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R. Azcón · M. Gómez · R. Tobar

Physiological and nutritional responses by *Lactuca Sativa* L. to nitrogen sources and mycorrhizal fungi under drought conditions

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Abstract We measured the growth, nutrition, and N assimilation of arbuscular-mycorrhizal and non-mycorrhizal lettuce (*Lactuca sativa* L.) as affected by forms of N and drought. Moisture was maintained at 80% water-holding capacity, and N was applied as NO_3^-, NH_4^+, or NO_3^-/NH_4^+ (3:1, 1:1, or 1:3). The growth of *Glomus fasciculatum*-colonized plants was comparable to that of uncolonized P-supplemented plants when N was provided as NH_4^+ or combined NO_3^-/NH_4^+. When N was supplied solely as NO_3^-, *G. fasciculatum*-colonized plants produced a higher yield than P-fertilized plants, suggesting that the uptake and/or assimilation of NO_3^- was particularly affected by mycorrhizal status in this water-limited situation. Nutrient availability, except Ca, was less limited for mycorrhizal plants than for P-fertilized plants. P fertilization increased the growth, glutamine synthetase activity, and protein content of lettuce to the same extent that *G. fasciculatum* colonization did when N was applied as NH_4^+. With NO_3^--fertilization, *G. fasciculatum*-colonized plants showed increased growth, nitrate reductase activity, and protein content compared to P-fertilizer treatment. Plants colonized by *G. mosseae* showed increased photosynthetic activity and proline acumulation, and these mechanisms may be important in adaptation by the plant to drought conditions. The present results confirmed that under drought conditions, the uptake or metabolism of N forms is particularly affected in mycorrhizal fungi-colonized plants, depending on the mycorrhizal endophyte and the N source added. Thus the significance of arbuscular-mycorrhizal fungus selection for plant growth in drought conditions is a consideration for management strategy.

R. Azcón (✉)[1] · M. Gómez · R. Tobar
Departments of Microbiology and Agroecology,
Estación Experimental des Zaidín (C.S.I.C.), Apartado 419,
E-18080 Granada, Spain

Present address:
[1] Departamento de Miocrobiología del suelo,
Estación Experimental del Zaidín (C.S.I.C.), Apartado 419,
E-18080 Granada, Spain

Introduction

Few reports deal with the effect of the arbuscular-mycorrhizal symbiosis on the net N gain that plants derive from the soil (Ames et al. 1983; Smith et al. 1985) and on the significance of the fungal species selected as a symbiont (Azcón et al. 1992). Limited studies have provided information from well-watered plants. Recent reports have focused on the use of NO_3^- and NH_4^+ by external hyphae of the arbuscular-mycorrhizal fungus *G. fasciculatum* (Tobar et al. 1994a,b). But no report has provided data on N uptake and metabolism in mycorrhizal systems formed by *G. mosseae* or *G. fasciculatum* using different N forms in soil under water stress conditions. We evaluated the physiological status of plants associated with different endophytes to determine whether N uptake from various sources in dry soil is only a direct mycelial effect or is also associated with changes in overall mycorrhizal behaviour.

Water stress and plant sensitivity to N forms may affect N uptake or N assimilation. Mycorrhizal symbiosis may be an effective strategy for drought tolerance by the plant, based on independent and/or complementary mechanisms. In general, soil moisture affects the movement of nutrients, especially those with a low diffusing capability (such as NH_4^+, and mycorrhizal hyphae help the plant to take up soil nutrients by increasing the root surface area (Barea 1991). This mechanism also promotes more efficient extraction of water from the soil (Hardie 1985; Faber et al. 1991).

During drought, plants suffer a reduction in photosynthesis and so one form of plant adaptation may be an increase in C assimilation (Gale and Zerony 1985). Mycorrhizae may increase plant resistance to water stress by increased root hydraulic conductivity (Safir et al. 1972), stomatal conductance of host leaves (Augé and Duan 1991), or by a fall in the leaf osmotic potential allowing the maintenance of greater turgor (Augé et al. 1986). Mycorrhizae have been shown to improve the P nutrition of a host under water stress (Fitter 1988). Bethlenfalvay et al. (1988) and Davies et al. (1992) have reported, however,

that mechanisms by which arbuscular-mycorrhizal fungi increase drought resistance are independent of plant P nutrition.

The mechanism by which arbuscular-mycorrhizal fungi increase the plant response to drought is still unclear. It has been suggested that N affects the plant response to water stress (Bennet et al. 1986; Morgan 1986). N affects osmotic regulation, cell wall elasticity, carbohydrate metabolism, and the synthesis of drought-induced substances in roots (Ögren 1985). However, the plant metabolism depends to some extent on the ionic form in which N is adsorbed. Different uptake processes and assimilation sites of NO_3^- and NH_4^+ (Bloom 1988) are known to affect physiological responses by plants (Van Beusichem et al. 1988). These factors may modify the plant sensitivity to drought.

In the present study we tested the responses by control, P-fertilized, or arbuscular-mycorrhizal fungi-colonized plants at 80% water-holding capacity to N amendments as NO_3^- or NH_4^+>, singly or combined at NO_3^-/NH_4^+ ratios of 3:1, 1:1, or 1:3. Growth, nutrition, and some physiological parameters were evaluated at the end of the experiment. Nitrate reductase and glutamine synthetase involved in NO_3^- or NH_4^+ assimilation were determined in order to obtain information on plant N metabolism under drought stress conditions. Proline accumulation in leaves and CO_2 assimilation were used as physiological indices of plant tolerance to drought (Gale and Zerony 1985; Naidoo 1986). We also assessed the percentage of arbuscular-mycorrhizal fungal infection with different N forms applied to *G. mosseae* and *G. fasciculatum*-colonized plants.

Materials and methods

Experimental design

The experiment had four treatments: non-mycorrhizal control, P-supplemented non-mycorrhizal plants, and *G. mosseae*- or *G. fasciculatum*-colonized plants. The N fertilizer consisted of 3 mmol N given as NO_3^-, NH_4^+, or NO_3^-/NH_4^+ (3:1, 1:1, or 1:3), which was applied weekly in each treatment. All treatments were replicated five times, with a total of 100 pots, and placed in a random complete block design with one plant per pot.

Host plant and soil inoculation

Sterilized seeds of lettuce (*Lactuca sativa* L. cv Romana) were sown in sterilized sand. Uniform seedlings were individually transplanted after 14 days to pots containing 500 g of a sterilized 1:1 (v/v) mixture of quartz sand and soil.

Soil collected from Granada (Spain) was sieved (2 mm), diluted with quartz sand, and autoclaved (100°C, 1 h on 3 consecutive days). This agricultural soil had a pH of 7.8, 2.07% organic matter, 0.1% total N, 4.6 μg NO_3^--N g^{-1}, 1.8 μg NH_4^+-N g^{-1}, 32 μg P g^{-1} ($NaHCO_3$-extractable P), 311.2 μg K g^{-1} (exchangeable), with 35.86% sand, 43.6% silt, and 20.54% clay.

One hundred pots were filled with a sterilized soil/sand mixture and the relevant ones were inoculated either with *G. mosseae* (Nicol. and Gerd.). Gerd. and Trappe or *G. fasciculatum* (Thaxter sensu Gerd.) Gerd. and Trappe. The mycorrhizal inoculum consisted of spores, mycelia, and mycorrhizal root fragments from a stock culture of each fungus with *Allium cepa* L. The inocula (5 g pot^{-1}) were placed directly below the seedling in the planting hole. A soil extract (2 ml pot^{-1} of soil/water at equal v/v filtered through Whatman no. 1 paper) was added to reintroduce the native microbial population except for propagules of Endogonaceae.

N application and P treatment

The plants were fertilized (10 ml $week^{-1}$ pot^{-1}) with a P-free nutrient solution (Hewitt 1952) modified to contain N and K in a 1:1 ratio to provide a total of 3 mmol N and K per pot.

The fertilizer solution was supplemented with NO_3^-, NH_4^+, or NO_3^-/NH_4^+ in ratios of 3:1, 1:1, or 1:3 (see above). N was added as $Ca(NO_3)_2$ and/or $(NH_4)_2SO_4$, and K as K_2SO_4. P as KH_2PO_4 (43.9 g l^{-1}; 5 ml pot^{-1}) was supplied weekly to one-half of the non-inoculated plants, giving a total P supplement of 100 mg g^{-1}. This rate was selected to match the effects on growth of the fungi, thus providing an appropriate control for the mycorrhizal plants.

Growth conditions

The plants were grown in a controlled environmental chamber with 50% relative humidity, day and night temperatures of 27°C and 18°C, respectively, and a photoperiod of 14 h. The photosynthetic photon flux density was 503 μmol m^{-2} s^{-1} as measured with a light meter (LICOR, model LI-188B). The pots were weighed daily and water was supplied accordingly to maintain 80% water-holding capacity in the soil/sand mixture throughout the experiment (Azcón et al. 1988). The plants were exposed to drought 20 days after emergence. Water stress was applied by weighing each pot and adding water (twice a day) to reach the calculated weight for the desired water regimen. In a preliminary experiment, three levels or irrigation were tested: 100, 80, and 70% water-holding capacity (Peters 1965). Wilting point was reached at 70% water-holding capacity, and at 80% plant growth decreased by 50% compared with 100% water-holding capacity.

Measurements

After 8 weeks the photosynthetic rate area was determined by a portable, integrated, infrared CO_2 analyzer (Analytical Development Company, model LCA-3). The leaf area was determined with an automatic leaf area meter (LICOR, model LI-3100). The in vitro activity of leaf nitrate reductase (EC 1.6.6.1) and glutamine synthetase (EC 6.3.1.2) was determined in (P-fertilized) non-mycorrhizal plants and in *G. fasciculatum*-mycorrhizal plants in the treatments supplied with 100% NO_3^- (nitrate reductase) or 100% NH_4^+ (glutamine synthetase). The determinations were made on fresh leaves picked 6 h after the beginning of the light period. The leaves (1 g) were frozen in liquid N_2 and pulverized with a mortar and pestle. Nitrate reductase was assayed by the method described by Kaiser and Lewis (1984) as modified by Caba et al. (1990), using the extraction buffer described by Azcón et al. (1992). Glutamine synthetase activity was determined as described by Cánovas et al. (1984) and Lillo (1984), except that the buffer for the reaction was 0.15 mmol imidazole-HCl (pH 7.8) containing 4 mmol ethylenediaminetetraacetic-Na_2. For the assay, the extraction buffer described by Azcón et al. (1992) was used. Protein was assayed according to Bradford (1976). Proline was determined by colorimetry (Bates 1973).

The plant leaves were weighed and dried in a forced-draught oven at 70°C for 1 day and ground in a Wiley Mill to pass a 0.5 mm mesh. The material was digested with H_2SO_4 (Wolf 1982). Concentrations of N and P were colorimetrically measured on a autoanalyzer according to the manufacturer's instructions (Technicon 1974). K was determined by flame photometry and Ca and Mg by atomic absorption spectrophotometry using a Perkin-Elmer 5000 spectrophotometer.

Mycorrhizal infection was assessed microscopically using the grid-line intersect method of Giovannetti and Mosse (1980), after staining by the procedure of Phillips and Hayman (1970).

The data were subjected to analysis of variance. Treatment and N-fertilizer differences were analysed by Duncan's multiple range test using the least significant difference method ($P \leq 0.05$).

Results

The fresh weight and foliar area of plants were stimulated by P fertilizer (except when NO_3^- was supplied as the only N source) or by arbuscular-mycorrhizal fungal colonization (Fig. 1). *G. mosseae* was the most effective endophyte regardless of the N form applied under these water-stressed conditions. When a 3:1 solution of NO_3^-/NH_4^+ was provided, both fungi affected plant growth similarly.

Plant growth was similar in the presence of P fertilizer or colonization by *G. fasciculatum* when N was supplemented solely as NH_4^+ or as NO_3^-/NH_4^+. In the presence of NO_3^- fertilizer, *G. fasciculatum*-colonized plants reached a higher yield than those fertilized with P, which suggests that this arbuscular-mycorrhizal fungus promotes NO_3^- uptake and/or metabolism.

Plant uptake of N and P was significantly increased by mycorrhizal colonization. P-supplemented plants did not take up more N and P than control plants fertilized with NO_3^- or NH_4^+ alone (Fig. 2).

Supplementation with NH_4^+ alone or NO_3^- alone significantly increased the N content in *G. mosseae*-compared with *G. fasciculatum*-colonized plants. But the plant N content was similar in the two mycorrhizal treatments when N was applied as combined NO_3^-/NH_4^+.

The shoot P concentration was higher in *G. mosseae*-inoculated plants than in *G. fasciculatum*-colonized plants when N fertilizer was supplied as NH_4^+ or NO_3^-/NH_4^+. P-fertilized plants took up less N when NO_3^- alone or NH_4^+ alone was the major N source.

K uptake by non-mycorrhizal P-fertilized lettuce plants was not increased over that of controls when NO_3^- or NH_4^+ was the major N source. However, NO_3^-/NH_4^+ at ratios of 3:1, 1:1, or 1:3 increased the K content of P-fertilized plants compared with controls and matched the K content of *G. fasciculatum*-colonized plants. *G. mosseae*-colonized plants had the highest K content, but *G. fasciculatum*-colonized plants had a comparable K content when NO_3^- or NH_4^+ was added (Fig. 3). Ca uptake, in contrast to the other nutrients evaluated, was not increased by fungal colonization, and fell to the lowest values in *G. fasciculatum*-infected plants (Fig. 3). P-fertilized plants amended with NO_3^-/NH_4^+ at 1:1 or 3:1 took up similar concentrations of Ca as *G. mosseae*-colonized plants. The Mg content, like the N, P and K contents, was increased in mycorrhizal plants compared to P-fertilized or control plants (Fig. 4).

CO_2 assimilation was higher in *G. mosseae*-colonized plants than in the other treatments irrespective of the N source (except with NO_3^- application). The photosynthetic rate in P-amended compared to *G. fasciculatum*-colonized plants was not significantly different or was increased by the non-mycorrhizal treatments (Fig. 4).

Proline accumulation in leaf tissue was improved in the mycorrhizal treatments, particularly for *G. mosseae*-colo-

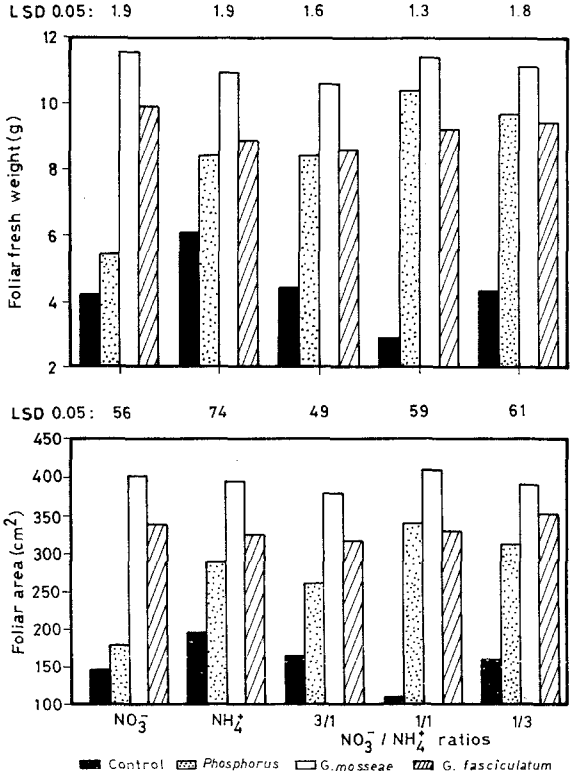

Fig. 1 Foliar fresh weight and foliar area of control, P-fertilized, *Glomus mosseae*-, or *G. fasciculatum*-colonized lettuce plants supplied with NO_3^-, NH_4^+, or NO_3^-/NH_4^+ at different ratios. DS 0.05 (Duncan's multiple range test $P \leq 0.05$). *LSD* least significant difference

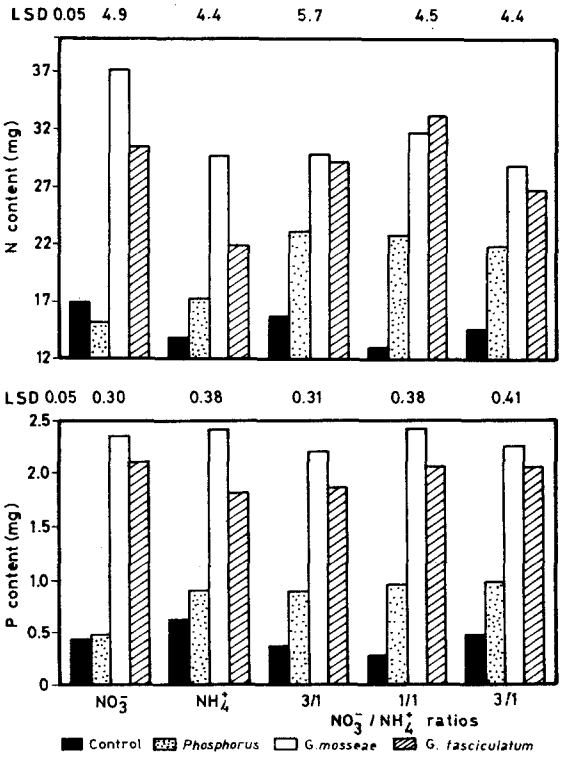

Fig. 2 Shoot N and P contents of control, P-fertilized, *Glomus mosseae*-, or *G. fasciculatum*-colonized lettuce plants supplied with NO_3^-, NH_4^+, or NO_3^-/NH_4^+ at different ratios. For further explanations, see Fig. 1

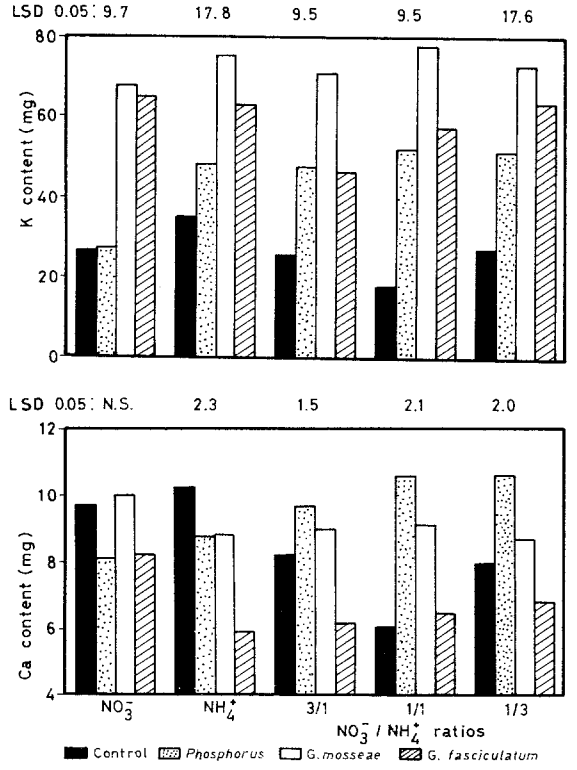

Fig. 3 Shoot K and Ca contents of control, P-fertilized, *Glomus mosseae*-, or *G. fasciculatum*-colonized lettuce plants supplied with NO_3^-, NH_4^+, or NO_3^-/NH_4^+ at different ratios. For further explanations, see Fig. 1

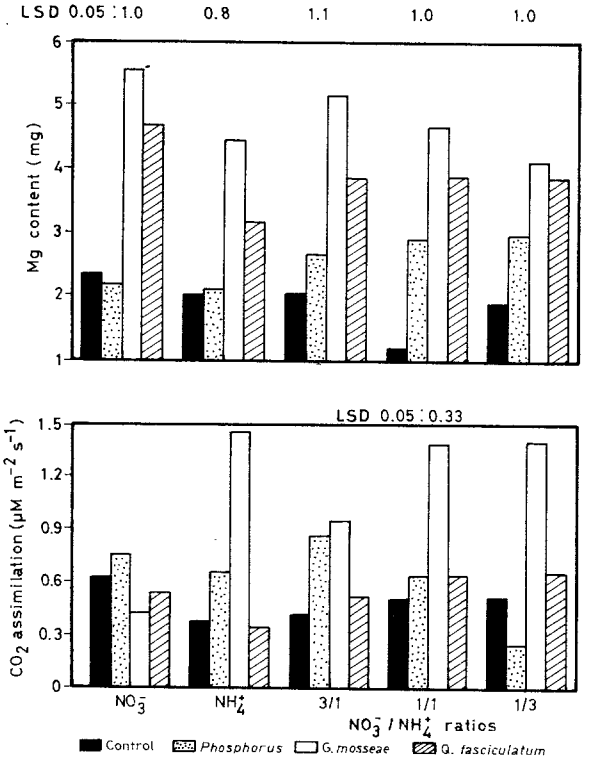

Fig. 4 Shoot Mg contents and CO_2 assimilation of control, P-fertilized, *Glomus mosseae*-, or *G. fasciculatum*-colonized lettuce plants supplied with NO_3^-, NH_4^+, or NO_3^-/NH_4^+ at different ratios. For further explanations, see Fig. 1

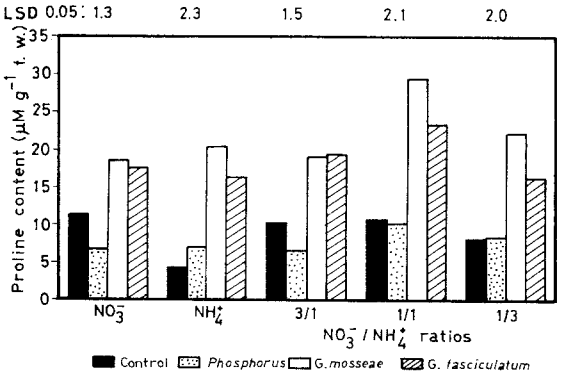

Fig. 5 Shoot proline content of control, P-fertilized, *Glomus mosseae*-, or *G. fasciculatum*-colonized lettuce plants supplied with NO_3^-, NH_4^+, or NO_3^-/NH_4^+ at different ratios. For further explanations, see Fig. 1

nized plants when NO_3^-/NH_4^+ was applied in a 1:1 ratio. Plants supplemented with NO_3^-/NH_4^+ at 1:1 accumulated the highest amounts of proline (Fig. 5). P-fertilized plants did not accumulate more proline than the control plants.

Leaf assimilative glutamine synthetase and nitrate reductase activity was increased in P-fertilized or mycorrhizal plants supplemented with NH_4^+ (glutamine synthetase) or NO_3^- (nitrate reductase) at 80% water-holding capacity compared with control plants (Tables 1, 2). Glutamine synthetase activity was stimulated to the same extent by P fertilizer as by mycorrhizal colonization. The effect of *G. fasciculatum* on nitrate reductase activity was greater than that of the P-fertilizer, and both treatments increased nitrate reductase more markedly than glutamine synthetase activity.

G. mosseae- and *G. fasciculatum*-colonized plants displayed similar degrees of mycorrhizal colonization and no significant differences were found among N sources (Table 3).

Table 1 Activity of glutamine synthetase (GS) in leaves of mycorrhizal, P-fertilized, and control lettuce plants. Results are means of five replicates. Means followed by the same letter are not significantly different ($P \leq 0.05$; Duncan's multiple range test)

Treatments	GS (μmol γ-glutamyl-hydroxamate h^{-1})	
	g^{-1} fresh weight	mg^{-1} protein
Control	19.25b	5.44b
P fertilizer	39.25a	6.53a
G. fasciculatum	32.12a	6.22a

Table 2 Activity of nitrate reductase (NR) in leaves of mycorrhizal, P-fertilized, and control lettuce plants. For further explanations, see Table 1

Treatments	NR (nmol NO_2^- h^{-1})	
	g^{-1} fresh weight	mg^{-1} protein
Control	10.14c	0.99c
P fertilizer	43.2b	4.2b
G. fasciculatum	58.6a	6.4a

Table 3 Effects of N source on percentage of arbuscular-mycorrhizal infection of lettuce plants inoculated with the mycorrhizal fungi, *Glomus mosseae* (*M*) or *G. fasciculatum* (*F*). Results are means of five replicates

	NO_3^-	NH_4^+	NO_3^-/NH_4^+ ratios		
			3:1	1:1	1:3
M	80.3	79.7	83.8	81.3	80.8
F	64.8	72.6	75.5	84.0	86.1

Discussion

For mycorrhizal plants, NO_3^- appeared to be the best N source in the experimental soil at the water limitation applied. For the control or P-fertilized plants, NH_4^+ performed best. These findings suggest that mycorrhizal fungi influence NO_3^- acquisition and/or assimilation by the symbiotic host plant. This contradicts the general assumption that arbuscular-mycorrhizal fungi do not affect the plant uptake of NO_3^- (Smith 1980; Oliver et al. 1983). The NO_3^- form is readily diffused in the soil and most reports do not acknowledge that mycorrhizal hyphae contribute to the supply of this ion to the plant. Nevertheless, a recent report (Tobar et al. 1994a) has provided evidence of hyphal transport of N from an NO_3^- source.

Although NO_3^- is likely to be the dominant form of inorganic N in many neutral-alkaline agricultural ecosystems, and is the most mobile N form (usually at least an order of magnitude more mobile than NH_4^+), it is often present in low concentrations and its diffusion is severely restricted in dry soils. Mycorrhizae with well developed extramatrical hyphae, however, increase the root surface area and thus are expected to aid the plant in the uptake of poorly diffused nutrients which become much less mobile in water-limited soils. In addition to promoting more efficient nutrient absorption around the root, mycorrhizal hyphae are able to increase water uptake from the soil, thus improving drought tolerance (Hardie 1985; Azcón et al. 1988; Davies et al. 1992). Both mechanisms actively provide nutritional advantages in general, and NO_3^- acquisition in particular, to mycorrhizal plants in water-stressed environments.

The manner in which mycorrhizal plants respond to added N in water-stressed soils is not clear and many interacting mechanisms may be involved. Nothing is known of the N form requirements nor of the mechanisms governing N metabolism under drought conditions. Factors regulating the uptake and assimilation of N from NH_4^+ or NO_3^- sources are still incompletely understood because much of the evidence concerning the effect of mycorrhizal fungi on N nutrition is indirect. Mechanisms that contribute to improved plant nutritional status, particularly P, have also been associated with mycorrhizal effects, improving NO_3^- nutrition and water relationships (Ames et al. 1983; Fitter 1988). Although it is difficult to produce comparable mycorrhizal and non-mycorrhizal plants, the preference displayed in the present study for NO_3^- by *G. fasciculatum*-colonized lettuce versus P-fertilized plants is an indication of the mycorrhizal effect on NO_3^-. This is apparently not a P-mediated effect. Because photosynthetic activity was higher in P-fertilized than in *G. fasciculatum*-colonized plants, this physiological parameter was not related to the mycorrhizal effect. This assumption is supported by the increased nitrate reductase assimilative activity found in mycorrhizal plants compared to P-fertilized plants, while glutamine synthetase activity remained similar in both mycorrhizal and P-fertilized treatments when NH_4^+ was added. Under well watered conditions the mycorrhizal responses to N fertilization also varied according to N source (Wyn and Storey 1981; Azcón et al. 1982; Smith et al. 1985; Azcón et al. 1992).

The increased photosynthetic activity and proline accumulation found in *G. mosseae*-colonized plants in the present study is an indication of the mycorrhizal ability to promote plant adaptation to drought resistance. The overall results from the present study confirm that N forms are more important for growth and nutrition in non-symbiotic than mycorrhizal plants. *G. fasciculatum*-colonized and P-fertilized plants grew similarly in the presence of any additional N form except NO_3^- alone. Several nutrients (N, P, and Mg) and the proline content increased in *G. fasciculatum*-colonized plants with water stress, while CO_2 was more efficiently assimilated in P-fertilized plants in general.

On the basis of these facts no clear correlations between nutritive and/or physiological abilities and drought resistance can be confirmed in mycorrhizal plants. Harley and Smith (1983) found no evidence of increased K uptake by plants infected with arbuscular-mycorrhizal fungi in spite of the low diffusion rate of this ion in the soil solution (Chapin 1980). However, the present results provide conclusive evidence that *G. mosseae* colonization directly promotes improved K uptake by plants under water stress. From these results, and in agreement with Davies et al. (1992) and Sanchez-Díaz et al. (1990), we conclude that mycorrhizal colonization appears to have an additive effect on nutrient availability and on physiological processes. The present results also show that it is possible to influence drought resistance and nutrient assimilation capacities of plants by selecting different fungal species as symbionts (Sarjala 1991).

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