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The role of plant size and nutrient concentrations in associations between *Medicago*, and *Rhizobium* and/or *Glomus*

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Abstract

The aim of this research was to carry out a critical study of the method of obtaining size equivalence between non-symbiotic alfalfa and alfalfa associated with *Glomus* and/or *Rhizobium* by applying fixed addition rates of nutrients to the non-symbiotic controls. The experimental design included three nutrient response curves in which the levels of added phosphorus and/or nitrogen were constant during the whole plant growth process: *1*) a phosphorus response curve, in order to compare the growth of double symbiotic plants with that of only-*Rhizobium* inoculated ones; *2*) a nitrogen response curve, that consisted of a comparison between the growth of double symbiotic alfalfa and four treatments associated only with *Glomus*; *3*) a phosphorus and nitrogen response curve, to compare the growth of non-inoculated alfalfa with that of double symbiotic plants. Although similar size was achieved among some treatments at harvest, shoot growth over time and nutrient concentrations in tissues differed, indicating that growth equivalence did not mean functional equivalence. A second experimental design was performed taking into account the establishment of microsymbionts for determining the adequate moment to add supplemental phosphorus and/or nitrogen. It included four treatments: *a*) double symbiotic plants (MR); *b*) plants inoculated with *Rhizobium* only (R); *c*) plants inoculated with *Glomus* only (M), and *d*) non-inoculated plants (N). Great similarity in terms of plant growth and nutrient contents in tissues were obtained. Moreover, symbiotic plants were able to produce similar dry matter than non-symbiotic ones under P and N limitations.

Additional key words: alfalfa, arbuscular mycorrhizal fungi, nitrogen, nitrogen-fixing bacteria, phosphorus, plant growth.

Introduction

In nutritional studies (Pacovsky *et al.* 1986, Eissenstat *et al.* 1993, Peng *et al.* 1993), as well as in research on nonnutritional effects involving symbiotic associations (Brown and Bethlenfalvay 1987, Syvertsen and Graham 1990) it is convenient to obtain non-symbiotic control plants of similar size to symbiotic ones in order to achieve comparable nutrient uptake rates by roots and nutrient concentrations in plant tissues. Several studies (Pacovsky *et al.* 1986, Brown and Bethlenfalvay 1987, Syvertsen and Graham 1990) have been conducted at low P supply for mycorrhizal plants, while additional P was supplied to non-mycorrhizal ones. Likewise, it has been necessary to add supplemental N to non-nodulated plants to achieve the same growth rate as nodulated ones (Brown and Bethlenfalvay 1987). In all these studies, non-mycorrhizal or non-nodulated plants received fixed amounts of additional P or N from planting until harvesting, which can be problematic, given the non-steady-state nature of mycorrhizal carbon consumption and P acquisition during and following the weeks of active root colonization (Eissenstat *et al.* 1993). Equivalence between mycorrhizal and non-mycorrhizal plants is easier to obtain growing both kinds of plants at high-P supply, provided that mycorrhizal colonization

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Abbreviations: MR - alfalfa plants associated with mycorrhizae and *Rhizobium*; M - plants inoculated only with mycorrhizae; R - plants inoculated only with *Rhizobium*; N - non-symbiotic plants.

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still occurs and benefits host development (Rousseau and Reid 1991, Eissenstat *et al.* 1993, Peng *et al.* 1993). However, in studies with legumes, the abundance of low P soils is an important fact to take into account. Nodulation and nitrogen fixation are severely impaired under P deficiency, so that dual infection with *Rhizobium sp.* and mycorrhizal fungi is an attractive approach for adapting legumes to P-deficient soils (Marschner 1991).

Two main purposes were pursued in this study. Firstly, to carry out a critical study of the method of obtaining size equivalence between non-symbiotic plants and plants associated with mycorrhizal fungus and/or nitrogen fixing bacteria by adding higher and fixed amounts of P and/or N to the non-symbiotic controls. Secondly, to design a nutritional model in which growth equivalence is achieved with low P supply in order to obtain an appropriate approach for studies under nutritional limitations.

Materials and methods

Alfalfa (*Medicago sativa* L. cv. Aragón) seeds were surface disinfected and germinated on wet filter paper in Petri dishes. Seedlings were planted in 25 × 10 cm pots (five seedlings per pot, and three pots per treatment) containing a mixture of washed vermiculite:sand (1:1 v/v). Plants were grown in a greenhouse with temperature and relative humidity varying within the maximum day/minimum night ranges of 30/15 °C, and 50/70 %, respectively, at 14-h photoperiod (natural daylight supplemented with fluorescent lamps *Sylvania DECOR 183*, Erlangen, Germany), providing a minimum photosynthetic photon flux density of around 300 - 400 $\mu mol\ m^{-2}\ s^{-1}$.

Inoculations with *Rhizobium meliloti* strain 102 F51 (*Nitragin Co.*, Milwaukee, USA) and *Glomus fasciculatum* (Taxter *sensu* Gerd.) Gerdemann and Trappe were imposed at planting. Mycorrhizal inoculum consisted of 6.0 g soil including root fragments, spores and hyphae.

Nutrients were repeatedly added (twice a week) as nutrient solutions (200 cm^3 in each addition). The basal nutrient solution consisted of 1.5 mM $CaCl_2$, 0.25 mM $MgSO_4$, 1 mM K_2SO_4, with micronutrients equivalent to one quarter strength Hoagland solution (Bethlenfalvay *et al.* 1985). The pH of each solution was adjusted to 6.9 with 0.01 M KOH. Phosphorus (K_2HPO_4) and nitrogen (NH_4NO_3) concentrations in the nutrient solutions varying among different experiments, and the differentiation of nutritional regimes was conducted by applying two methods:

1) This method included a phosphorus (P) response curve, a nitrogen (N) response curve, and a P and N response curve. P and/or N addition rates were constant from planting until harvesting. For P response curve determination the growth of alfalfa inoculated with *G. fasciculatum*, and *R. meliloti* (MR) was compared with that of alfalfa plants inoculated only with *R. meliloti* and supplemented with 0.02, 0.05, 0.10 or 0.20 mM K_2HPO_4, and 0.5mM NH_4NO_3 (Table 1). For N response curve determination five treatments were used, all of them colonized by *G. fasciculatum*, but only one (MR) also inoculated with *R. meliloti*. Plants inoculated only with *G. fasciculatum* received various N concentrations in the nutrient solution (Table 1). Both, P response curve and N response curve were carried out simultaneously. For P and N response curve the growth of double symbiotic MR plants was compared with that of six groups of non-symbiotic plants. P and N supplements were chosen according to the results obtained in the P response curve and N response curve (Table 1).

Table 1. Concentrations [mM] of P (K_2HPO_4) and N (NH_4NO_3) at various treatments.

	P	N		P	N		P	N
MR	0.02	0.5	MR	0.02	0.5	MR	0.02	0.50
R + 0.02P	0.02	0.5	M + 0.5N	0.02	0.5	0.1P/1.5N	0.10	1.50
R + 0.05P	0.05	0.5	M + 1.0N	0.02	1.0	0.1P/1.75N	0.10	1.75
R + 0.1P	0.10	0.5	M + 1.5N	0.02	1.5	0.1P/2.0N	0.10	2.00
R + 0.2P	0.20	0.5	M + 2.0N	0.02	2.0	0.2P/1.5N	0.20	1.50
						0.2P/1.75N	0.20	1.75
						0.2P/2.0N	0.20	2.00

2) The growth rate of MR plants was compared with that of three groups of alfalfa plants: only colonized by AM fungi (M), only associated with *Rhizobium* (R) and non-symbiotic plants (N). During the first month, plants received basal nutrient solution described previously supplemented with 0.02 mM K_2HPO_4 and 1 mM NH_4NO_3, until the endophytes were established. When 30-d-old, the concentration of nutrients (N and P) were varied as follows: 0.18 mM K_2HPO_4 and 2 mM NH_4NO_3 (N plants), 0.35 mM K_2HPO_4 and 0 mM NH_4NO_3

(R plants), 0.02 mM K_2HPO_4 and 3 mM NH_4NO_3 (M plants), and 0.02 mM K_2HPO_4 and 0 mM NH_4NO_3 (MR plants).

All plants were harvested 60 d after planting. Shoot height was measured from the epicotyl to the stem top. Biomass was separated into leaves, stems, roots and, when associated with *Rhizobium*, nodules. Plant organs were dried at 80 °C for 48 h and weighed. Leaf area was measured by using an automatic leaf area meter (*LI-3000 model, Li-COR*, Lincoln, USA). Total N was measured by Kjeldahl method and plant P content was determined according to Allen *et al.* (1976).

Root samples were cleared and stained as described by Phillips and Hayman (1970). Mycorrhizal colonization was determined by examining 80 - 100 1-cm root segments under the microscope. Data are expressed as percentage of infection (Hayman *et al.* 1976).

Means ± SE (n = 7 - 9) were calculated, and their differences tested for significance by using a LSD technique with the Student's t-test.

Results and discussion

Plant growth and endophyte establishment: In the P response curve (Table 2), the growth of MR plants was compared with that of four groups of plants *Rhizobium*-inoculated but not associated with mycorrhizal fungus, which received P in the nutrient solution. Shoot height, as well as leaf, stem and root dry matter and number of plant leaves and stems were similar in MR, R + 0.1P and R + 0.2P treatments, and significantly higher than those measured in R + 0.02P and R + 0.05P plants. The best nodulation was observed in mycorrhizal MR plants and in non-mycorrhizal ones which received the highest supply of P (R + 0.2P). The lack of nodulation observed in R + 0.02P and R + 0.05P treatments could be due to the low phosphorus concentration in the nutrient solution (O'Hara *et al.* 1988).

Table 2. Phosphorus response curve. Shoot height, leaf, root and stem dry matter, number of stems and leaves, total leaf area per plant, number of nodules per plant and mycorrhizal infection of *Medicago sativa* associated with *Rhizobium* and AM fungi (MR), and four alfalfa treatments only *Rhizobium*-inoculated, which received increasing P concentrations in nutrient solution. ND: Not detected. Measurements were made when plants were 60-d-old. Within each column, means (n = 8 - 15) followed by the same letter are not significantly different (P>0.05).

Treatments	Shoot height [cm]	Leaf d.m. [g plant^{-1}]	Root d.m. [g plant^{-1}]	Stem d.m. [g plant^{-1}]	Stems [plant^{-1}]	Leaves [plant^{-1}]	Leaf area [cm^2 plant^{-1}]	Nodules [plant^{-1}]	Mycorrhizal infection [%]
MR	30.46 a	0.085 a	0.253 a	0.113 a	2.000 ac	21.33 a	35.22 a	13.24 a	48.89 a
R + 0.02P	20.07 b	0.051 b	0.129 b	0.055 b	1.366 b	12.13 b	14.60 b	ND	ND
R + 0.05P	19.85 b	0.059 b	0.137 b	0.059 b	1.560 ab	15.07 b	17.74 b	ND	ND
R + 0.1P	27.73 a	0.089 a	0.196 c	0.116 a	2.066 c	20.40 a	32.69 ac	1.13 b	ND
R + 0.2P	28.12 a	0.085 a	0.199 c	0.111 a	2.466 c	22.53 a	30.54 c	10.00 a	ND

Shoot of double symbiotic (MR) plants grew much faster than non-mycorrhizal ones between days 43 and 58, but shoot height of MR plants was significantly lower than that of only *Rhizobium* inoculated ones from day 15 until day 40 after planting (Fig. 1*A*). These results may indicate that fungal establishment took place during the first 40 d after planting and its functionality was not complete until day 40. An initial growth depression of mycorrhizal plants followed by growth enhancement has been reported several times (Bethlenfalvay *et al.* 1982, Koide 1985) and such negative effects on plant growth may be a common occurrence during initial stages of colonization (Koide and Elliot 1989). The change from negative to positive effect on host plant growth has been found to occur at the midpoint in the ontogeny of this symbiotic association (Bethlenfalvay *et al.* 1982). Moreover, the carbon economy also depends on plant ontogeny and nutritional state (Eissenstat *et al.* 1993). In addition, plants are generally more sensitive to the external nutrient concentration during the first two-three weeks after germination (Wild *et al.* 1987), including external P concentration (Breeze *et al.* 1984).

In the N response curve (Table 3), MR plant growth was compared with that of four groups of N-fed plants, non-*Rhizobium* inoculated and colonized by *Glomus fasciculatum*. Growth results of M + 0.5N and M + 1N treatments will not be discussed because nodulation could not be avoided. Similarly, Ligero *et al.* (1986) found nodules in alfalfa plants fed with nitrate concentrations lower or equal than 2.0 mM. In our case, the *Rhizobium* could be introduced with the mycorrhizal inoculum despite the previous tetracycline 0.01 % treatment applied to the mycorrhizal inoculum. Shoot height, leaf and stem dry matter and total leaf area of MR plants was comparable to that of M + 2N ones, and significantly higher than in M + 1.5N.

There was no difference among shoot growth of MR plants and N-fed M + 1.5N and M + 2N treatments

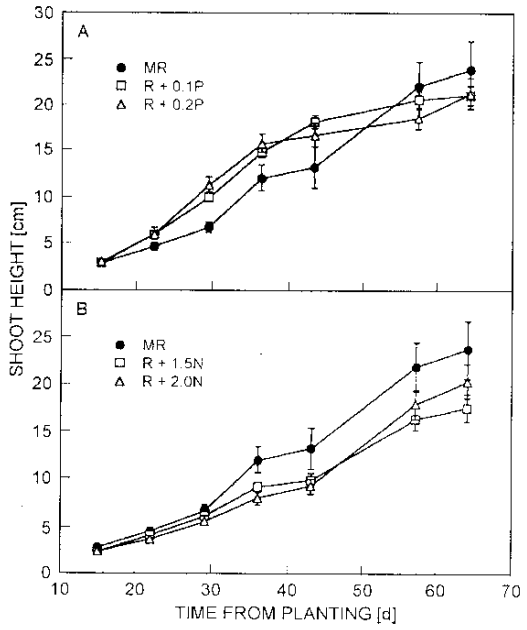

Fig. 1. Shoot height over time of double symbiotic MR (*closed circles*) alfalfa plants and the *Rhizobium*-inoculated R + 0.1P (*open squares*) and R + 0.2P (*open triangles*) treatments (*A*) or the *Glomus*-inoculated M + 1.5N (*open squares*) and M + 2.0N (*open triangles*) treatments (*B*).

between days 15 and 30, because all of them were associated with *Glomus* (Fig. 1*B*). However, MR plants showed higher shoot growth than M + 1.5N and M + 2N from day 30, which could be due to an optimal nodule functionality. During the first 30 d after transplanting, N addition had not an effect on plant growth as evident as P did.

In the P and N response curve (Table 4), we found that size of MR plants was only comparable to that of non-symbiotic 0.1P/1.5N ones.

Some visual parameters can provide information on plant growth process. In fact, the relative growth rate is a linear function of the internal concentration of some nutrients such as N (Agren 1985). We found high correlations between number of stems and stem mass ($r = 0.804$, $P < 0.001$), number of leaves and total leaf mass ($r = 0.942$, $P < 0.001$) and shoot height and stem mass ($r = 0.807$, $P < 0.001$) at harvest. There was also a good relationship between number of leaves and total leaf area ($r = 0.961$, $P < 0.001$).

The second method applied to achieve comparable plant size and nutrient concentrations in tissues was carried out taking into account the visual plant growth parameters described above as well as endophyte establishment according to results obtained in the P, N, and in the P and N response curves. The four groups of plants studied (MR, R, M and N) had similar shoot and root dry matter and number of leaves per plant (Table 5).

Table 3. Nitrogen response curve. MR and four alfalfa treatments only *Glomus*-inoculated, which received increasing N concentrations in nutrient solution. Otherwise as for Table 2.

Treatments	Shoot height [cm]	Leaf d.m. [g plant^{-1}]	Root d.m. [g plant^{-1}]	Stem d.m. [g plant^{-1}]	Stems [plant^{-1}]	Leaves [plant^{-1}]	Leaf area [cm^2 plant^{-1}]	Nodules [plant^{-1}]	Mycorrhizal infection [%]
MR	30.46 a	0.085 a	0.253 a	0.113 a	2.000 ac	21.33 a	35.22 a	13.24 a	48.89 a
M + 0.5N	24.61 ab	0.080 a	0.146 bc	0.086 b	2.357 a	19.43 ab	32.30 a	11.27 a	6.84 b
M + 1.0N	25.33 ab	0.075 a	0.116 b	0.083 b	2.133 a	17.33 bc	25.70 b	10.58 a	22.23 bc
M + 1.5N	20.85 b	0.052 b	0.097 b	0.053 c	1.646 b	14.38 c	16.44 c	ND	51.06 a
M + 2.0N	25.50 ab	0.090 a	0.195 c	0.103 ab	1.950 ab	17.92 bc	32.86 a	ND	26.31 c

Table 4. Phosphorus and nitrogen response curve. MR and six non symbiotic alfalfa treatments, which received increasing P and N concentrations in nutrient solution. Otherwise as for Table 2.

Treatments	Shoot height [cm]	Leaf d.m. [g plant^{-1}]	Root d.m. [g plant^{-1}]	Stem d.m. [g plant^{-1}]	Stems [plant^{-1}]	Leaves [plant^{-1}]	Leaf area [cm^2 plant^{-1}]	Nodules [plant^{-1}]	Mycorrhizal infection [%]
MR	25.65 a	0.094 a	0.176 a	0.112 a	1.750 a	17.56 a	28.96 a	15.32 a	45.12 a
0.1P/1.5N	25.25 ac	0.095 a	0.259 b	0.121 b	2.333 b	19.90 ab	33.30 a	ND	ND
0.1P/1.75N	34.10 b	0.146 b	0.281 bc	0.188 b	2.133 ab	22.40 b	51.52 b	ND	ND
0.1P/2N	28.38 c	0.151 b	0.312 c	0.187 b	2.400 b	24.60 b	49.54 b	ND	ND
0.2P/1.5N	33.18 b	0.154 b	0.342 c	0.198 b	2.533 b	25.53 bd	55.36 b	ND	ND
0.2P/1.75N	34.91 b	0.214 c	0.426 d	0.257 c	3.133 c	32.18 c	79.05 c	ND	ND
0.2P/2N	32.20 b	0.197 c	0.464 d	0.258 c	2.533 b	28.87 cd	72.37 c	ND	ND

Nutrient concentrations in tissues: Differences in the shoot growth during the development of MR, R + 0.2P and M + 2N treatments may indicate that these plants could be in a different stage of development despite their similar size.

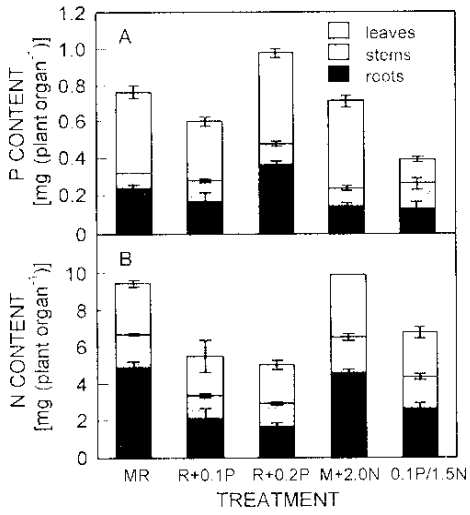

Fig. 2. Phosphorus (*A*) and nitrogen (*B*) contents in leaves, stems and roots of double symbiotic (MR), non-*Glomus*-inoculated (R + 0.1P and R + 0.2P), non-*Rhizobium*-inoculated (M + 2.0N) and non-symbiotic (0.1P/1.5N) alfalfa plants. Measurements were made when plants were 60 d old. Values are means ± SE (n = 8 - 15).

Growth equivalence between mycorrhizal and non-mycorrhizal plants does not necessary mean equivalence in terms of functional parameters such as leaf development, dry matter partitioning and nutrient assimilation (Pacovsky *et al.* 1986). In fact, the nutrient levels differed among some of the comparable plants at harvest. For instance, the N content found in shoots of MR and R + 0.2P plants (Fig. 2*B*) was different in spite of their similar dry matter (Table 2). Likewise, 0.1P/1.5N plants showed lower phosphorus content in leaves than MR ones (Fig. 2*A*), although their leaf dry matter were not significantly different (Table 4).

By contrast, when the nutrient regimes among different treatments were differentiated only after the establishment of the microsymbionts, it was possible to obtain a closer similarity in terms of P and N concentrations, both in leaves and roots (Figs. 3*A*,*B*).

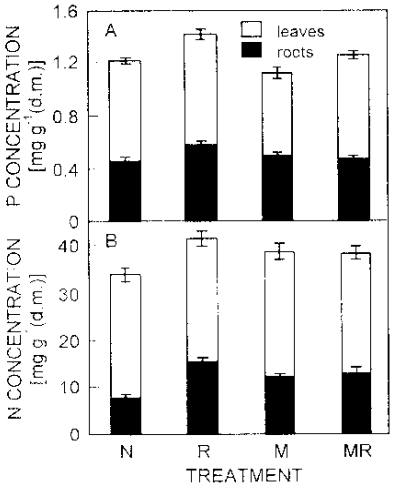

Fig. 3. Phosphorus (A) and nitrogen (B) concentrations in leaves and roots of double symbiotic (MR), *Rhizobium*-inoculated (R), *Glomus*-inoculated (M) and non-symbiotic (N) alfalfa plants. Measurements were made when plants were 60-d-old. Values are means + SE (n = 7 - 9).

In conclusion, in nutritional studies involving mycorrhizal fungi and nitrogen fixing bacteria, it is of interest to carry out P and N response curves to achieve similar growth in both non-symbiotic and symbiotic plants. However, the endophyte establishment must be taken into account for determining the adequate moment to differentiate the nutrient regimes. The capability of symbiotic plants to produce similar dry matter than non-symbiotic ones under P and N limitations demonstrates the increasing importance of these associations in sustainable agriculture.

Table 5. Shoot height (cm) and dry matter (g), root dry matter (g) and number of stems and leaves per plant of double symbiotic (MR), *Rhizobium*-inoculated (R), *Glomus*-inoculated (M) and non-symbiotic (N) alfalfa plants. ND: Not detected. Measurements were made when plants were 60 d old. Values are means (n = 7 - 9). Otherwise as for Table 1.

Treatments	Shoot height [cm]	Shoot d.m. [g plant^{-1}]	Root d.m. [g plant^{-1}]	Stems [plant^{-1}]	Leaves [plant^{-1}]	Nodule d.m. [g plant^{-1}]	Mycorrhizal infection [%]
MR	46.70 a	1.26 a	4.43 a	5.09 a	44.88 a	0.023 a	48.80 a
R	55.81 b	1.54 a	3.89 a	4.94 ab	44.00 a	0.081 b	ND
M	52.42 ab	1.35 a	4.01 a	4.64 ab	40.38 a	ND	32.60 b
N	51.35 ab	1.47 a	4.78 a	4.08 b	33.33 a	ND	ND

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