *G. deserticola*-colonized ones under well watered conditions, with a reduction in NRA of 79% when the plants were subjected to drought stress. Under well-watered conditions the P-fertilized plants showed similar or higher growth and P content than the *G. mosseae* and *G. fasciculatum* mycorrhizal ones, the NRA being lower in P-fertilized than in AM plants. These results suggest that either the AM fungi increase the NRA in the host plant (regardless of the P content) or the AM fungi have such enzymatic activity *per se*. Besides, under the experimental conditions, plants colonized by different AM fungi showed different NR activities. It was concluded that drought stress decreased NRA, but much less in mycorrhizal than in uninoculated plants. This effect may be a factor in the drought tolerance of mycorrhizal plants.

Keywords: Arbuscular mycorrhiza; Drought; NR; N-assimilation

1. Introduction

Nitrate is the main nitrogen source for much of the higher plants that feed most of the world. In fact, in most soils the major part of the inorganic N pool consists of nitrate because of rapid nitrification of ammonium mineralized from organic material or applied to the soil as fertilizers (Schmidt, 1982). The global rate of nitrate assimilation by plants is roughly 2×10^{13} kg nitrogen per year (Guerrero et al., 1981). This would be about in maximun of 10 fold greater than the rate of biological N_2 fixation. Thus, the process of nitrate assimilation is of fundamental biological importance.

NR is the first enzyme in the nitrate assimilation pathway and probably represents the rate-limiting

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step in this process (Campbell, 1988). So, the conversion of nitrate to ammonia in plants is a process with two enzymatic steps. The first one is a two electron reduction of nitrate to nitrite, catalyzed by NR. The second step is a six electron reduction of nitrite to ammonium, catalyzed by nitrite reductase (Hoff et al., 1992).

Nitrate can be reduced inside the AM fungal cells by the assimilatory reduction pathway, implying that AM fungi have the gene set for assimilatory nitrate reduction (Kaldorf et al., 1994). This fact is important because many microorganisms, even *Escherichia coli* (Stewart, 1988), cannot perform assimilatory nitrate reduction. Nitrate mobilized from soils by an AM fungus could be transferred directly as the anion to the root cells where reduction could proceed. However, it has been proposed that spores of AM fungi possess NR (Smith et al., 1985; Sundaresan et al., 1988). The ability of mycorrhizal roots to utilize nitrogen sources has been attributed, in most cases, to an indirect effect associated with an improved phosphorus nutrition (Oliver et al., 1983) because this enzyme requires phosphate which may complex with the molybdenum of the enzyme thereby facilitating its reduction (Hageman and Reed, 1980).

Previously, Azcón et al. (1992) studied the effect of mycorrhization on plant growth and nitrogen metabolism (including NRA) of lettuce plants cultivated under well-watered conditions. However, NR levels fluctuate in response to environmental conditions such as temperature, pH, CO_2, light, nitrogen source and water potential (Guerrero et al., 1981). Thus NR activity may decrease in leaves experencing dehydration caused by drought stress (Aparicio-Tejo and Sanchez-Diaz, 1982; Sanchez-Diaz and Aguirreolea, 1993) because of a lower flux of nitrate from the roots to the leaves; NR is probably the best characterized example of a plant enzyme induced by its substrate (nitrate). NR is thus responsive to the metabolic and physiological status of plants and can be used as a reporter to indicate stress or other changes in plant physiology, including drought (Srivastava, 1980).

The objectives of this study were to determine if drought stress affects the NR activity in mycorrhizal plants in the same way as in similar sized P-fertilized control and in unfertilized control ones, and to ascertain if the maintenance of high levels of this enzymatic activity is involved in the drought tolerance of mycorrhizal plants.

2. Materials and methods

2.1. Experimental design

The experiment used was a randomized complete block design. The factors were: AM colonization (3 AM isolates or two nonmycorrhizal treatments, one P-fertilized non-AM and one unfertilized non-AM control) and soil water potential (well watered or drought stressed). Five replicates per treatment were carried out, thus making a total of 50 experimental units.

2.2. Soil and biological materials

The loamy soil was collected from the grounds of the Estación Experimental del Zaidín (Granada), sieved (2 mm), diluted with quartz sand (1/1 v/v) and autoclaved (100°C, 1 h on 3 consecutive days). The soil had: pH 8.1; 1.8% organic matter; and the following nutrient concentrations (mg kg^{-1}): N, 2.5; P ($NaHCO_3$ – extractable P), 6.24; K, 132; and a texture made up of 35.8% sand; 46.3% silt and 20.5% clay. Pots were filled with 500 g of sterilized soil/sand (1/1, v/v) mixture

Mycorrhizal inoculum from each endophyte was multiplied in an open pot culture of *Lactuca sativa* L. and consisted of soil, spores, hyphae and AM root fragments. The AM species, belonging to the collection of the Estación Experimental del Zaidín (Ruiz-Lozano et al., 1995), were *Glomus deserticola* (Trappe. Bloss. and Menge), *G. fasciculatum* (Thax. and Gerd.) Gerd. and Trappe or *G. mosseae* (Nicol. and Gerd.) Gerd and Trappe. Five grams of each inoculum, having similar characteristics (an average of 30 spores g^{-1} and 75% of infected root), were placed directly below the seeds of *Lactuca sativa* L. cv. Romana. Nonmycorrhizal treatments received the same amount of autoclaved inoculum together with a 2 ml aliquot of a filtrate (< 20 μm) of the AM inoculum in order to provide a general microbial population free of AM propagules. Four seeds were sown and thinned, after emergence, to one seedling per pot.

2.3. Growth conditions

Plants were grown in a controlled environmental chamber with 70–80% RH, day/night temperatures of 25/15°C, and a photoperiod of 16 h. Photosynthetic photon flux (PPF) was 500 μmol m^{-2} s^{-1}, as measured with a light meter (LICOR, Lincoln, NE, USA, model LI-188B).

Water was supplied daily to maintain constant soil water potential close to −0.04 MPa during the first six weeks of plant growth. At this stage half of the plants were allowed to dry until soil water potential reached −0.17 MPa for further six weeks.

Throughout the experiment, plants received 10 ml Hewitt's nutrient solution lacking P (Hewitt, 1952) each week. Nonmycorrhizal P-fertilized plants received P as KH_2PO_4 (7 mg P pot^{-1} $week^{-1}$). This P application was selected from a previous experiment to match growth and P concentration of AM plants.

2.4. Determinations

At harvest (12 wks after planting), the root system was separated from the shoot, and dry weights were recorded. Shoot water content was calculated as $[(\text{fresh mass} - \text{dry mass})/_{\text{fresh mass}}] \times 100$. Concentrations of foliar N (micro-Kjeldahl) and P (Olsen and Dean, 1965) were determined by colorimetry on a Technicon auto-analyzer, (Anon., 1974).

The percentage of mycorrhizal root infection was estimated by visual observation of mycorrhizal infection after clearing washed roots in 10% KOH and staining with 0.05% trypan blue in lactophenol (v/v), according to Phillips and Hayman (1970). Quantification was performed using the grid-line intersect method (Giovannetti and Mosse, 1980).

Soil water potential was determined by a pressure plate apparatus (Soilmoisture Equipment Corp. 15 Bar Ceramic Plate Extractor, Cat. No 1500) and soil water content was measured by weighing the soil before and after drying at 110°C for 24 h (Bethlenfalvay et al., 1990).

2.4.1. Preparation of crude extracts

Leaves (1 g fresh weight) from mycorrhizal and nonmycorrhizal plants were harvested 6 h after the commencement of the light period (Murphy, 1984) and homogenized in 10 ml of a medium containing 50 mM tris (hydroxymethyl)-aminomethane (pH 8.0), 3 mM EDTA-Na, 250 mM sucrose, 1 μM $Na_2MoO_4(H_2O)_2$, 5 μM flavin adenine dinucleotide (FAD), 2 mM dithiothreitol (DTT), 1.5 mM phenylmethyl-sulfonyfluorid (PMSF) and 10 mM cysteine, with 0.5% (w/v) insoluble polyvinylpolypirrolidone (PVPP) in a Sorvall omni-mixer (3 min at 10000 rpm). Homogenates were filtered through 4 layers of nylon cloth, centrifuged at 30000 g for 20 min at 2°C and the supernatants were used for assaying the nitrate reductase (NR, EC 1.6.6.1) activity.

2.4.2. Nitrate reductase activity and protein assay

An *in vitro* method of nitrate reductase assay was used because, once the enzyme had been extracted, no further production of the enzyme could be induced by the phosphate in the assay medium, as might have occurred if an in vivo method had been used. Besides, Hageman and Reed (1980) found that *in vitro* NRA assays give results up to ten times higher than in vivo assays on the same plant material. The difference has been attributed to the lack of NADH in living tissues.

NRA was measured according to Becana et al. (1985) as modified by Caba et al. (1990). Protein content was determined by the method of Bradford (1976), using BSA (fraction V) to standardize the assay.

2.5. Statistics

Data were subjected to analysis of variance (ANOVA) with AM treatments and water status as factors. When the main effects were significant ($P < 0.05$), differences among means were evaluated for significance by Duncan's multiple range test (Duncan, 1955) in an orthogonal design. For the percentage values an Arcsin transformation was made before the statistical analysis.

3. Results

When the plants were grown under well-watered conditions, the shoot dry weight was higher in AM plants than in unfertilized non-AM ones. Plants fertilized with P made the same growth as *G. fascicula-*

Table 1
Shoot and root dry weight (g), shoot water content (%) and radical colonization (%) of mycorrhizal (*G. mosseae*, *G. fasciculatum* or *G. desertícola*) or uninoculated (control and P-fertilized) lettuce plants grown under well-watered (−0.04 MPa) or drought stressed (−0.17 MPa) conditions.

Treatments	Dry weight		Water content	AM colonization
	Shoot	Root		
−0.04 MPa				
Control	2.7d	1.2b	82.3b	0c
P-fertilized	4.2b	1.2b	83.8a	0c
G.mosseae	3.6c	1.5a	84.6a	81.9b
G. fasciculatum	4.2b	1.6a	84.8a	82.7b
G. desertícola	4.6a	1.4a	84.1a	92.5a
−0.17 MPa				
Control	0.6f	0.3e	74.7d	0c
P-fertilized	1.4e	0.5d	77.9c	0c
G. mosseae	2.6d	0.7cd	82.3b	80.1b
G. fasciculatum	2.8d	0.8c	81.9b	80.0b
G. desertícola	3.9bc	0.9c	81.6b	90.2a

Whitin each column, means followed by the same letter are not significantly different ($P < 0.05$) using Duncan's multiple range test ($n = 5$).

tum-colonized plants and both treatments increased yield compared to *G. mosseae* ones. Plants colonized by *G. deserticola* showed the highest growth (Table 1). The water deficit (−0.17 MPa) decreased the plant growth in all treatments. However, although in uninoculated plants this decrease was 78% (control) and 67% (P-fertilized), in AM plants it was only 38% (*G. mosseae*), 33% (*G. fasciculatum*) and 15% (*G. deserticola*) (Table 1). The root dry weight (Table 1) showed a similar trend to the shoot dry weight but there were no significant differences among the three mycorrhizal treatments.

At −0.04 MPa of soil water potential, the shoot water content (Table 1) was similar for mycorrhizal and P-fertilized plants, with unfertilized control plants having the lowest water content. The mycorrhizal plants had slightly decreased water content as a consequence of water stress (nearly 3 units percent as average), with more pronounced decreasing (9%, control and 7%, P-fertilized) in uninoculated plants.

The three AM fungi actively colonized the radicale system of the host plants and the water stress application did not affect this parameter, *G. deserticola* being the most colonizing fungus (Table 1).

The shoot NRA (Table 2) showed differences between mycorrhizal and nonmycorrhizal plants both under well-watered and drought stressed conditions. Thus, at −0.04 MPa (well-watered) control plants had 57% less total NRA than *G. deserticola*-colonized ones. Drought stress (−0.17 MPa) decreased this enzymatic activity in all the treatments, particularly in control plants. The decrease in the total activity of uninoculated plants was 57% (control) and 40% (P-fertilized) but in mycorrhizal plants it was only 18% (*G. fasciculatum*) and 13% (*G. desertícola*). In the case of *G. mosseae*, the NRA reduction was as high as in control plants (42%). In spite of this detrimental effect of drought stress on the NRA mycorrhizal plants also showed, at this water level, more activity than non-AM ones. In fact, control plants had 79% less NRA relative to *G. deserticola*-colonized ones. In the case of specific NRA (Table 2), the results are quite similar to those on total NRA.

The protein concentration was the same in all the treatments, regardless of mycorrhizal presence or water regime.

Data on N uptake (Table 3) showed that plants grown under well-watered conditions took up N more efficiently than stressed ones. Only *G. deserticola*-colonized plants had greater N content than the

Table 2
Total NR activity (mol NO^{2-} $gFW^{-1}h^{-1}$), specific NR activity (mol NO^{2-} mg^{-1}) and protein content ($mg\,ml^{-1}$) of mycorrhizal (*G. mosseae*, *G. fasciculatum* or *G. desertícola*) or uninoculated (control and P-fertilized) lettuce plants grown under well-watered (−0.04 MPa) or drought stressed (−0.17 MPa) conditions.

Treatments	Total NR ($\times 10^{-7}$)	Specific NR ($\times 10^{-3}$)	Proteins ($\times 10^{-3}$)
−0.04 MPa			
Control	18.2d	10.1cd	0.18a
P-fertilized	19.2d	11.3c	0.17a
G. mosseae	36.8b	21.6b	0.17a
G. fasciculatum	25.6c	15.1c	0.17a
G. desertícola	42.2a	24.8a	0.17a
−0.17 MPa			
Control	7.8f	4.6d	0.17a
P-fertilized	11.6e	7.3d	0.16a
G. mosseae	21.4d	12.6c	0.17a
G. fasciculatum	21.0d	12.4c	0.17a
G. desertícola	36.6b	21.5b	0.17a

Whitin each column, means followed by the same letter are not significantly different ($P < 0.05$) using Duncan's multiple range test ($n = 5$).

Table 3
N and P concentration (%) and total content (mg plant^{-1}) of mycorrhizal (*G. mosseae*, *G. fasciculatum* or *G. desertícola*) or uninoculated (control and P-fertilized) lettuce plants grown under well-watered (−0.04 MPa) or drought stressed (−0.17 MPa) conditions.

Treatment	Nitrogen		Phosphorus	
	%	content	%	content
−0.04 MPa				
Control	1.2c	31.5c	0.04f	2.8e
P-fertilized	0.8d	34.0c	0.14bc	6.2b
G. mosseae	0.9d	31.8c	0.09e	3.5d
G. fasciculatum	0.8d	34.2c	0.16ab	6.6b
G. desertícola	1.1c	49.7a	0.19a	7.8a
−0.17 MPa				
Control	1.8a	18.5e	0.10de	1.1f
P-fertilized	1.4b	18.7e	0.10de	1.4f
G. mosseae	0.8d	21.0de	0.10de	2.8e
G. fasciculatum	0.9d	24.8d	0.15bc	4.2c
G. desertícola	1.1c	42.4b	0.12cd	4.6c

Whitin each column, means followed by the same letter are not significantly different ($P < 0.05$) using Duncan's multiple range test ($n = 5$).

rest of the treatments. The negative effect of drought on this nutrient was lower in mycorrhizal plants (particularly in those colonized by *G. deserticola*) than in uninoculated ones.

Under well-watered conditions (−0.04 MPa) P-fertilized plants equalized the P concentration and content (Table 3) with plants inoculated with *G. fasciculatum*. Both treatments had a greater P concentration and content than plants colonized by *G. mosseae* and, obviously, unfertilized non AM plants. However, under drought stress conditions the P fertilization was ineffective and P-fertilized plants only equalized the P concentration with *G. mosseae*-colonized plants. So, the P nutrition was lower in this P-amended treatment than in plants colonized by *G. fasciculatum* or *G. deserticola*.

4. Discussion

NR is found in most plants, especially when nitrate is the nitrogen source. Nitrate reduction takes place in green tissues and in roots of plants. However, NRA is highest in plant leaves (Campbell, 1988). For this reason, the present study used such tissue to determine the NRA.

NR seems to be highly sensitive to the metabolic and physiological plant status, so it can be used as a stress index (Srivastava, 1980). However, the active contribution of mycorrhizae to nitrate uptake and assimilation has been recently evidenced (Azcón et al., 1992; Tobar et al., 1994; Cuenca and Azcón, 1994).

Results show that mycorrhizal plants had higher NRA than the uninoculated treatments. This finding agrees with results by Tobar et al. (1994) found, under reduced soil water availability, improved N uptake and assimilation by external hyphae of AM fungus. This finding indicates an especial capability of mycorrhizal plants to assimilate nitrate when the environmental conditions are limiting for vegetative development and it should result in higher biomass production as well as higher N content (Venkataramana et al., 1987). The present study tried to relate parameters such as NRA, plant development and plant N content. The treatment with the highest NRA (*G. deserticola*) also showed the highest biomass and N content under both well-watered and drought stressed conditions. In contrast, the uninoculated treatments decreased considerably plant growth, NRA and, consequently, the plant N content, as a consequence of drought.

The fact that mycorrhizal plants had higher NRA than nonmycorrhizal ones can be related to the phosphate requirements of this enzyme (Hageman and Reed, 1980). However, the present study included a P-fertilized control and, under well-watered conditions, this treatment showed higher (*G. mosseae*) or equal (*G. fasciculatum*) growth and P content as some of the mycorrhizal ones. Nevertheless, the NRA was lower than in mycorrhizal plants. These results indicate that either the AM fungi increases the NRA in the host plant (regardless of the P content) or the AM fungi have such enzymatic activity, *per se*. Both possibilities have been proposed (Ho and Trappe, 1980; Plassard et al., 1985). The capacity to assimilate nitrate is possessed by certain bacteria, some fungi, and virtually all algae and higher plants (Hoff et al., 1992). Most higher plants and algae contain NADH-specific NR (EC 1.6.6.1). A NAD(P)H-bispecific NR (EC 1.6.6.2.) has been identified in a limited number of higher plants. The third NR type, NADPH-specific (EC 1.6.6.3), is present in fungi, but has not been found in higher plants (Guerrero et al., 1981; Hoff et al., 1992).

It is known that NRA decreases in leaves exposed to dehydration because of a lower flux of nitrate from roots to the leaves (Aparicio-Tejo and Sanchez-Diaz, 1982; Sanchez-Diaz and Aguirreolea, 1993). This fact may also help explain the higher NRA in mycorrhizal than in nonmycorrhizal plants, because the plant water content was considerably higher in the AM plants, probably as a result of the better plant water relations in those plants (Ruiz-Lozano et al., 1995).

Vézina et al. (1989) found, in ectomycorrhizas, that mycorrhizal infection altered the enzymatic activities involved in nitrogen assimilation by pine trees and that the nature of these changes depended on the fungal associate. Similar results were found by Sarjala (1990). The preent results are also consistent with the finding by Sundaresan et al. (1988) that the NRA differs in various AM fungal species. They showed that all 12 AM fungi tested reduced nitrate to nitrite but the efficiency of reduction was significant in only three of the isolates. Under the experimental conditions of the present work, plants colonized by each AM fungus also showed different NR activities which was not related with their particular ability to improve growth and nutrition (particularly in the case of *G. mosseae*-colonized plants).

In conclusion, drought stress decreased the NRA but it was maintained considerably higher in mycorrhizal than in uninoculated plants, this fact being related to the increase in growth and N content (higher drought tolerance) of mycorrhizal plants, particularly when *G. deserticola* was the colonizing fungus.

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