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Influence of arbuscular mycorrhizae and a genetically modified strain of *Sinorhizobium* on growth, nitrate reductase activity and protein content in shoots and roots of *Medicago sativa* as affected by nitrogen concentrations

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Abstract

This study examined the effect of nitrogen fertilization (0, 4 or 8 mmol N added kg^{-1} soil) on plant growth, nitrate reductase activity (NR) (EC 1.6.6.1) and protein content in *Medicago sativa*. Plants were inoculated with two strains of *Sinorhizobium meliloti* [the wild type (WT) strain GR4 and its genetically modified (GM) derivative GR4(pCK3)]. In combination with these inoculations, the arbuscular mycorrhizal (AM) fungus, *Glomus mosseae*, was compared to a non-mycorrhizal control supplemented with phosphate. The effects of AM on plant growth were greatest when no nitrogen was added to the soil. Nitrogen fertilization reduced these effects according to the *S. meliloti* strain involved. Growth responses of mycorrhizal plants coinoculated with the GM strain were affected less negatively than those of mycorrhizal plants associated with the WT strain. These results were not related to differential colonization by AM. Increasing nitrogen concentrations reduced mycorrhizal infection and nodule formation was drastically inhibited in mycorrhizal plants by the addition of 4 and 8 mmol N kg^{-1} soil. Nevertheless, AM symbiosis increased nodule formation in the absence of N fertilization. In non-mycorrhizal plants, however, N fertilizer application did not significantly affect shoot and root growth or nodule formation. Although the P content was higher in P-fertilized, non-mycorrhizal plants than in mycorrhizal ones AM colonization significantly improved P use efficiency. The N content and use efficiency were also highest in mycorrhizal plants. However, the most relevant result regarding NR activity was the varying distributions in mycorrhizal plant shoots and roots as affected by the particular *Sinorhizobium* strain. The root portion was enhanced by the GM strain, while the WT strain increased the shoot portion. This change in the distribution pattern of root and shoot NR activities was unaffected by N concentrations in the soil. Protein content was substantially higher in mycorrhizal plants. The protein distribution pattern in shoot and root was also highly influenced by mycorrhizal colonization, which enhanced the root portion. Increased nitrogen supply lowered the protein content in both shoot and root tissues. This effect was greatest in AM colonized plants. These results suggest that high N fertilization levels are detrimental to mycorrhizal legume plants. However, under stress conditions (e.g. high N levels) mycorrhizal legumes nodulated by the GM *Sinorhizobium* strain displayed a physiological response which was better than those nodulated by the WT strain.

Keywords: Arbuscular mycorrhizae; Genetically modified microorganisms; *Medicago sativa*; Nitrate reductase activity; *Sinorhizobium meliloti*

1. Introduction

Beneficial microbial inoculants (biofertilizers, phytostimulators and biological control agents) are presently attracting more attention in the context of sustainable agriculture. This is a consequence of the need to solve health and environmental problems caused by the excessive use of agrochemical compounds in traditional agriculture. *Rhizobium* is a key factor in the establishment and development of legumes, because biological N_2 fixation improves their growth and nutrition. Recent research has led to the identification and characterization of genotypes with either superior nodulation competitiveness (Sanjuán and Olivares, 1991) or higher N_2 fixation capacity (Cannon et al., 1988). From a more practical point of view, special attention should be given for assessing the efficacy in these aspects of genetically modified (GM) organisms compared with their wild types (WT) within a range of environmental conditions that affect the establishment and functioning of the *Rhizobium*–legume symbiosis. Under these conditions, interactions with beneficial and ubiquitous soil microbial groups [e.g. arbuscular mycorrhizal (AM) fungi] are also particularly relevant. The presence of combined N in soil is one important environmental factor that interferes with both nodulation and AM colonization.

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Biological N_2 fixation is presumed to be the most significant source of N for legumes. However, N fertilization substantially increases legume growth, which may indicate that symbiotic nitrogen fixation does not provide enough nitrogen for maximum yield (Buttery et al., 1987). N fertilization is a common agricultural procedure where soil N content is suboptimal.

Nitrogen availability is important for the rapid establishment of legumes, and low N applications improve growth and stimulate N_2 fixation until the latter fixation can provide adequate N for plant development (Müller et al., 1993). N fertilizer rates exceeding those that stimulate the symbiotic process do however have a detrimental effect on nodulation and N_2 fixation (Müller et al., 1993). The threshold for the depressing effects of mineral N on the biological N_2-fixing process is determined by environmental factors, such as rhizobial strains (Awonaike et al., 1980; Senaratne et al., 1987). *Rhizobium*–mycorrhizal interaction is also related to which strains are involved (Azcón et al., 1992).

In most soils, most of the inorganic N pool consists of nitrate and is the main nitrogen source for many higher plants. This is due to either the application of nitrate as a fertilizer or to the rapid nitrification of ammonium mineralized from organic material (Schmidt, 1982). Because nitrate reductase (NR) (EC 1.6.6.1) is the first enzyme in the nitrate assimilation pathway, and is probably the rate-limiting step in this process (Campbell, 1988), its activity regulates N nutrition of plants (Vogel and Dawson, 1991). Mycorrhizal colonization affects the development and function of N_2-fixing systems, and mycorrhizal symbiosis also affects N uptake and metabolism in colonized plants (Johansen et al., 1994; Azcón and Tobar, 1998; Hawkins and George, 1999). The effect of AM symbiosis on N nutrition and metabolism may determine the response of nodulated legumes to N fertilization, both directly and indirectly. However, soil N concentrations could be the main determinant of mycorrhizal effects, since nodulation and AM colonization are interactive processes in legume roots.

On the basis of previous research, the *Sinorhizobium* GM strain compared to the WT strain increased AM colonization and nutrient uptake in plants colonized with *G. mosseae* (Tobar et al., 1996). The present study examines the symbiotic interactions between *Medicago sativa*/*G. mosseae*/*Sinorhizobium meliloti* [wild type GR4 strain and its genetically modified derivative, GR4(pCK3) strain] under greenhouse conditions at different concentrations of mineral N. Our objective was to determine the effect of increasing nitrogen fertilization on growth, NR activity and protein content in single or dual inoculated *Medicago* plants.

2. Materials and methods

2.1. Experimental design

The experiment was designed as a complete randomized block with three factors. The first factor, AM fungi treatments, consisted of two levels, a *G. mosseae* inoculated treatment and a 100 $\mu g\ g^{-1}$ phosphorus-supplemented non-mycorrhizal treatment. The second factor, N addition to soil, consisted of three levels (0, 4 or 8 mmol N kg^{-1} soil). Finally, the third factor, *Sinorhizobium* inoculation, consisted of two levels: the inoculation of either the WT *S. meliloti* strain GR4 or the GM *S. meliloti* strain GR4(pCK3). The GM strain contained the plasmid pCK3, which carries the *Klebsiella pneumoniae nif* A gene, constitutively expressed from a kanamycin gene promoter (Sanjuán and Olivares, 1991). These 12 treatments were replicated five times, giving a total of 60 pots.

2.2. Host plant and soil inoculation

Alfalfa (*Medicago sativa* L., cv. Aragon) seeds were sterilized in a 10% commercial bleach (3.5% sodium hypochlorite as active agent) solution for 30 min, then washed five times with sterile water to remove any trace of chemical that could interfere with seed germination. Surface sterilized seeds were sown in 500 ml pots. Mycorrhizal inoculum consisted of spores, soil, hyphae and AM root fragments from a stock culture of the AM fungus *G. mosseae* (BEG 12) grown with *Lactuca sativa* L. Ten grams of inoculum with an average 75% root infection were placed directly under the seed in each pot. The inoculum of both strains of *Sinorhizobium* (WT and GM) were grown in TY medium (tryptone 0.5%, $CaCl_2$ 0.05%, yeast extract 0.3%) and applied (1 ml containing 10^8 cells per pot) at sowing time in both mycorrhizal and non-mycorrhizal treatments.

2.3. Growth conditions

Plants were grown for 75 days under growth chamber conditions (27 and 18 °C, day/night, relative humidity of 50%, 14 h photoperiod). Photosynthetic photon flux density (PPFD) was 500 $\mu mol\ m^{-2}\ s^{-1}$. Water was supplied by weighing pots on a daily basis in order to maintain the required water capacity of the test soil/sand mixture (nearly 100% of the water holding capacity) throughout the experiment.

2.4. Soil characteristics and phosphorus and nitrogen fertilization

Soil was collected in the province of Granada (Spain) and 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 et al., 1954). The soil was sieved (2 mm), diluted with quartz sand (5/2, v/v) and pasteurized (100 °C, 1 h per day for 3 consecutive days).

Pots were filled with 500 g of sterilized soil/sand mixture. Phosphorus was weekly applied as KH_2PO_4 to non-mycorrhizal plants over the first growth period (5 weeks).

Table 1
Shoot and root dry weight of *M. sativa* in response to AMF colonization (NM, non-mycorrhizal but supplemented with 100 μg P g^{-1} soil; M, mycorrhizal), *Sinorhizobium* inoculation (WT, wild type; GM, genetically modified) and addition of N to soil (0, 4 and 8 mmol N added kg^{-1} soil). LSD, least significance difference ($P \leq 0.05$)

N added (mmol kg^{-1} soil)	Shoot dry weight (mg)				Root dry weight (mg)			
	NM		M		NM		M	
	WT	GM	WT	GM	WT	GM	WT	GM
0	162.5	178.0	400.0	382.0	137.5	130.0	546.0	690.0
4	194.0	160.0	336.0	342.0	128.0	110.0	482.0	460.0
8	180.0	162.0	218.0	280.0	126.0	116.0	194.0	284.0
$LSD_{0.05}$	89.53				120.26			

This was done to provide a final concentration of 100 μg P added g^{-1} soil.

Nitrogen was applied by mixing an appropriate NH_4NO_3 solution with soil before sowing. This was done to provide 0, 4 or 8 mmol N added kg^{-1} soil.

From the third to the seventh week each plant received 10 ml per week of a nutrient solution (Hewitt, 1952) in which N and P were eliminated.

2.5. Measurements

Plants were harvested after 75 days of growth. Shoot and root weights were recorded after drying at 70 °C to constant weight. N and P concentrations were measured after Kjeldahl digestion or molybdenum blue procedures, respectively (Lachica et al., 1973).

Shoot N and P use efficiency, defined as the amount of biomass produced per unit of N or P in plant tissues, were determined as the ratio of shoot dry weight (milligram) produced per milligram of total shoot N or P content.

AM colonization percentages were assessed microscopically using the gridline intersect technique (Giovanetti and Mosse, 1980). Root samples were stained following Phillips and Hayman (1970) and using trypan blue in lactic acid. Total AM colonization was calculated in terms of AM colonization percentage and root dry weight.

In vitro assay of root and shoot NR activity (EC 1.6.6.1) was carried out as described by Kaiser and Lewis (1984) and Becana et al. (1985) and modified by Caba et al. (1990). Determinations were carried out using fresh leaves or root tissues harvested 6 h after the commencement of the light period. Detached plant material [root or shoot (1 g)] was frozen in liquid N_2 and ground with mortar and pestle. The powder was extracted for 5 min with 2 ml of the following buffer: 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 (FAD), 2 mM dithiothreitol (DTT), 1.5 mM phenylmethyl-sulfonylfluoride (PMSF) and 10 mM cysteine, with 3% (w/v) insoluble polyvinylpolypyrrolidone in a mortar and pestle (5 min). Homogenates were filtered through miracloth and centrifuged (3000 g) for 5 min at 4 °C. The supernatant was collected and centrifuged (30,000 g) for 20 min at 4 °C. Finally, the supernatant was decanted and kept on ice.

Protein was assayed according to Bradford (1976), using bovine serum albumine (BSA, fraction V) to standardize the assay.

Results were analyzed by three-way analysis of variance (ANOVA 3). Significance was determined according to Fisher's protected least significance difference (PLSD). Five replicates were used for calculation. $P \leq 0.05$ represented statistically significant difference. Percentage values were analyzed following arcsin square-root transformation.

3. Results

Neither *Sinorhizobium* strains (WT vs. GM) nor nitrogen applications influenced the growth of non-mycorrhizal alfalfa plants (Table 1). Alfalfa shoot and root weights were increased by mycorrhizal colonization, particularly under the lowest nitrogen concentrations. Addition of N negatively affected mycorrhizal plants since shoot and root biomass decreased with increasing N concentrations. This was more evident in WT inoculated plants where increases in plant growth produced by mycorrhizal colonization (versus P-fertilized non-mycorrhizal plants) disappeared at the highest N treatment (8 mmol N added kg^{-1} soil) (Table 1).

P content in P-fertilized non-mycorrhizal plants was higher at the highest N concentrations than in mycorrhizal plants. No significant differences in P content were observed between plants inoculated with the WT or GM strains (Table 2). Mycorrhizal plants displayed the highest N acquisition, and this effect was particularly significant under the lowest N treatments (0 and 4 mmol N added kg^{-1} soil). As a consequence of these effects, the P/N ratio was lower in mycorrhizal plants (Table 2). This indicates mycorrhizal preference for the uptake of N rather than for P, particularly under the highest amount of N. No differences in the P/N ratio between *Sinorhizobium* strains were observed (Table 2).

P and N use efficiencies at all N concentrations and mycorrhizal–*Sinorhizobium* combinations were highest in mycorrhizal plants (Table 3).

Table 2
Nutritional plant response (P and N content in shoots and P/N ratio) to AMF colonization (NM, non-mycorrhizal but supplemented with 100 μg P g^{-1} soil; M, mycorrhizal), *Sinorhizobium* inoculation (WT, wild type; GM, genetically modified) and addition of N to soil (0, 4 and 8 mmol N added kg^{-1} soil). LSD, least significance difference ($P \leq 0.05$)

N added (mmol kg^{-1} soil)	Shoot P content (mg $plant^{-1}$)				Shoot N content (mg $plant^{-1}$)				P/N ratio			
	NM		M		NM		M		NM		M	
	WT	GM	WT	GM	WT	GM	WT	GM	WT	GM	WT	GM
0	0.47	0.50	0.43	0.36	4.86	5.00	8.84	9.00	0.10	0.10	0.05	0.04
4	0.59	0.47	0.28	0.28	6.52	4.94	9.21	9.60	0.09	0.09	0.03	0.03
8	0.62	0.46	0.14	0.19	5.94	5.88	6.48	7.74	0.10	0.08	0.02	0.03
$LSD_{0.05}$	0.20				2.48				0.008			

Neither rhizobial treatments nor nitrogen applications significantly affected the percentage of mycorrhizal colonization (Table 4). However, the GM *Sinorhizobium* strain had a stimulating effect on total AM colonization, although only at the lowest N treatment (Table 4). The highest N concentration severely reduced total AM colonization. Similarly, increasing soil N concentrations drastically inhibited nodulation in mycorrhizal plants. Mycorrhizal plants did however favor nodule formation for both *Sinorhizobium* strains when no nitrogen was added (Table 4).

Plants inoculated with *Sinorhizobium* WT strain required a supply of 8 mmol N added kg^{-1} soil in order to show maximal shoot and root NR activity (Fig. 1). At this N concentration, mycorrhizal shoot NR activity increased by 263% in comparison with P-fertilized plants. In plants inoculated with the GM *Sinorhizobium* strain, 4 mmol N added kg^{-1} soil was sufficient for maximal shoot and root NR activity. Amounts of over than 4 mmol N added kg^{-1} soil negatively affected this value, particularly in roots (Fig. 1). In mycorrhizal treatments, NR activity in shoots was higher in WT inoculated plants, whereas in roots it was higher in GM inoculated plants (Fig. 1). In contrast, the opposite pattern was displayed by P-fertilized non-mycorrhizal plants. This specific effect of the *Sinorhizobium* strains influenced the relative shoot and root NR distribution in both mycorrhizal and non-mycorrhizal plants (Fig. 1). In fact, in non-mycorrhizal plants inoculated with the GM strain, only a slight proportion of NR activity was located in the root (<1%), whereas this proportion was much higher (15–20%) with the WT strain. On the other hand, in mycorrhizal plants inoculated with the GM strain, the root NR proportion increased substantially. In this treatment root NR activity reached values of 74% (0 mmol N added kg^{-1} soil), 44% (4 mmol N added kg^{-1} soil) and 15% (8 mmol N added kg^{-1} soil). However, the WT strain significantly reduced this proportion in mycorrhizal roots [34% (0 mmol N added kg^{-1} soil), 6% (4 mmol N added kg^{-1} soil) and 7% (8 mmol N added kg^{-1} soil)] (Fig. 1). Specific NR activities in shoot and root of *Sinorhizobium* (WT or GM) inoculated plants showed a similar trend (Table 5).

The protein content (Fig. 2) was increased by mycorrhizal colonization, particularly in root tissue, where a five-fold increase was observed (0 and 4 mmol N added kg^{-1} soil) compared with P-supplemented non-mycorrhizal plants (Fig. 2). An increase in the nitrogen supply significantly lowered the protein content in mycorrhizal shoot and root tissues. Shoot/root protein distribution was also influenced by mycorrhizal colonization, which favored the root proportion, particularly at the lowest N concentrations (Fig. 2). Neither of the *Sinorhizobium* strains had any major effect on this pattern.

4. Discussion

Although the N fertilizer was added to the soil as

Table 3
Effect of AMF colonization (NM, non-mycorrhizal but supplemented with 100 μg P g^{-1} soil; M, mycorrhizal), *Sinorhizobium* inoculation (WT, wild type; GM, genetically modified) and addition of N to soil (0, 4 and 8 mmol N added kg^{-1} soil) on P and N use efficiency. LSD, least significance difference ($P \leq 0.05$)

N added (mmol kg^{-1} soil)	P use efficiency				N use efficiency			
	NM		M		NM		M	
	WT	GM	WT	GM	WT	GM	WT	GM
0	345	370	953	1079	33.1	37.5	45.8	42.8
4	321	344	1238	1247	29.4	32.4	36.4	35.6
8	296	372	1668	1452	29.9	28.9	34.1	36.1
$LSD_{0.05}$	146				5.1			

Table 4
Effect of AMF colonization (NM, non-mycorrhizal but supplemented with 100 μg P g^{-1} soil; M, mycorrhizal), *Sinorhizobium* inoculation (WT, wild type; GM, genetically modified) and addition of N to soil (0, 4 and 8 mmol N added kg^{-1} soil) on percent and total AM colonization and nodule number. LSD, least significance difference ($P \leq 0.05$)

N added (mmol kg^{-1} soil)	% AM (Asin square-root transformed)		Total AM		Nodule number			
	M		M		NM		M	
	WT	GM	WT	GM	WT	GM	WT	GM
0	18.7	23.3	178.3	271.5	4	8	20	30
4	21.1	27.3	177.9	199.8	6	2	2	0
8	22.6	19.4	77.1	90.2	4	2	0	0
$LSD_{0.05}$	5.3		63.0		9			

NH_4NO_3, the alkaline soil pH favored nitrification (Johansen et al., 1993). This result was also found in a previous assay carried out under the same experimental conditions as those of the present study. It can thus be assumed that the effects observed were the consequence of nitrate rather than ammonium ions.

The tripartite symbiotic association in legumes, with nodule-forming soil bacteria and AM fungi, acts synergistically. This is the case because the two microsymbionts improved phosphorus and nitrogen acquisition, respectively, under limiting nutrient conditions for plant growth. With low N concentrations (0 and 4 mmol N added kg^{-1} soil) both rhizobial strains behaved similarly in that they affected shoot and root growth of mycorrhizal and non-mycorrhizal plants. Mycorrhizal colonization increased plant growth and nodule formation. Nevertheless, the highest N added concentration substantially reduced plant response to AM colonization. At a supraoptimal N concentration (8 mmol N added kg^{-1} soil), however, the *G. mosseae* + GM treatment was the most effective in counteracting the detrimental N effect. Since nodule formation was inhibited at this N concentration, the effect of the GM strain at 8 mmol N added kg^{-1} soil could be ascribed to the bacterium as a free-living microorganism. The beneficial effects of tripartite symbiosis have been attributed to a superior colonizing ability or to increased symbiont activity. However, the plant growth-promoting rhizobacterial (PGPR) effect of the rhizobial strains in legume plants is a new aspect. This PGPR effect was only apparent in mycorrhizal plants. In a recent study (Galleguillos et al., 2000), a similar effect was observed with the dual GM–*G. mosseae* inoculation in a non-legume plant. This interaction led to larger shoot biomass and longer lateral root lengths in lettuce plants.

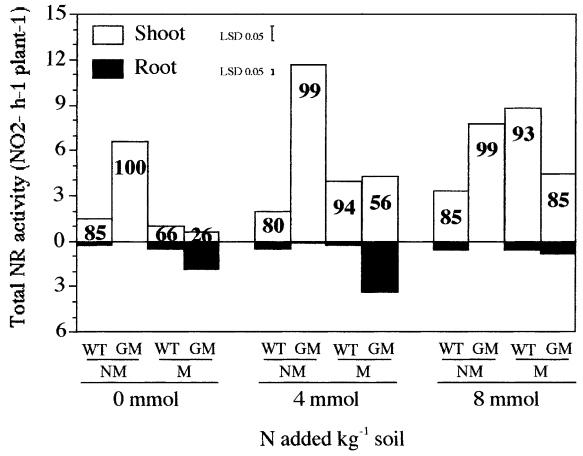

Fig. 1. Effect of AMF colonization (NM, non-mycorrhizal but supplemented with 100 μg P g^{-1} soil; M, mycorrhizal), *Sinorhizobium* inoculation (WT, wild type; GM, genetically modified) and addition of N to soil (0, 4 and 8 mmol N added kg^{-1} soil) on total nitrate reductase activity (nmol NO_2^- h^{-1} $plant^{-1}$) and the relative distribution of NR activity between shoot and root. *Numbers* indicate the relative proportion of shoot NR activity.

The present results confirm previous findings (Tobar et al., 1996) regarding AM fungal infectivity: the positive effect of GM increasing AM colonization at the lowest N level has again been observed. As a consequence of N fertilization, the differential effects of the rhizobial strains (WT vs. GM) on AM colonization disappeared. Nevertheless, plant growth was highest in the supraoptimal N treatment (8 mmol N added kg^{-1} soil) with *G. mosseae* + GM. The behavior of the GM strain in non-nodulated AM plants could be interpreted as an effect on mycorrhizal activity which alleviates stress and makes the legume more tolerant to adverse N effect. In order to understand the potential of the associations under limiting conditions (e.g. high N fertilization) it is necessary that the particular efficiency of each symbiotic partner be determined.

In mycorrhizal plants, nodule formation did not occur when N was added to soil (4 and 8 mmol N added kg^{-1} soil). The fact that fungal hyphae facilitate the uptake of N ions by mycorrhizal roots (Johansen et al., 1993; Bago et al., 1996) may account for the detrimental mycorrhizal effect on nodule formation at high N concentrations. However, this is probably the result of an indirect effect of mycorrhiza on N ion uptake since the highest N content in mycorrhizal plants was observed at 0 and 4 mmol added N. These differences may be correlated with those in plant P content, which decreased as N supply increased. The fact that NO_3^- reduction and protein synthesis was higher in mycorrhizal than in non-mycorrhizal plants could be

Table 5
Effect of AMF colonization (NM, non-mycorrhizal but supplemented with 100 μg P g^{-1} soil; M, mycorrhizal), *Sinorhizobium* inoculation (WT, wild type; GM, genetically modified) and addition of N to soil (0, 4 and 8 mmol N added kg^{-1} soil) on specific nitrate reductase (mmol NO_2^- h^{-1} mg $protein^{-1}$) activity in shoots and roots of *Medicago sativa*. LSD, least significance difference ($P \leq 0.05$)

N added (mmol kg^{-1} soil)	Specific shoot NR activity				Specific root NR activity			
	NM		M		NM		M	
	WT	GM	WT	GM	WT	GM	WT	GM
0	0.47	1.86	0.18	0.12	0.29	0.00	0.11	0.44
4	0.55	3.82	0.80	0.88	0.47	0.21	0.05	1.03
8	1.17	2.58	2.62	1.14	0.69	0.07	0.27	0.43
$LSD_{0.05}$	0.14				0.10			

limiting the capacity for nodule formation due to the large carbohydrate requirement for both NO_3^- reduction and protein synthesis.

The effect of WT or GM single or dual (AM) inoculation was tested on NR activity in shoot and root. Although NR distribution in roots and shoots was influenced by nitrate supply (Wallance and Pate, 1965), this pattern was also affected by the specific microbial interactions. NR activity was lower in the roots of GM inoculated than in WT inoculated non-AM plants. Conversely, in mycorrhizal plants inoculated with the GM strain, NR activity was higher in the roots than in plants inoculated with the WT strain. This trend remained the same in nodulated (0 mmol N added kg^{-1} soil) and non-nodulated plants (4 and 8 mmol N added kg^{-1} soil). Hence, these particular microbial effects on NR activity should be attributed to the biological interaction, but not at the symbiotic stage (in the case of *Sinorhizobium*). Changes in N assimilation sites have a physiological relevance in that they affect metabolism of C and osmoregulation in plants (Ögren, 1985). The distribution of NR in the *G. mosseae*–WT strain interaction could

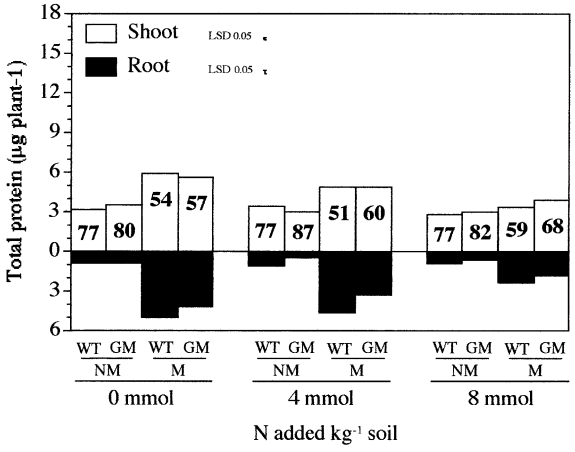

Fig. 2. Effect of AMF colonization (NM, non-mycorrhizal but supplemented with 100 μg P g^{-1} soil; M, mycorrhizal), *Sinorhizobium* inoculation (WT, wild type; GM, genetically modified) and addition of N to soil (0, 4 and 8 mmol N added kg^{-1} soil) on total protein (μg $plant^{-1}$) and the relative distribution of protein between shoot and root. *Numbers* indicate the relative proportion of shoot protein content.

be interpreted as the result of different carbon utilizations and, possibly, as a limitation in the supply of photosynthate to the root (Wallance, 1986). On the other hand, the fungal NR activity itself (Kaldorf et al., 1994, 1998) could have been stimulated in interaction with the GM strain of *Sinorhizobium*, and this may have accounted for the observed increases in root NR activity. These results suggest that the behavior of the endophyte, *G. mosseae*, varied with each of the *Sinorhizobium* strains (WT or GM), and affected some physiological and biochemical plant aspects in a common host. The regulation of microbial–plant interactions involves a combination of mechanisms that differ with the environmental conditions. However, based on the available data, it is difficult to infer which causes account for the effects of the specific endophyte couples.

The effects of *Sinorhizobium*–mycorrhizal interactions were thought to be restricted to the infective and nutritional stages of mutual symbiosis on the plant (Barea et al., 1992). However, these unexpected results demonstrate that the interaction between both microorganisms (AM and *Sinorhizobium*) occurs even in non-nodulating plants. The way in which the saprophytic bacterial state affects the functioning of the symbiotic mycorrhizal system remains an open question. Although the dynamics of mycorrhizal infections were not determined here, unpublished results have shown that neither of the *Sinorhizobium* strains causes a faster spread of AM colonization, which could explain the differential mycorrhizal behavior in the specific microbial interactions.

Based on our experimental data, we suggest that factor(s) produced by *Sinorhizobium* specifically influence the microbial interactions. This is the case regardless of the rhizobial symbiotic status and of whether or not plant responses are mediated by the *Sinorhizobium* strains. The possible plant growth-stimulating effect of the free-living saprophytic stage of *S. meliloti* observed in the highest N treatment (8 mmol N added kg^{-1} soil) is an interesting aspect of growth response which requires further study. This ability, as well as the N-fixing process in nodulated *Medicago* plants, could be of ecological importance for the sustainability of agroecosystems.

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