Activity of nitrate reductase and glutamine synthetase in shoot and root of mycorrhizal *Allium cepa*
Effect of drought stress

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Received 3 July 1996; received in revised form 4 October 1996; accepted 4 October 1996

**Abstract**

The effect of arbuscular-mycorrhizal (AM) fungus *Glomus fasciculatum* on growth and N form assimilation was measured on onion (*Allium cepa*) grown under well-watered (−0.04 MPa) or drought conditions (−0.17 MPa). Two uninoculated control treatments, one provided with phosphate, were also addressed. These three treatments were supplemented with 2.0 mM nitrogen as nitrate and ammonium in a 1:1 ratio. Shoots and root weights, percentage of root colonized and glutamine synthetase (GS) (EC 6.3.1.2.) and nitrate reductase (NR) (EC 1.6.6.1) activity in shoot and root tissue were determined when water was maintained at (−0.04 MPa) or (−0.17 MPa) in the growth medium. The growth of *G. fasciculatum*-colonized plants was comparable to that of uncolonized P-supplemented plants under well-watered or drought conditions but mycorrhizal plants reached a higher specific and total GS activity in shoots and roots than P-fertilized plants growth at −0.04 MPa. The mycorrhizal effect on GS activity under water stress (−0.17 mPa) was evident only in roots being comparable to that found in P-fertilized plants. The proportion of GS in roots was increased in AM plants under whatever soil water conditions. The most marked increasing effect of AM-colonization on NR activity was in root tissue. Under water limitations the effectiveness of *G. fasciculatum* increasing NR activity in plant was enhanced. The proportion of nitrate assimilation into root was increased in AM plants particularly under well-watered conditions. Mycorrhizal modifications in the GS and NR distribution into root and shoot compartments may account for some physiological effect from mycorrhizal colonization. These results are further evidence of a direct effect on absorption, translocation and assimilation of both N forms by the endomycorrhizal system. That mycorrhizal plants can utilize nitrate form more efficiently than ammonium under drought conditions is consistent with more recent studies on the AM effect on N uptake from a neutral-alkaline soil. Results here presented suggest that either AM fungi increase the nitrogen forms assimilation in the host plant (regardless of P content) or the AM fungi have such enzymatic activities per se. This last assumption is supported by the relative high increase of NR and GS activities found in the roots of mycorrhizal plants. Nevertheless while NR was maintained increased in mycorrhizal roots under water stress the GS activity was not affected. This suggests the AM ability to provide an active nitrate acquisition in particular in water stressed environment. The different proportion of nitrate and ammonium assimilation into shoot and root compartments may account to modify physiological mycorrhizal responses related to plant sensitivity to drought. © 1998 Elsevier Science Ireland Ltd. All rights reserved.
Keywords: Arbuscular mycorrhiza; Nitrogen assimilation; Drought stress

1. Introduction

Some recent reports inform on the role of arbuscular-mycorrhizal fungi on nitrogen metabolism and in the utilization of different forms of soil nitrogen [2,3]. These two studies were carried out separately under well-watered or drought conditions. The results confirmed that the uptake and metabolism of N forms is particularly affected in mycorrhizal colonized plants depending on the mycorrhizal endophyte and the N source added. Other reports have focused on the use of NO$_3^-$ or NH$_4^+$ by external hyphae of the AM fungus _G. fasciculatum_ [27,28]. These ions mobilized from soils by an AM fungus are transferred directly to the root cells where assimilatory reduction could proceed. Nitrate reduction and ammonium assimilation takes place in green tissue and in roots of plants. In previous assay carried out in this laboratory the effect of AM fungi on NR and GS activities were only observed in shoot tissue at the end of the growth period. However, in the same way that uptake processes varied in AM plants the assimilation sites of N forms may be affected by mycorrhizal colonization and such aspects are known to affect physiological responses by plants [29]. Separation of nitrate and ammonium assimilation into shoot and root compartments may have relevance on plant physiology. In fact, the large carbohydrate requirement of nitrate reduction in mycorrhizal roots may be a factor limiting the ability of roots for such enzymatic processes. In leaves, nitrate reduction and CO$_2$ reduction compete for reductants and ATP from photosynthesis [23]. This competition may have important ecological consequences for the adaptation of plants to the limiting conditions. Ammonium assimilation in roots has a large carbohydrate requirements because of the need for carbon skeleton in the synthesis of amino acids and amides. The pattern of N sources assimilation may change in mycorrhizal plants not only by the fungal activity itself into the root but also by the different allocation of photosynthesis products from their non-mycorrhizal counterparts [24].

The objective of this study were to assess values of nitrate reductase and glutamine synthetase involved in NO$_3^-$ or NH$_4^+$ assimilation in shoots and roots of control, mycorrhizal and non-mycorrhizal but P-fertilized onion plants of comparable size as AM plants. The response of mycorrhizal colonization on the allocation and levels of enzymatic activities related to plant nitrogen assimilation was evaluated under well-watered and drought conditions.

2. Materials and methods

2.1. Experimental design

The experiments have three treatments: Non-mycorrhizal control, P-supplemented non-mycorrhizal plants and _Glomus fasciculatum_ colonized plants. The nitrogen fertilization consisted of 2 mM N given as NO$_3^-$:NH$_4^+$ in a 1:1 ratio. The test plant was onion and the cropping time was 35 days. For each treatment, one-half of the plants were maintained at a soil water potential of (-0.04 MPa) (field capacity) and the other half plants were subjected to drought conditions (-0.17 MPa). Treatments were replicated ten times given a total of 60 pots placed in a randomized block design.

2.2. Host plant and soil inoculation

Seeds of onion were sown in sterilized sand and then uniform seedlings were transplanted after two weeks to pots containing 1000 g of a sterilized 5:2 (v/v) mixture of soil and sand. The soil was collected from Granada (Spain); sieved (2 mm), diluted with quartz sand and autoclaved (100°C, 1 h during 3 consecutive days) and then reinoculated with a soil filtrate containing its own microbiota except arbuscular mycorrhizal propagules. This soil filtrate was obtained by suspending 100 g of the experimental soil in 1 l of sterile water. After shaking and decanting, the suspension was filtered (Whatman u.s) twice.
The main characteristics of the agricultural soil used were: pH 7.8; 2.07% organic matter; 0.1% N total, 4.6 μg NO₃⁻ – N; 1.8 μg NH₄⁺ -N/g, 32 μg P/g (NaHCO₃ extractable P), 311, 2 μg K/kg, 35.86% sand, 43.6 loam and 30.54 clay. Pots were filled with sterilized soil/sand mixture and twenty of them were inoculated with *Glomus fasciculatum*. Mycorrhizal inoculum consisted of spores, soil, hyphae and AM root fragments from a stock culture of the fungus with *Allium cepa* L. The AM fungal specie used, belonging to the collection of the Estación Experimental del Zaidín, was *Glomus fasciculatum* (Tax. and Gerd) Gerd. and Trappe. 10 g of inoculum having on average of 30 spores/g and 75% of infected root was placed directly below the seedlings.

2.3. Nitrogen application and phosphorus treatments

Plants were fertilized (20 ml/week) with P-free nutrients solutions (Hewitt 1952) [15] modified to contain N and K in a 1/1 ratio and so provide a total supply of 2 mM N and K per pot. Nitrogen was added as Ca (NO₃)₂ and (NH₂)₂SO₄ and K as K₂SO₄. P as KH₂PO₄ (100 μg/g) was supplied to half or non-inoculated plants. This rate was selected to match the effect of the fungus on plant growth there by being an appropriate control for the mycorrhizal plants.

2.4. Growth conditions

Plants were grown in a controlled environmental chamber under conditions of 50% RH, day and night temperatures of 27 and 18°C, respectively, and a photoperiod of 14 h. Photosynthetic photon flux density (PPFD) was 503 μmol/m² per s as measured with a lightmeter (LICOR, model LI-188B). Water was supplied by daily weighing to maintain the required water capacity of the test soil/sand mixture throughout the experiment. Half of the plants were maintained with a soil water potential of −0.04 MPa and the other half were allowed to dry until soil water potential reached −0.17 MPa.

2.5. Determinations

At harvest, 35 days after planting onion plants the root system was separated from the shoot and weights were recorded.

The percentage of VA mycorrhizal infection was microscopically assessed using the gridline intersect method of Giovannetti and Mosse (1980) [12], after staining by the procedure of Phillips and Hayman (1970) [21].

In vitro activities of root and shoot nitrate reductase (NR, EC 1.6.6.1), and glutamine synthetase (GS, EC 6.3.1.2) were determined in control, P-fertilized non-mycorrhizal plants and in mycorrhizal plants. Determinations were made on fresh leaves or root tissue harvested 6 h after the on set of the light period [20]. Detached plant material root or shoot (1 g) were frozen in liquid N₂ and pulverized with a mortar and pestle. The powder was extracted for 3 min with 3 ml of one of the following buffers. For the assay of GS, the buffer contained: 100 mM maleic acid (PH 6.8), 100 mM sucrose, 2% (v/v) β-mercaptoethanol, 15% (v/v) ethylene glycol and 1–5 mM phenylmethylsulphonylfluoride (PMSF), with 5% (w/v) insoluble polyvinylpolypyrrolidone (PVPP). For assay of NR the buffer contained: 50 mM Tris(hydroxymethyl)-aminomethane (pH 8.0), 3 mM EDTA, 250 mM sucrose, 1 μM Na₂MoO₄(H₂), 5 μM faldin adenine dinucleotide (FAD), 2 mM dithiothreitol (DTT), 1.5 mM phenylmethyl-sulfonylfluoride (PMSF) and 10 mM cysteine, with 5% (w/v) insoluble PVPP in a omni-mixer (3 min at 10000 rpm). Homogenates were filtered through miracloth and cenrifuged at 30000 × g for 20 min at 4°C and the supernatant was decanted and kept on ice. In vitro assay of NR was as described by Kaise and Lewis (1984) [16] and Becana, Aparicio-Tejo and Sánchez-Díaz, (1985) [4], as modified by Caba et al. (1990) [8]. In vitro GS activity was determined using the methods described by Lillo (1984) [19] and Canovas, Valpuesta and Nuñez de Castro (1984) [9], except that the buffer for the reaction was imidazol-HCl 0.15 mM (pH 7.8) containing 4 mM EDTA-Na₂. Protein was assayed according to Bradford (1976) [7], using BSA (fraction V) to standardize the assay.
The proportion of NR and GS activities allocated into shoots and roots compartment (number inside the bars, Figs. 1–3 and Fig. 4) was determined making 100 the shoot plus root enzymatic activity evaluated in each treatment and calculating the percentage that was into root or shoot tissue.

Data were subjected to a randomized block analysis of variance. Treatments were differentiated with Duncan’s multiple range test [11] by the least significant difference method (LSD $P \leq 0.05$).

3. Results

The fresh weight of onion leaves were increased by arbuscular-mycorrhizal colonization or by $P$ fertilization under both water conditions assayed in the present study. Weight of roots were not affected by these treatments. Plant growth responses were similar in the presence of $P$-fertilizer or AM colonization under well-watered or drought conditions. Mycorrhizal colonization was slightly repressed under water limitation in the medium (Table 1).

Figs. 1 and 2 show the total nitrate reductase and glutamine synthetase activities by plant and
Table 1
Shoot and roots weight and mycorrhizal infection of control, P-fertilized or *G. fasciculatum*-colonized onion plants grown under well-watered (–0.04 MPa) and drought (–0.17 MPa) conditions

<table>
<thead>
<tr>
<th>Treatments</th>
<th>0.04 MPa</th>
<th></th>
<th></th>
<th>0.17 MPa</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Shoots</td>
<td>Roots</td>
<td>AM(%)</td>
<td>Shoots</td>
<td>Roots</td>
<td>AM(%)</td>
</tr>
<tr>
<td>Control</td>
<td>0.90b</td>
<td>0.32a</td>
<td>—</td>
<td>0.77b</td>
<td>0.27a</td>
<td>—</td>
</tr>
<tr>
<td>P-fertilized</td>
<td>2.09a</td>
<td>0.35a</td>
<td>—</td>
<td>1.14a</td>
<td>0.31a</td>
<td>—</td>
</tr>
<tr>
<td><em>G. fasciculatum</em></td>
<td>2.45a</td>
<td>0.32a</td>
<td>83.3</td>
<td>1.12a</td>
<td>0.37a</td>
<td>76.2</td>
</tr>
</tbody>
</table>

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

their distribution (%) into shoot and root according to treatments and the water status in the soil.

The effects of *G. fasciculatum* on nitrate reductase activity was greater than that of the P-fertilizer. The biological treatment increased nitrate reductase in roots more markedly than in shoots under well watered conditions. Under water limitation the efficiency of mycorrhizal-colonization increasing NR activity in plant was widely enhanced (Fig. 1).

Shoot and root GS activity were stimulated in P-fertilized or AM plants compared with the controls under well-watered conditions. *G. fasciculatum* colonized plants reached a higher GS activity than those fertilized with P particularly in the root tissue. GS activity in shoot was increased by 21% and by 59% over control by P-fertilization or AM colonization respectively. The effect in the roots of these treatments were even greater compared with the control being GS activity increased 47% in P-supplied and 185% in AM plants (Fig. 2). These results are an indication on the ammonium metabolism promotion by the mycorrhizal fungus (Figs. 1 and 2).

The relative GS distribution between shoot and root shows that most GS activity was located in the shoot. Mycorrhizal colonization increased the proportion of GS allocated in roots (Fig. 2). Nevertheless in relation to GS activity tested in plants subjected to drought at – 0.17 MPa it was only increased in shoot of P-fertilized plants compared with control plants (Fig. 2). The GS activity in root of plants grown under water stress were higher in mycorrhizal plants than in P-fertilized ones (Fig. 2).

With regards to the NR activity this was higher in roots than GS was and the AM colonization increased the NR proportion allocated in the root particularly under well-watered conditions (Fig. 1).

Specific NR activity was increased by 13% in shoots and 17% in roots over controls by improvement the phosphate supply. This effect was increased by 22% only in shoots under stress conditions. Nevertheless the effect of *G. fasciculatum* was even greater compared with the control being 17% for shoot and 43% for root at –0.04 MPa and 52% (shoots) and 19% (roots) at –0.17 MPa (Fig. 3).

The GS specific activity was highly increased by the biological treatment in plant roots developed under well watered conditions (Fig. 4). Under such conditions P-fertilization was also effective in increasing this parameter in shoots and roots but less than the mycorrhizal colonization.

Specific GS activity was not affected by treatments under water stress situation. Specific GS under sufficient water supply was increased by both mycorrhizal colonization and by P-fertilization. In *G. fasciculatum* infected plants this effect was particularly noticeable (Fig. 4).

4. Discussion

After 35 days of plant growth phosphate fertilization had a similar effect on plant biomass production as mycorrhizal colonization did. At this early stage of plant growth the mycorrhizal colonization was fully developed in onion roots
although the full potential of AM effect on growth probably will be expressed in a later period. The high colonizing ability of *G. fasciculatum* allowed the determinations of NR and GS activities in AM colonized and noncolonized young root tissue. In previous experiments using the root material collected at the end of the experiment (60-days-old plants) negligible enzymatic activities were detected.

The presence of AM fungi in the root altered NR and GS activities in the shoots [2,3]. However, according to the results from the present study the increase of these activities in roots as consequence of mycorrhization was considerably higher than in shoots. These observations could demonstrate that fungal biomass allocated in the roots posses these enzymatic activities per se. Sundaresan et al. (1988) [26] reported that spores of AM fungi possessed NR activity. This fact has been further confirmed by Kraldor et al (1994) [18] testing that nitrate can be reduced by AM fungal cells. Smith et al (1985) [25] tested GS activity in fungal tissue from young mycorrhizal roots.

The fact that mycorrhizal plants had higher NR and GS activities than non-mycorrhizal ones can be related to the phosphate requirements of these enzymes [14]. However, in the present study the effect of *G. fasciculatum* on NR and GS activities cannot be attributed to this indirect effect since P-fertilized plants showed equal growth as the mycorrhizal ones.

Results indicate that both mycorrhizal infection and increased phosphate in soil are associated with increased NR and GS activities. In this sense the mycorrhizal effect could be interpreted as an indirect response to the improved phosphorus nutrition. As expected the mycorrhizal effect on enzymatic activities in shoots could be consistent with a P-mediated effect but the highest increases of NR and GS activities in roots as compared to the shoots seem to be consequence of a fungal effect per se. These results reinforce those reported by Smith et al. (1985) [25] on the mycorrhizal contribution to GS activity in the symbiotic system.

There are no previous reports on the activity of these enzymes in AM roots under water limited conditions. The large carbohydrate requirement for mycorrhization in the roots ought to be certainly one of the factors limiting the roots for NR and GS assimilation. But the present results show the opposite AM effect in spite of nitrate reduction and CO₂ reduction compete for reductants and ATP from photosynthesis [23]. The increased photosynthetic activity found in AM plants [2,3,5] is an indication of the mycorrhizal ability to promote plant adaptation to drought resistance. The enhanced CO₂ assimilation protected various enzyme systems. This is a mechanism for stress tolerance of AM plants against a range of perturbing effects. Such mycorrhizal effects appear to be the results of additive mechanisms involving nutrient availability and physiological processes [22].

Results from this study confirm the previous report by Azcón et al. (1992, 1996) [2,3] on lettuce, that *G. fasciculatum* enhances NR activity in plants more markedly than GS activity. Particularly under stress conditions. In fact, Azcón et al. (1996) [3] found an increased nitrate reductase activity under drought conditions in mycorrhizal plants compared to P-fertilized plants, while glutamine synthetase activity remained similar in both mycorrhizal and P-fertilized treatments. Experiments were developed on the same neutral alkaline soil in both studies. Mycorrhizal effect on assimilates NR and GS activities were previously tested in leaves of plant supplemented with ammonium only (GS) or nitrate only (NR) but in the present study similar tendencies were observed using a mixture nitrate, ammonium in 1:1 ratio. Under well watered conditions the mycorrhizal responses to N fertilization also varied according to N sources [1,25,30].

Regarding the relative NR or GS distribution (%) between shoots and roots in unstressed plants data show that in roots, NR activity is higher compared to the GS activity. The maximum for both GS and NR activities occurred in AM plants being the activities in root particularly increased. These results contrast with the fact that mycorrhizal roots require a extra C amount for fungal development [24]. The fact that assimilation of ammonium places a carbon stress on plant roots [17] may account for the reduced GS activity in AM-stressed roots.
The evidence of hyphal transport of N from a nitrate source under water limitation [27] indicates that mycorrhizal-colonization can be important for N-nutrition of plants grown in relatively dry soils [10]. The present results on the NR increased activity in mycorrhizal plants supporting the previous ones because the mycorrhizal effect on N-nitrate uptake is here reflected in an increased of N-nitrate assimilation activity. Different assimilation sites of nitrate and ammonium are known to modify physiological responses related to plant sensitivity to drought [6,13,29]. The information here reported supports the importance of arbuscular mycorrhization in neutral-alkaline soils where nitrate is the predominant nitrogen form and water potential is normally a plant growth limit- ing in most of mediterranean soils.

Acknowledgements

This study was supported by CICYT Spain (Project AGR 91-0605-CO2-01).

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