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Uptake and metabolism of nitrate in mycorrhizal plants as affected by water availability and N concentration in soil

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Summary

We compare the effect of arbuscular mycorrhizal (AM) colonization and PO_4^{-3} fertilization on nitrate assimilation, plant growth and proline content in lettuce plants growing under well-watered (−0.04 MPa) or drought (−0.17 MPa) conditions. We also tested how AM-colonization and PO_4^{-3} fertilization influenced N uptake (^{15}N) and the percentage of N derived from the fertilizer (% NdfF) by plants under a concentration gradient of N in soil. Growth of mycorrhizal plants was comparable with that of P-fertilized plants only under well-watered conditions. Shoot nitrogen content, proline and nitrate reductase activity were greater in AM than in P-fertilized plants under drought. The addition of 100 μg g^{-1} P to the soil did not replace the AM effect under drought. Under well-watered conditions, AM plants showed similar (at 3 mmol N), greater (at 6 mmol N) or lesser (at 9 mmol N) %NdfF than P-fertilized plants. Comparing a control (without AM inoculation) to AM plants, differences in % NdfF ranged from 138% (3 mmol N) to 22.6% (6 mmol N) whereas no differences were found at 9 mmol N. In comparison with P fertilization, mycorrhizal effects on %NdfF were only evident at the lowest N levels, which indicated a regulatory mechanism for N uptake in AM plants affected by N availability in the soil. At the highest N level, P-fertilized plants showed the greatest %NdfF. In conclusion, AM symbiosis is important for N acquisition and N fertilizer utilization but this beneficial mycorrhizal effect on N nutrition is reduced under large quantities of N fertilizer.

Introduction

In many areas, plants are often exposed to drought stress, which decreases plant productivity. Water limitation reduces nutrient diffusion in soil and leads to a reduced root absortion capacity in crop plants. It has been proposed that arbuscular mycorrhizal (AM) symbiosis increases plant growth and drought tolerance by enhancing nutrient acquisition, increasing plant gas-exchange and improving osmoregulation (Ruíz-Lozano *et al*., 1995). Mycorrhizal hyphae have access to a greater volume of soil and can absorb and translocate slow diffusing nutrients and water to their associated plants (Ruíz-Lozano & Azcón, 1995). Thus, drought stress and nutrient availability for plant growth can be improved by AM symbiosis.

Nitrate constitutes the major part of the inorganic N pool in most soils because of the rapid nitrification of ammonium mineralized from organic materials or applied to the soil as fertilizers (Schmidt, 1982). However nitrate ions are very mobile in the soil solution and AM fungi may therefore be of less importance for N-nutrition in soils. It has long been thought that AM fungi do not contribute significantly to nitrate uptake by plants. Nevertheless, considering the great demand for N by plants and that under certain conditions, such as drought stress, nitrate difussion is reduced, the effect of mycorrhizal colonization on N uptake can become very important (Tobar *et al*., 1994; Azcón *et al*., 1996; Ruíz-Lozano & Azcón, 1996; Azcón & Tobar, 1998; Azcón *et al*., 2001). Some studies have demonstrated significant N enrichment in mycorrhizal-colonized non-leguminous plants such as tomato (Mäder *et al*., 2000), cucumber (Johansen *et al*., 1994; Johansen, 1999), lettuce (Hepper, 1983; Tobar *et al*., 1994; Azcón *et al*., 1996; Ruíz-Lozano & Azcón, 1996), onion (Azcón & Tobar, 1998), and wheat in comparison with non-mycorrhizal plants. All these studies confirm the capacity of extra-radical mycorrhizal mycelia to acquire N. In fact Mäder *et al*., (2000) determined a flux of ^{15}N nearly three times greater in AM roots than in non-mycorrhizal roots.

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Nitrate reductase (NR) is the first enzyme in the nitrate assimilation pathway and probably represents the rate-limiting step in this process (Campbell, 1988) and NR activity decreases in leaves affected by drought stress (Sánchez-Díaz & Aguirreolea, 1993; Azcón *et al*., 1996; Ruíz-Lozano & Azcón, 1996; Azcón & Tobar, 1998).

The nitrate mobilized from soil by AM fungal mycelia might be transferred directly as an anion to the root cells where reduction could take place. Besides, nitrate can also be reduced inside the AM fungal cells by the assimilatory reduction pathway, which implies that AM fungi have the gene-set for assimilatory nitrate reduction (Kaldorf *et al*., 1994). Nevertheless, the ability of AM-colonized roots to increase the utilization of nitrogen has been attributed, in most cases, to an indirect effect associated with phosphorus nutrition because this enzyme requires phosphate, which in turn may form a complex with molybdenum and thereby facilitate nitrate reduction (Hageman & Reed, 1980).

Nitrogen transport by extra-radical AM mycelium is regulated by the concentrations of this nutrient in the host (Johansen *et al*., 1994; Azcón *et al*., 2001). In these studies, tracer amounts of ^{15}N-labelled nitrate were used as a direct method for determining the proportion of N taken up by the plant from a fertilizer and the influence of microbial treatments on it (Azcón *et al*., 2001).

The aims of the present study were: (i) to compare the effectiveness of PO_4^{-3} fertilization or AM inoculation on nitrate assimilation under well-watered and drought conditions; and (ii) to evaluate the ability of AM colonized or PO_4^{-3} fertilized plants to take up N under different levels of N in the soil.

Materials and methods

We tested the ability of the mycorrhizal fungus *Glomus intraradices* (BEG 123) from our collection (EEZ) to take up N from a neutral-alkaline medium in two microcosm experiments.

1 Plant N (NO^-_3) uptake and N assimilation activity under drought stress and well-watered conditions was compared in non-mycorrhizal control plants, fertilized or not with 100 μg g^{-1} available-P and plants inoculated with *G. intraradices*.

2 The dynamics of *G. intraradices*-colonization was determined in four successive harvests (15, 25, 35 and 45 days after of inoculation) in order to select the best time for ^{15}N application when lettuce plants were AM colonized at an optimum level.

For the investigation of when the mycorrhizal contribution to N uptake was more important we used a concentration gradient of N in soil ranging from low (3 mmol) to medium (6 mmol) or high (9 mmol). In this experiment, ^{15}N applied when AM colonization was well established allowed us to determine the contribution of the AM fungus to the percentage of nitrogen derived from the N fertilizer applied (%NdfF). Two non-mycorrhizal control treatments, amended or not with PO_4^{-3}, were also included.

Experimental design

Experiment 1. We had three treatments: (i) non-fertilized, non-mycorrhizal control, (ii) P-fertilized, non-mycorrhizal control, and (iii) plants colonized by *Glomus intraradices*. Treatments were replicated 10 times (one plant per pot), giving a total of 30 pots placed in a randomized block design. For each treatment, half of the pots were maintained at a soil water potential of −0.04 MPa, and the other half of the pots were subjected to drought conditions (−0.17 MPa).

In order to select the best time for N^{15} application, we needed to know the development of AM-colonization with time. Sequential harvests during the growing period allowed us to determine when mycorrhizal lettuce plants had reached the maximum rate of active AM colonization. Thus, 20 additional pots inoculated with *G. intraradices* were used to determine the dynamics of AM-colonization in lettuce plants with five replicates harvested at 15, 25, 35 and 45 days after inoculation.

Experiment 2. This consisted of a randomized block with two factors: (i) microbial treatment, consisting of inoculation with a fungal strain of *Glomus intraradices*, non-fertilized and P-fertilized non-mycorrhizal control; (ii) three N levels supplied to the growth medium as calcium nitrate (3, 6 or 9 mmol N). Five replicates per treatment were prepared to give a total of 45 pots (one plant per pot).

In this experiment, the percentage of plant N derived from labelled fertilizer (%NdfF) was determined using ^{15}N tracer. The fertilizer Ca $(^{15}NO_3)_2$, 10 atom % excess was applied 45 days after inoculation (period previously selected by sequential harvests at which plants showed maximum active AM colonization).

Soil characteristics and microbial soil inoculation

A loamy agricultural soil was collected, from Granada province in Spain, for both experiments, The main characteristics (given on a dry weight basis) of the soil used were: pH (water) 7.8; 2.07% organic matter; 0.1% total N, 4.6 μg NO_3^--N g^{-1}soil, 1.8 μg NH_4^+-N g^{-1} soil, 32 μg P ($NaHCO_3$-extractable P) g^{-1}soil, 311.2 μg K g^{-1}soil. The soil contained 35.8% sand, 43.6% silt, and 20.5% clay. Sieved (2 mm) soil was diluted with quartz sand (4:6 v/v) to improve soil texture and sterilized (100°C, 1 hour for 3 consecutive days). Pots of 500 g of capacity were used for all the experiments. According to those characteristics the soil was classified as a loam (Baldwin *et al*., 1938).

The mycorrhizal inoculum used in both experiments was *G. intraradices* (BEG, 123) (Schenk and Smith). It was propagated in an open pot culture of *Lactuca sativa* L. and consisted of soil, spores, hyphae and AM root fragments. Ten grams of

this inoculum, having an average of 30 spores g^{-1} and 75% of colonized root pieces, were used in each AM inoculated pot. The inoculum was placed directly below the seeds of *Lactuca sativa* L. cv. Romana. Non-mycorrhizal 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. The inoculum filtrate was obtained by suspending 100 g mycorrhizal inoculum in 600 ml sterile water. The suspension was filtered (Whatman No 1) after shaking and decanting. Four seeds were sown and thinned to one seedling per pot after emergence.

Half of the non-inoculated plants received KH_2PO_4, (as 100 μg g^{-1} P). This rate has been previously used to match the effect of the AM fungus on plant growth, giving an appropriate control for the mycorrhizal plants.

Growth conditions

In both experiments, plants were grown in a controlled environment chamber at 50% RH, day and night temperatures of 27°C and 18°C, respectively, and a photoperiod of 14 hours. Photosynthetic photon flux density (PPFD) was 503 μmol m^{-2} s^{-1} as measured with a lightmeter (LICOR, model LI-188B, Lincoln, NE, USA). Pots were watered to constant weight to maintain the required water capacity of the test soil/sand mixture throughout the experiment. In the first 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 the soil water potential reached −0.17 MPa. In the second experiment, pots were watered daily to maintain constant soil water potential close to −0.06 MPa until harvest.

Plants were fertilized (20 ml $week^{-1}$) with a P-free nutrient solution (Hewitt, 1952) modified to provide a total supply of 3 mmol N (first experiment) or 3, 6 and 9 mmol N in the second experiment. Nitrogen was added as $Ca(NO_3)_2$.

Plants were harvested 60 days (first experiment) or 75 days (second experiment) after sowing.

Nitrogen (^{15}N) application

In the second experiment, tracer amounts of ^{15}N-labelled fertilizer were applied as an aqueous solution (10 ml of $Ca(^{15}NO_3)_2$, 10 atom % ^{15}N excess) to each pot, thereby providing a total of 2.5 mg kg^{-1} N. The ^{15}N was applied when AM-colonization had reached an optimum level of activity, 45 days after plant sowing.

Determinations

In both experiments, soil water potential was determined by a pressure plate apparatus (Soil Moisture Equipment, 15 Bar Ceramic Plate Extractor, Cat. N. 1500, Santa Barbara, CA, USA) and soil water content measured by weighing the soil before and after drying at 110°C for 24 hours (Bethlenfalvay *et al*., 1990).

Plants were dried for 48 hours at 70°C and the shoot dry weight (SDW) was recorded. The shoot tissue was analysed for N content (first experiment) and for N and P content (second experiment) (Lachica *et al*., 1973). Mycorrhizal root colonization was measured after staining (Phillips & Hayman, 1970) and the percentage of total colonized root length calculated using the gridline-intersect technique (Giovannetti & Mosse, 1980).

In the first experiment, nitrate reductase activity (NRA) was measured according to Kaiser & Lewis, (1984) and Becana *et al*. (1985), as modified by Caba *et al*., (1990). Leaf nitrate reductase (NR, EC 1.6.6.1) was determined (on 1 g fresh weight) of mycorrhizal and non-mycorrhizal shoot tissue harvested 6 hours after the beginning of the light period (Murphy, 1984). The leaves were 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% (g $litre^{-1}$) insoluble polyvinylpolypirrolidone (PVPP) in a Sorvall omni-mixer (3 minutes at full speed). The homogenates were filtered through four layers of nylon cloth, centrifuged at 30 000 ***g*** for 20 minutes at 2°C and the supernatants were used for the NR assay. The NR assay system consisted of 100 mM phosphate buffer (pH 7.5), 30 mM $NaNO_3$, 0.2 mM NADH and 100 μml of extract, in the final volume of 2 ml. After incubating the tubes at 30°C for 30 minutes, the reaction was terminated by keeping the tubes in a boiling water bath for 5 minutes. The nitrite produced was measured at 540 nm after colour development. The colour was developed by adding 1 ml each of 1% sulfanilamide in 1 M HCl and 0.01% N-(1-naphtyl)-ethylene diamine hydrochloride, to a suitable amount of aliquot.

Protein content was determined by the method of Bradford (1976), using BSA (fraction V) to standardize the assay. Free protein or proline was extracted in 50 mM phosphate buffer (pH 7.8) (4 ml) from 0.3 g leaf tissue from three independent plants (two leaves per plant) previously immersed in liquid N_2 and stored at −80°C, as according to Bates *et al*., (1973). Proline amount was determined by spectrophotometric analysis at 515 nm, using the ninhydrin reaction (Bates *et al*., 1973).

In the 20 additional pots, fungal succinate dehydrogenase (SDH) and alkaline phosphatase (ALP-ase) were determined at each harvest period to evaluate the dynamics of AM colonization by the methods of Smith & Gianinazzi-Pearson, (1990) and Tisserant *et al*., (1993) respectively.

In experiment 2, the N isotopic composition of plant shoots was determined by mass spectrometry (Fiedler & Proksch, 1975). The % excess ^{15}N was calculated by subtracting 0.366 (^{15}N natural abundance) from the measured % atoms of ^{15}N. The ^{15}N enrichment of the plant sample is then related to the amount of N taken up from the ^{15}N-labelled fertilizer. The

percentage of plant N derived from the labelled fertilizer (NdfF) was calculated according to Zapata (1990) from:

$$\%\text{NdfF} = \frac{\text{excess}\,\%\,15\text{N atoms in plant sample}}{\text{excess}\,\%\,15\text{N atoms in labelled fertilizer}} \times 100. \quad (1)$$

Statistical analysis

The data were subjected to analysis of variance. Differences between means were analysed by Duncan's multiple range test ($P \leq 0.05$). For the percentage values, the data were arcsin square transformed before statistical analysis.

Results

Experiment 1

G. intraradices-colonized plants had the greatest biomass over P-fertilized plants under the −0.17 MPa water regime (Table 1). Mycorrhizal lettuce growing without water limitation (−0.04 MPa) reached a similar shoot biomass to the P-fertilized non-mycorrhizal plants. Nevertheless, the growth of P-fertilized plants was reduced to a greater extent by water limitation than AM-colonized plants. Shoot dry weight from control plants was the least, irrespective of whether plants were grown under well-watered or drought conditions. Drought decreased the biomass of P-fertilized plants by 65% and of mycorrhizal plants by 42% (Table 1).

AM-colonization was slightly depressed under water limitation, although AM colonization still increased plant growth over the control and P-fertilized treatments under drought conditions (Table 1).

Nitrogen content by lettuce plants was significantly increased by AM colonization by 21% when compared with P-fertilized plants under drought conditions (Table 2). At −0.17 MPa, N content was reduced by 48% in P-fertilized plants and 28% in AM plants when compared with well-watered plants. Lettuce N uptake was poor in response to P-fertilizer application when water was limited (−0.17 MPa) whereas AM symbiosis had maximum N content and achieved increased growth (Tables 1, 2).

Table 1 Shoot dry weight and AM colonization (%) in non-mycorrhizal (control or P-fertilized) and mycorrhizal lettuce plants grown under well-watered (−0.04 MPa) or drought stress (−0.17 MPa) conditions

	Shoot dry weight/g		AM colonization/%	
Treatments	−0.04 MPa	−0.17 MPa	−0.04 MPa	−0.17 MPa
Control	8.1 b	4.2 d	—	—
PO_4^{-3} fertilized	15.8 a	5.5 c	—	—
G. intraradices	16.8 a	9.8 b	85.0 a	65.0 b

Means not followed by a common letter differ significantly ($P \leq 0.05$) from each other (Duncań s multiple range test).

Similarly, mycorrhizal plants accumulated substantially more proline than non-mycorrhizal plants irrespective of the water potential in the growing medium (Table 2). Shoot proline content was increased only under drought in the control plants.

The N/proline ratio decreased as a consequence of AM-colonization (Table 2). The effect of mycorrhizae on proline accumulation was stronger than on N uptake.

The P-fertilized plants showed a greater N/proline ratio than AM plants but smaller ratio compared with control plants. This ratio did not significantly change in uncolonized plants with drought conditions whereas in the non-mycorrhizal plants, P-fertilized or not, this ratio decreased (Table 2).

Shoot nitrate reductase (NR) activity was greater in mycorrhizal than in non-mycorrhizal plants (Table 3). P-fertilization was effective in increasing this value compared with the control plants but to a lesser extent than AM-colonization. Differences in NR activities according to treatments applied were greater under drought conditions (Table 3).

The greatest protein contents were measured in AM-colonized plants regardless of water availability in soil (Table 3). Protein content was affected only by watering conditions in the control plants.

Table 4 shows the development of AM colonization by *G. intraradices* in lettuce roots. The percentage of AM colonization increased with time and reached an optimum level at 35 days of inoculation. Colonization by *G. intraradices* developed slowly after 15 days of inoculation. The vital stains succinate-dehydrogenase (SDH) and alkaline phosphatase (ALP) allowed the fungal activity to be assessed in AM colonized roots. SDH activity was first observed after 15 days and ALP activity after 25 days of inoculation. Both SDH and ALP activities reached their maximum levels after 35 days of inoculation with no further change after 45 days of inoculation.

Experiment 2

The positive effect of P-fertilization and AM-colonization on N and P plant contents was evident at all N levels compared with the controls. The AM plants matched the effect of P-fertilization on N uptake only at the medium (6 mmol N) N level (Figure 1a). Phosphorus content was similar in mycorrhizal and P-fertilized plants only at the greatest N supply (9 mmol) whereas it was larger in mycorrhizal plants at the other two N levels applied (Figure 1b). At 9 mmol N, the inoculation with *G. intraradices* was as effective as P fertilization in terms of P uptake but less effective in terms of N uptake. This mycorrhizal effect on N acquisition at the greatest N level (9 mmol) can not be explained by less AM-colonization at this N level because there were no differences in the ability of *G. intraradices* to colonize lettuce roots at the three N levels (Figure 1c).

Mycorrhizal colonization was as effective at 3 and 6 mmol N as P fertilization in increasing NdfF (Figure 1d). Differences in %NdfF between AM-infected and non-colonized control plants ranged from 138% (3 mmol N) to 22.6% (6 mmol N).

Table 2 Nitrogen content (mg plant^{-1}) and proline (μmol ml^{-1}) content and N/proline ratio in non-mycorrhizal (control or P-fertilized) or mycorrhizal lettuce plants grown under well-watered (−0.04 MPa) or drought stress (−0.17 MPa) conditions

	N		Proline		N/proline	
Treatments	WW[a] −0.04 MPa	Drought −0.17 MPa	WW[a] −0.04 MPa	Drought −0.17 MPa	WW[a] −0.04 MPa	Drought −0.17 MPa
Control	30.4 ab	17.0 b	27.5 c	48.3 b	1.10 a	0.35 c
PO_4^{-3} fertilized	32.0 a	16.5 b	52.1 b	41.2 b	0.61 b	0.40 c
G. intraradices	28.0 b	20.0 a	174.7 a	166.6 a	0.16 d	0.12 d

[a]Well-watered.
Means not followed by a common letter differ significantly ($P \leq 0.05$) from each other (Duncańs multiple range test).

In contrast, at 9 mmol N, AM colonization affected NdfF similarly to unfertilized controls. Thus the beneficial mycorrhizal effect on %NdfF was evident at the smallest N application.

Discussion

There have been several investigations of the role of AM fungi in nitrogen metabolism and the utilization of different forms of soil nitrogen by them (Mäder *et al*., 2000). Previous studies have also shown that NO_3^- appeared to be the best N source for AM plants in a neutral-alkaline soil and particularly under drought stress conditions (Azcón *et al*., 1996). Water deficiency is widespread on earth and the importance of AM colonization to improve plant growth and nutrition under dry conditions has been studied (Ruíz-Lozano *et al*., 1995).

In the present study, the amount of P-fertilizer previously applied to non-inoculated plants to match plant growth (100 μg g^{-1} P) did not compensate for the positive effect of mycorrhizae under drought conditions. We found differences in the effectiveness of the AM-fungus under the two watering regimes as it could totally replace the effects of P application under well-watered conditions but not under drought. Perhaps the amount of the P applied to soil was insufficient to match AM responses under drought.

The fact that water stress produced a smaller reduction in shoot biomass in mycorrhizal plants is an indication of greater nutrient and water uptake in mycorrhizal plants under such conditions.

Improved N nutrition can result from a better exploitation of soil N and greater N uptake in mycorrhizal roots. Mass flow has been recognized as the process by which nitrate is taken up by roots. However, the direct uptake and transport of nitrate by extraradical AM mycelium have been confirmed by means of ^{15}N under non-stressed and water-stressed conditions (Tobar *et al*., 1994), but these previous studies were carried out under N-limiting conditions.

The high level of mycorrhizal colonization and the improved N nutrition found in the drought-stressed treatment is an indication of the good adaptation to drought of this isolate of *G. intraradices*, as previously reported by Ruíz-Lozano *et al*., (1995). Improved water uptake in AM-colonized plants via extraradical mycelial development has also been reported (Hardie, 1985; Ruíz-Lozano & Azcón, 1995).

The increase in N uptake at 3 mmol N in AM plants compared with P-fertilized plants was only evident under water limitation. The accumulation of solutes such as proline should decrease cellular osmotic potential, thus allowing for greater water retention during drought (Saliendra & Meinzer, 1991). Thus, a greater accumulation of proline could provide the leaf with an osmotic mechanism to prevent water loss. The great proline accumulation in *G. intraradices*-colonized plants observed in experiment 1 may have protected cellular metabolism by controlling cell

Table 3 Shoot nitrate reductase activity (NR, mmol hour^{-1} NO_2 g^{-1} fresh weight) and protein content (mg) of non-mycorrhizal (control or P-fertilized) or mycorrhizal lettuce plants grown under well-watered (−0.04 MPa) or drought stress (−0.17 MPa) conditions

	NR/g^{-1} fresh wt		Protein/mg	
Treatments	−0.04 MPa	−0.17 MPa	−0.04 MPa	−0.17 MPa
Control	35.0 c	10.1 c	4.0 b	1.0 c
PO_4^{-3} fertilized	45.0 b	43.0 b	4.5 b	4.2 b
G. intraradices	54.0 a	58.6 a	6.6 a	6.4 a

Means not followed by a common letter differ significantly ($P \leq 0.05$) from each other (Duncan's multiple range test).

Table 4 Dynamics of AM-colonization (following trypan blue (TB), SDH and ALP staining) at four harvest times from 15 to 45 days after *G. intraradices* inoculation

	AM colonization/%			
	Days			
Staining methods	15	25	35	45
TB	15 c	40 b	60 a	65 a
SDH	2 c	25 b	50 a	52 a
ALP	—	10 b	45 a	47 a

Means not followed by a common letter differ significantly ($P \leq 0.05$) from each other (Duncan's multiple range test).

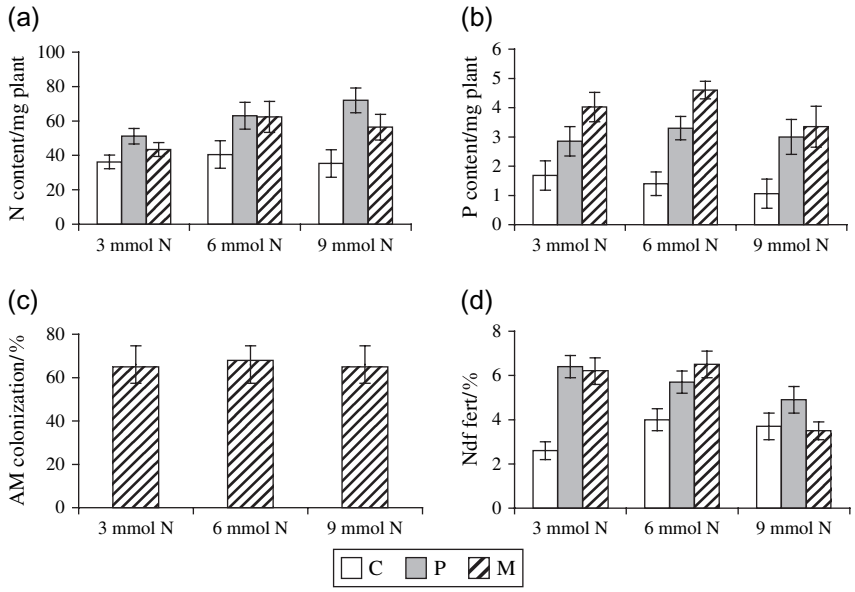

Figure 1 Nutrients (N, P) content (a, b), percentage of AM colonization (c) and percentage of plant N derived from fertilizer (% NdfF) (d) in non mycorrhizal (control (C) or PO_4^{-3}-fertilized (P)) or mycorrhizal (M) lettuce plants growing under three N levels. Bars are standard errors ($n = 5$).

turgor and by avoiding protein denaturalization (see Aspinall & Paleg, 1981). Nevertheless, proline accumulation in leaf tissue was observed in mycorrhizal plants under both water regimes. Therefore, in water-stressed plants, the greater accumulation of proline in AM plants was not only in response to the greater N content in these plants. In mycorrhizal non-stressed plants, although having an N content similar to the P-fertilized plants, the proline accumulation changed markedly between the two treatments. The accumulation of proline did not appear to be affected by N content in the plant as might be expected. The improvement of N nutrition by AM plants under drought stress over P-fertilized plants seemed not to be related to the effect of AM inoculation on proline accumulation as *G. intraradices* showed a similar effect under both water conditions. The greatest accumulation of this solute by AM-colonized plants may be related to the ability of the fungi to acquire water (Ruíz-Lozano & Azcón, 1995) and this may be the main mycorrhizal mechanism involved in the observed effect. The benefit that plants obtain from AM symbiosis in terms of growth and N nutrition under water-stress conditions is more than that obtained under well-watered conditions when compared with the non-mycorrhizal controls or P-fertilized plants.

Nitrate reductase activity was stimulated to a greater degree by AM colonization than by P fertilization, particularly under drought conditions, but the difference between these treatments was not correlated with N uptake of these treatments. Perhaps root NR activities (not determined here) could better explain this relationship.

It remains to be answered whether, or not, the increase in N acquisition could be directly (by fungal enzymes) or indirectly (by stimulation of the host enzymes) related to fungal colonization. Both mechanisms might be involved and could explain better N uptake by AM plants.

Previous reports have shown that the Trypan blue staining of roots is a good indicator of fungal establishment into the root (fungal colonization) but not of symbiotic efficiency (Smith & Gianinazzi-Pearson, 1990). The use of histo/cytochemical staining has been associated with effective AM colonization (Tisserant *et al*., 1993). Vital (SDH) and metabolically active (ALP) staining techniques provide valuable information on the fungal activity. Thus, 45 days after inoculation, having nearly 50% of SDH and ALP activity (the largest values determined) and similar values to those found after 35 days, which indicate a plateau, was the time selected as the best time for ^{15}N application in the second experiment.

The two experiments to test the ability of *G. intraradices* to improve plant N nutrition were not carried out simultaneously and the results are not comparable from a quantitative point of view, but they are complementary and useful in drawing some general conclusions about the role of AM fungi in N nutrition under different ecological and practical situations. The use of ^{15}N methodologies allowed us to assess the influence of AM-colonization on %NdfF and optimum amount of N fertilizer applied for the greatest mycorrhizal effect on these values. At the two lowest N fertilization levels (3 and 6 mmol) mycorrhizal plants have smaller or similar N contents to P-fertilized plants. Nevertheless, the highest N level (9 mmol N) significantly reduced %NdfFert but this effect was particularly great in AM-colonized plants. The application of 9 mmol N revealed a possible regulatory mechanism for AM mycelia on N uptake and it was affected by the level of N in the medium as previously suggested by Johansen *et al*., (1994).

In fact, the regulation of N uptake (Ndf fert) by the extraradical mycorrhizal mycelium seems to depend on nitrogen availability in the medium in spite of the AM colonization not being affected.

In P-fertilized plants on the other hand, the application of 6 mmol N resulted in a similar %NdfF as in mycorrhizal plants. Nevertheless, an application of 9 mmol N increased N content and did not affect %NdfF only in P-fertilized plants. Thus, the benefit of the AM fungus on N plant nutrition decreased when the N concentration in the medium was more than 6 mmol N under these experimental conditions.

The present results provide conclusive evidence that AM-colonization promotes %NdfF under poor N supply (3 mmol N). This AM activity increased particularly under drought conditions. The effect of the inoculation with an AM fungus in improving nitrogen metabolism in the plant as well as N uptake from soil, as found in our study, confirms and extends previous findings (Tobar *et al*., 1994; Azcón *et al*., 1996; Azcón & Tobar, 1998; Azcón *et al*., 2001).

In many areas, a common situation is that plants grow under nutrient (N, P) and water limitation. Our results confirm that AM-colonization plays an important role in plant N uptake and it can result in a greater ability of AM plants to use the fertilizer applied. Physiological and morphological root characteristics and the acquisition of nutrients by roots represent the most important role in nutrient use efficiency, particularly in AM-colonized roots.

Results from this study increase the knowledge of how nitrogen fertilizers may be better managed in terms of the level of application, thereby decreasing pollution and the cost associated with nitrogen fertilization. Thus, the improved management of fertilizer application and AM fungi has practical and ecological interest. The importance of the AM symbiosis to improve plant N nutrition in natural systems as well as to reduce the use of fertilizers in low input sustainable agriculture is of great interest. This aspect, in addition to the other non-nutritional factors, such as the role of AM fungus in improving plant tolerance to environmental stresses (Ruíz-Lozano & Azcón, 1995; Ruíz-Lozano *et al*., 1995), needs to be considered in sustainable systems.

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