Biol Fertil Soils (2001) 34:57–63
DOI 10.1007/s003740100373

ORIGINAL PAPER

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Impact of soil nitrogen concentration on *Glomus* spp.-*Sinorhizobium* interactions as affecting growth, nitrate reductase activity and protein content of *Medicago sativa*

Received: 13 November 2000 / Published online: 17 May 2001

Abstract Our objective was to evaluate how increasing levels of N in the medium (0, 4, 8 and 16 mmol N added kg^{-1} soil) affect the interaction between *Sinorhizobium* and arbuscular mycorrhiza (AM) fungi in the tripartite symbiosis with *Medicago sativa*. Growth response, nutrient acquisition, protein content, and nitrate reductase (NR) activity were measured both in plant shoots and roots. Results showed that N levels in soil did not affect mycorrhizal colonization but they strongly influenced nodulation, particularly of mycorrhizal plants. Mycorrhizal colonization was required for a proper nodulation when no N was applied to soil. In contrast, the addition of 4 mmol N kg^{-1} soil reduced nodulation only in mycorrhizal plants and 8 mmol N added kg^{-1} soil allowed nodule formation only in non-mycorrhizal plants. Nodulation was totally inhibited in all treatments with the addition of 16 mmol N added kg^{-1} soil. N addition enhanced NR activity in all the treatments, while AM colonization increased the proportion of NR allocated to roots. This effect was more pronounced under the lowest N levels in the medium. The two AM fungal species showed different distribution pattern of enzymatic activities in plant tissues indicating specific physiological traits. Protein content as well as the relative proportion of protein in roots were greatly increased after mycorrhizal colonization. *Glomus intraradices*-colonized plants had the highest protein content in shoot and root. Mycorrhizal effects on growth, N acquisition and biochemical variables cannot be interpreted as an indirect P-mediated effect since P content was lower in mycorrhizal plants than in those which were P fertilized. Mycorrhizal colonization increased the N content in plants irrespective of the N level, but the effectiveness of AM fungi on plant N acquisition depended on the AM fungus involved, *G. intraradices* being the most effective, particularly at the highest N rate. N_2 fixation, enhanced by AM colonization, contributed to N acquisition when a moderate N quantity was available in the soil. Nevertheless, under a high N amount the nodulating process and/or fixing capacity by *Sinorhizobium* was reduced in AM plants. In contrast, the AM fungal mycelium from a particular mycorrhizal fungus may continue to contribute efficiently to the N uptake from the soil even at high N levels. These results demonstrate the particular sensitivity of AM fungal species in terms of their growth and/or function to increasing N amounts in the medium. A selection of AM fungi used to address specific environmental conditions, such as N fertilization regimes comparable to those used in agronomic practices, is required for a better use of N applied to soil.

Keywords Ammonium nitrate · Arbuscular mycorrhizas · *Sinorhizobium* · Nitrate reductase · *Medicago sativa*

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Introduction

The establishment of a dual symbiosis [arbuscular mycorrhizal (AM) and rhizobial nodules] in legume roots is of great interest for the sustainability of agroecosystems. The microorganisms contribute substantially to the P and N requirements of the plant and, therefore, the inputs of synthetic fertilizers required for optimal plant growth can be significantly reduced with a considerable benefit for the environment (Barea et al. 1997).

The interactions between AM fungi and *Rhizobium* spp. with their common host legumes have been the subject of many studies (Barea et al. 1997). However, little is known of the effect that N fertilizers have on the tripartite symbiosis, including changes in biochemical reactions and growth responses.

N is a major nutrient affecting both nodulation and N_2 fixation, and also AM colonization (Subba Rao and Krhisna 1988; Patterson et al. 1991; Johansen et al. 1994). NO_3^- ions are very mobile in soil solution and, therefore, AM fungi were thought not to contribute sig-

nificantly to the NO_3^- uptake of plants (Ames et al. 1983; Barea et al. 1987). However, some reports (Azcón et al. 1992; Johansen et al. 1993; Cuenca and Azcón 1994; Johansen et al. 1994; Azcón et al. 1996) demonstrate that AM colonization can improve NO_3^- acquisition. In fact, the use of ^{15}N under both stress and non-stress conditions showed direct uptake and transport of NO_3^- through the extraradical mycelium of AM fungi (Johansen et al. 1994; Tobar et al. 1994a, 1994b). In addition, an increase in nitrate reductase (NR) activity has been shown for both roots and shoots of AM plants (Tobar et al. 1994b; Ruíz-Lozano and Azcón 1996; Azcón and Tobar 1998). Moreover, NO_3^- has been suggested to be the preferential N source for AM fungi (Azcón et al. 1996). NR (EC 1.6.6.1) is the first enzyme in the NO_3^- assimilation pathway. It probably represents the rate-limiting step of this process (Campbell 1988) and regulates the N nutrition of plants (Vogel and Dawson 1991). Interestingly, there is genetic evidence of fungal NR genes in AM fungi (Kaldorf et al. 1998).

When the N level in a plant is high, a considerable amount of C is required for amino acid and protein synthesis, and consequently less C is available for transport to the nodules. Such feedback control mechanisms enable leguminous plants to regulate N_2 fixation rates according to the demand for combined N. In this way the plant restricts the expenditure of additional respiratory cost due to N_2 fixation to those conditions in which the external supply of N is the growth-limiting factor (Vance et al. 1983). AM colonization may alter this feedback control since it increases the root C demand at the same time that it increases the N level in the plant (Pacovsky 1989). However, AM colonization can improve photosynthetic processes (Azcón et al. 1992, 1996; Ruíz-Lozano and Azcón 1994). The tripartite symbiosis in legumes benefits plant growth and nutrition under N-limiting conditions leading to a physiologically balanced interaction between the microsymbionts and the plant, which is required for their mutual functional compatibility. However, these general trends may change under moderate to high N concentrations.

The objective of this study was to determine how AM fungi influence N uptake by *Sinorhizobium*-inoculated plants growing under a gradient of N concentrations. We investigated how they can affect N_2 fixation and how this is regulated by changes in the NR activity of the plants. The results determine the concentration of N in the medium required for an optimal interactive microbial (*Rhizobium*-AM fungus) effect for maximizing legume N nutrition.

Materials and methods

Experimental design

The experiment was a pot assay set up as a complete randomized block design with two factors. One factor, AM treatments, contained two AM fungal inoculation treatment, i.e. *Glomus mosseae* (BEG12) and *G. intraradices* (LPA8), and a 100 µg P g^{-1} soil supplemented non-mycorrhizal treatment. The second factor, N addition to soil, contained four levels (0, 4, 8 or 16 mmol N added kg^{-1} soil). All plants were inoculated with a wild type *Sinorhizobium meliloti* strain GR4 (Casadesús and Olivares 1979). These 12 treatments were replicated 5 times for a total of 60 pots.

Soil characteristics and P and N application

The test soil was collected from Granada Province, Spain. It was a reddish-brown calcareous type (42.0% clay, 39.8% loam, 18.2% sand) with pH 7.4; 1.23% organic matter; 4.5 mg P kg^{-1} soil extractable with 0.5 M $NaHCO_3^-$ (Olsen P). The soil was sieved (2-mm pore size), diluted with quartz sand (5/2, v/v) and steam sterilized (100°C, 1 h, 3 consecutive days), and then inoculated with a soil and AM inoculum filtrate [10 ml pot^{-1} of a soil/water mixture (1/1, v/v) filtered through a Whatman no. 1 paper] to reintroduce the normal microbiota except AM propagules.

Pots were filled with 500 g sterilized soil/sand mixture. P was gradually applied as KH_2PO_4 (100 µg P g^{-1} soil) to non-mycorrhizal plants in the first 5 weeks of plant growth. N was supplied by mixing an appropriate solution of NO_3NH_4 with soil before sowing, to provide a total of 0, 4, 8 or 16 mmol N added kg^{-1} soil.

Each plant received 10 ml weekly (from the third week until the seventh week) of Hewitt's nutrient solution (Hewitt 1952) lacking N and P.

Host plant and inoculation treatments

Alfalfa (*Medicago sativa* L., cultivar Aragón) surface-sterilized seeds were sown in pots of 500 ml (1 seed/pot). The mycorrhizal inoculum consisted of loose soil containing *G. mosseae* or *G. intraradices* spores, hyphae and lettuce root fragments colonized to approximately 80% by *G. mosseae* and 90% by *G. intraradices*. Ten grams per pot of inoculum were placed directly below the planting hole. The inoculum of *S. meliloti* was grown in Ty medium (tryptone 0.5%, yeast extract 0.3%, $CaCl_2$ 0.05%) and applied (1 ml containing 10^8 cells per pot) on the roots of the seedling of both mycorrhizal and non-mycorrhizal plants.

Plants were grown for 75 days under controlled environmental chamber conditions with temperatures of 27°C/18°C, day/night respectively, a relative humidity of 50% and a photoperiod of 14 h. Photosynthetic photon flux density was 503 µmol m^{-2} s^{-1}. Water lost was supplied daily by top watering to maintain soil moisture close to 100% field capacity.

Determinations

After harvest, shoot and root weights were recorded and the shoot tissues analysed for N and P (Lachica et al. 1973).

The percentage of AM colonization was microscopically assessed using the gridline intersect technique (Giovanetti and Mosse 1980) after staining the root samples according to Phillips and Hayman (1970), using lactic acid to avoid using phenol.

In vitro assay of NR was done as described by Kaiser and Lewis (1984) and Becana et al. (1985), as modified by Caba et al. (1990). Root and shoot NR activities (EC 1.6.6.1), were determined on fresh leaves or root tissue harvested 6 h after the commencement of the light period. Detached root or shoot (1 g), was frozen in liquid N_2 and pulverized with a mortar and pestle. The powder was extracted for 5 min with 2 ml of a buffer consisting of 50 mM TRIS(hydroxymethyl)-aminomethane (pH 8.0), 3 mM EDTA, 250 mM sucrose, 1 µM $Na_2MoO_4(H_2)_2$, 5 µM flavin adenine dinucleotide, 2 mM dithiothreitol, 1.5 mM phenylmethyl-sulphonylfluoride and 10 mM cysteine, with 3% (w/v) insoluble PVPP in a pestle (5 min). Homogenates were filtered through miracloth and centrifuged at 3,000 *g* for 5 min at 4°C. The supernatant was collected and centrifuged again at 30,000 *g* for 20 min at 4°C, and the resulting supernatant was decanted and kept on ice. Protein was assayed according to Bradford (1976), using bovine serum albumin (fraction V) to standardize the assay.

The statistical significance of the differences between treatments was determined by two-way ANOVA. Five replicates were used for calculations. Significance was determined according to Fisher's protected least significance difference. In the case of percentage values these are represented after arcsin square-root (×/100) transformation.

Results

The rate of mycorrhizal colonization by the two AM fungi was not affected by the N level in the medium (Fig. 1A). Mycorrhizal colonization greatly enhanced nodule production when no N was added (Fig. 1B). In contrast, the number of nodules formed was highly influenced by the availability of N in the soil, particularly in mycorrhizal plants. In P-fertilized control plants an amount of 16 mmol N added kg^{-1} soil was required to inhibit nodule formation. In AM-colonized plants, 4 mmol N added kg^{-1} soil reduced the nodule number, and a higher amount of N (8 and 16 mmol N added kg^{-1} soil) totally inhibited nodulation.

The benefit of the mycorrhiza to the shoot and root biomass generally decreased as the N concentration in the medium increased (Fig. 2A, B). *G. intraradices* was the most effective AM fungus at increasing alfalfa growth (by 304% and 216% over the non-mycorrhizal controls, at the lowest and highest N levels, respectively). Plant growth of P-supplemented and *G. mosseae*-colonized plants was similar at 8 and 16 mmol N added kg^{-1} soil, but *G. mosseae* was effective with low levels of N in the medium (Fig. 2A, B).

The P content in P-fertilized non-mycorrhizal plants was higher than in mycorrhizal plants at 4 and 8 mmol N added kg^{-1} (Fig. 2C). *G. intraradices* enhanced N plant acquisition, increasing the N content in plants regardless of the N availability in the growth medium (Fig. 2D).

N addition increased shoot NR activity. This effect was more evident in mycorrhizal plants (Fig. 3A). The NR activity in roots of *G. mosseae*-colonized plants was similar to that found in P-supplemented non-mycorrhizal plants, and did not change with increased N supply. Nevertheless, *G. intraradices*-colonized plants had the highest NR activity in roots, and this even increased when the N concentration in the medium increased (Fig. 3B). Most of the NR activity was in the shoot (Fig. 3C), but proportions of NR activity in the shoot and root changed according to biological treatments and N level. In the soil without N addition, mycorrhizal colonization, particularly by *G. intraradices*, increased the proportion of NR allocated to roots. *G. mosseae*-colonized plants has less NR in roots as N levels were increased. In contrast, *G. intraradices*-colonized plants had a higher

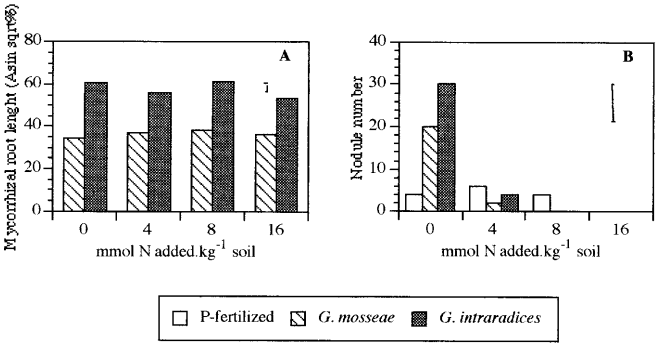

Fig. 1A, B Mean values of symbiotic variables. **A** Percentage of mycorrhizal root length; **B** number of nodules. *Bars* represent Fisher's protected least significance difference (PLSD) (P≤0.05)

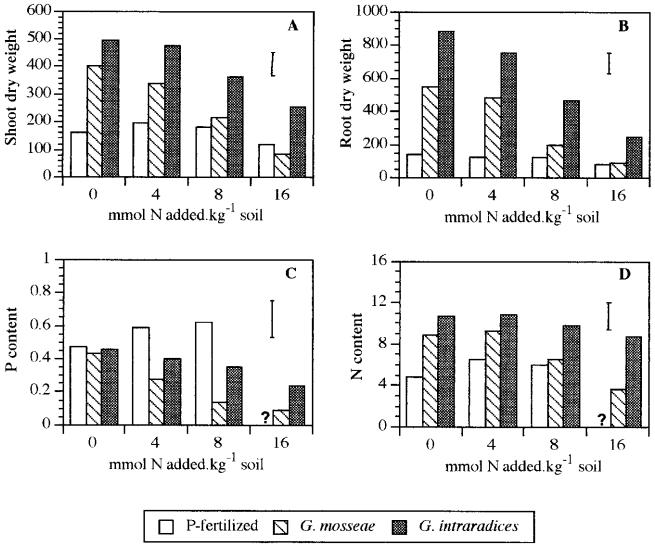

Fig. 2A–D Mean values of plant growth and nutritional variables. **A** Shoot dry weight (mg $plant^{-1}$); **B** root dry weight (mg $plant^{-1}$); **C** shoot P content (mg $plant^{-1}$); **D** shoot N content (mg $plant^{-1}$). *Bars* represent PLSD (P≤0.05). *?* Insufficient material for analysis

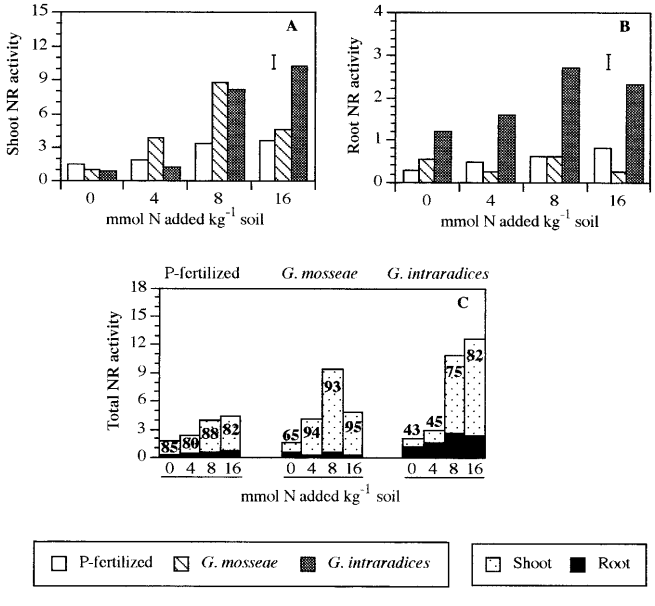

Fig. 3A–C Nitrate reductase (NR) activity in plants. **A** Shoot NR activity (nmol h^{-1} $plant^{-1}$); **B** root NR activity (nmol h^{-1} $plant^{-1}$); **C** total NR activity per plant and relative proportion shoot/root. *Numbers* indicate the relative proportion of shoot NR activity; *bars* represent PLSD (*P*≤0.05)

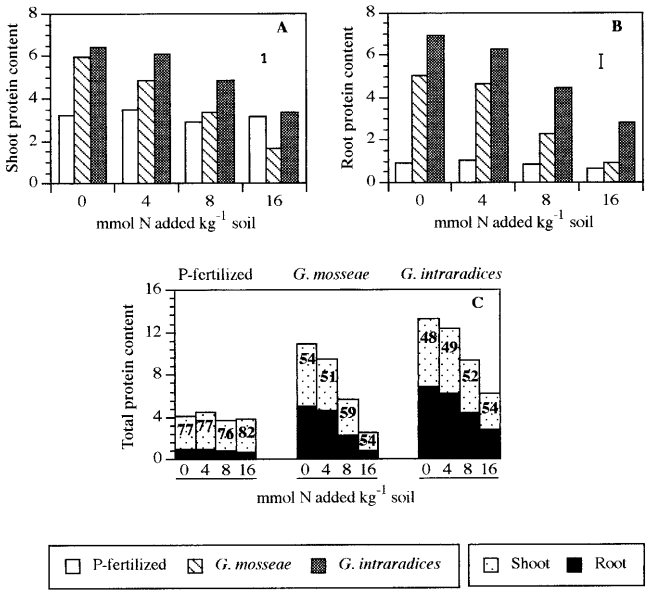

Fig. 4A–C Protein content in plants. **A** Shoot protein content (µg plant^{-1}); **B** root protein content (µg plant^{-1}); **C** total protein content per plant and relative proportion shoot/root. *Numbers* indicate the relative proportion of shoot protein content; *bars* represent PLSD ($P \leq 0.05$)

proportional NR activity in roots, irrespective of the N level, and at 0 and 4 mmol N added kg^{-1} soil most of their NR activity was located in the roots.

The greatest mycorrhizal effect on total (shoot plus root) NR activity was found under 16 mmol N added kg^{-1} soil for *G. intraradices*-colonized plants. To maximize NR shoot and root activities, the two AM fungi required different N amounts (Fig. 3C): 8 mmol N added kg^{-1} soil in the case of *G. mosseae*-colonized plants and 16 mmol N added kg^{-1} soil for *G. intraradices*-colonized plants.

Protein content was higher in mycorrhizal plants, particularly those inoculated with *G. intraradices* (Fig. 4). Protein content was not affected by N supply in P-fertilized non-mycorrhizal plants but decreased in mycorrhizal ones as the N level increased.

Discussion

Recent studies have demonstrated a significant N enrichment of mycorrhizal non-leguminous plants, such as tomato (Mäder et al. 2000), cucumber (Johansen et al. 1993, 1994) and lettuce (Tobar et al. 1994a, 1994b), compared to non-mycorrhizal plants. These studies confirm the capacity of extraradical AM hyphae (besides a better exploitation of soil nutrients) to acquire N.

It was estimated that in mycorrhizal roots, the flux of ^{15}N was nearly 3 times higher than in non-mycorrhizal roots (Mäder et al. 2000). Obviously this fact might account for the performance of rhizobia on mycorrhizal leguminous plants, particularly when the medium was supplied with N. In fact, results from this study showed that the N level applied was a determinant of this. Thus, the effect of the microbial (AM fungi-*Rhizobium*) interaction on the N acquisition of the legume was negatively affected by the increasing amounts of N fertilizer applied.

Nodulation and AM colonization are interactive processes in roots of legumes related to the N nutrition of the host plant. Most available information on this interaction concerns positive responses between both types of endophytes which result in the enhanced formation and function of symbiotic structures (Barea et al. 1997). In this study, high N concentration in soil did not affect the AM colonization rate, agreeing with some previous findings (Patterson et al. 1991; Johansen et al. 1994; Mäder et al. 2000). However, this effect cannot be generalized since different fungus/host plant/soil combinations may produce different responses to N fertilization (Azcón et al. 1982), probably related with different P uptake according to the partners involved. On the other hand, high doses of N application greatly reduced nodule formation, particularly in AM-colonized plants where nodulation became zero at 8 and 16 mmol N added kg^{-1} soil, independent of the AM endophyte involved. These results support a positive mutual effect on symbiotic developments (nodules and AM infection) but only when no N was added. Such an effect was not correlated with either differences in the colonization rate that did not change with increasing N supply, or a higher N content per plant, but NO_3^- reduction and protein synthesis and P content under a high N nutrition could be involved in such an effect, as is discussed below.

N acquisition by *G. mosseae*-colonized plants decreased in response to a N increase in the soil. This effect has been previously described by Johansen et al. (1994) and Mäder et al. (2000) These authors hypothesized that AM hyphal N transport might be regulated according to the N fertilizer content of the host plant: they found that a high N status in mycorrhizal plants led to a decrease in the ^{15}N recovery to about half of the recovery in plants grown at low N levels. But in this study, in the case of *G. intraradices* colonization, N acquisition by plants was not reduced at the highest N levels applied. This means that the hyphal N transport was regulated not only according to the N content in the host plant, but also the colonizing AM fungus, indicating specific physiological behaviour of the different AM fungi. In fact, values of NR activity also differed according to the colonizing endophyte, as there were not only quantitative but also qualitative differences between them in the pattern of shoot/root distribution.

In addition to the effect of mycorrhizal activity on plant N acquisition, some studies have found an increased NR activity both in the shoot (Azcón et al. 1992; Cliquet and Stewart 1993; Azcón et al. 1996; Ruíz-Lozano and Azcón 1996; Azcón and Tobar 1998) and in the root (Sarjala 1991; Cliquet and Stewart 1993; Azcón and Tobar 1998) of mycorrhizal plants. This can be seen as an index of NO_3^- assimilation which provides evidence on the increase in the N metabolism in AM plants.

Although in our experiment the N fertilizer was added to the growth medium as NH_4NO_3, the alkaline soil pH favoured nitrification (Johansen et al. 1993). This was shown in a previous assay carried out under the same experimental conditions as in the present study. Thus, it can be assumed that the observed effects were due to the NO_3^- rather than the NH_4^+ ions.

In many plants NO_3^- reduction takes place mainly in the leaves. The contribution of the root to the reduction of NO_3^- seems to be specially intense during the early stages of plant development (Hoff et al. 1992; Azcón and Tobar 1998). In the present study, NR activities were measured after 75 days of plant growth. The proportion of NO_3^- reduction carried out in roots and shoots (and their relative distribution, as percentages) depends on factors such as the level of NO_3^- supply and the C economy of plants. The C economy of AM plants seems to be different according to the AM fungal species associated with them. In fact, in *G. mosseae*-colonized roots, the large carbohydrate requirement for NO_3^- reduction in the roots seems to be certainly one of the factors limiting the capacity for NO_3^- reduction and nodule formation. However, in the case of *G. intraradices*-colonized roots it seems that the carbohydrate supply to the roots did not affect NO_3^- reduction in this tissue, although it affected nodule formation.

A predominantly root-based reduction of NO_3^- occurred in mycorrhizal plants in the absence of N application. This relative proportion decreased as N increased in the soil. Sarjala (1991) described this effect as a consequence of the saturation of NR activity in roots at higher NO_3^- concentrations. Therefore, this ion is translocated from root to shoot, increasing the NR activity in the shoot. The capacity of saturation for the enzyme NR varied depending on the AM fungi involved, since *G. intraradices*-colonized plants showed a predominantly shoot-based reduction of NO_3^- at >8 mmol N added kg^{-1} soil, whereas *G. mosseae*-colonized plants did at >4 mmol N added kg^{-1} soil. Moreover, the effect of AM fungi on this enzymatic activity in roots in comparison to that in the shoots could be a consequence of fungal NR activity. In fact, Kaldorf et al. (1994, 1998) described assimilatory NR activity in mycorrhizal fungi. The differences observed in mycorrhizal treatments could be the result of different patterns of expression of NR activity in the two fungi assayed.

Different N-uptake processes and assimilation sites (root and/or shoot) are known to affect physiological plant responses (Van Beusichem et al. 1988), since N affects osmotic regulation in plant, cell-wall elasticity, carbohydrate metabolism and synthesis of drought-induced substances in roots (Ögren 1985). Here, as in previous experiments (Azcón et al. 1992, 1996) it is necessary to underline the importance of the selection of appropriate AM fungal species for a particular system. Host root carbohydrates are required by the microsymbiont as sources of nutrition and energy. Kucey and Paul (1982) estimated that 10% of root carbohydrates were consumed by the metabolic activity of AM fungi in colonized roots. However, physiological differences (C requirements) of the fungus involved may influence this general C estimate. Differences in the balance between their photosynthetic and nutritive interchanging mechanisms are the basis for specific plant responses to a specific endophyte.

Factors regulating the uptake and assimilation of NO_3^- are still poorly understood because most of the evidence concerning the effect of AM symbiosis on N nutrition is indirect since the PO_4^{3-} requirements of NR (Hageman and Reed 1980) suggest a P-mediated effect. But in this study, as in previous ones (Azcón et al. 1996), the mechanisms by which AM fungi increased NR activity are independent of P plant nutrition, because non-mycorrhizal but P-fertilized plants showed the highest P content. A possible explanation for decreased P uptake in mycorrhizal plants under high N nutrition might link the process with rhizospheric pH changes caused by the amount of NO_3^- being reduced in the shoot and the root (Leidi and Rodríguez-Navarro 2000). The excess of OH^- generated by this reduction is released as HCO_3^- by roots and thereby increases the rhizosphere pH. In general, the increase in rhizosphere pH would decrease P uptake, which has an optimum pH of between 5 and 6, and at the same time might favour the formation of relatively insoluble P compounds (Leidi and Rodríguez-Navarro 2000). The lower concentration of P in shoots might explain the decreased growth of mycorrhizal plants in response to the increase in the NO_3^- supply.

The relative distribution of protein between the shoot and root demonstrated that mycorrhizal plants possessed a higher root/shoot protein content than non-mycorrhizal ones. This effect has been attributed to quantitative and qualitative changes in the protein composition of mycorrhizal roots (Dumas-Gaudot et al. 1990; Berta et al. 1995; Wright and Upadhyaya 1996; Benabdellah et al. 1998).

So, the reduction in nodule formation when >4 mmol N kg^{-1} soil was added to soil could be due to the competition between symbionts for the limited supply of photosynthates, especially considering that a large proportion of C is required by the plant for amino acids and protein synthesis under high N nutrition (Harper and Gibson 1984). NO_3^- reduction and CO_2 reduction compete for electron donors and ATP from photosynthesis (Smith 1980). In fact, the large carbohydrate requirement for supporting increased NR assimilation and protein synthesis in AM-colonized plants, as well as extended AM colonization, certainly is a limiting factor for nodule formation in mycorrhizal roots. Nevertheless, greater shoot biomass and thus, a larger photosynthetic rate in AM plants, may partially compensate for these extra C requirements in mycorrhizal N-fed plants. The inhibition of nodulation by a high N supply could be based on the fact that the energetic requirements for nodulation seem not to be satisfied under high N levels. This indicates a greater sensitivity of the nodulation process to a lack of C compounds as was previously evidenced

(Ruíz-Lozano and Azcón 1993), although a P-mediated effect as previously discussed could also be involved since nodule formation and N_2 fixation, which have a greater P requirement than NO_3^- assimilation, would be constrained by limited P availability and further by the accumulation of soluble N compounds around the roots.

The results reported here support the belief that AM fungi play an important role in the N nutrition of plants growing at a wide range of N levels in the soil, since the NR activity responsible for NO_3^- assimilation was greatly increased by AM colonization over a range of N levels. Relative NR distribution (root/shoot), as affected by the AM treatments, is known to change physiological plant responses that may be related to the specific sensitivity of AM legume for nodule formation at high N levels. Nevertheless, colonizing mycorrhizae supplied the plant's N requirement, even in the absence of nodules, at all N levels tested, in a more effective way than nodulated non-mycorrhizal plants. The contribution of external AM hyphae to the supply of NO_3^- to plants has been demonstrated (Tobar et al. 1994a, 1994b; Johansen et al. 1993, 1994; Mäder et al. 2000). Thus, mycorrhizal symbiosis may be an effective strategy for N uptake in the form of NO_3^- which is common in Mediterranean soils.

These results demonstrate differences among two AM fungal species for growth and/or function under increasing N concentrations. The selection of AM fungi to address specific environmental conditions, such as N fertilization regimes similar to those used in agronomic practice, seems to be required for a better utilization of N applied to soil. Studies are in progress on this topic to give additional information which is required for a better understanding of this process.

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