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Effect of Mycorrhizal Inoculation on Nodule Initiation, Activity and Contribution to Legume Productivity

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Abstract

The effect of a mycorrhitic fungus, *Glomus macrocarpum* Tul. and Tul. on early stages of *Rhizobium*-legume interaction was studied in soil with a low P concentration, under controlled conditions. Establishment of vesicular-arbuscular mycorrhiza in the roots of *Medicago sativa* L. and *Trifolium alexandrinum* L. was associated with growth improvement and increased dry weight (73% and 22%, respectively) of dual-inoculated plants as compared to plants inoculated with *Rhizobium* alone. In *M. sativa* and *T. alexandrinum* mycorrhizal establishment was accompanied by increased N (240% and 20%, respectively) and P (380% and 9%, respectively) content. Generally, fewer and larger nodules were formed on mycorrhizal as compared to non-mycorrhizal plants. Nodule formation showed specific pattern along the root system. Number of nodules formed on aerial part of the root system in mycorrhizal compared to non-mycorrhizal plants did not differ significantly. However, on the subterranean portion of the root system, mycorrhizal plants showed significant reduction in nodule number. In mycorrhizal plants, nitrogen fixation activity (ARA) was significantly higher on a per-plant basis and on a nodule mass basis than in non-mycorrhizal. It

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is suggested that mycorrhizal establishment affects nodule development on the lower parts of the root. Apparently, this effect is an indirect response of the plant.

Keywords: nitrogen fixation, VAM, nodulation, Rhizobium spp.

1. Introduction

There is considerable accumulated information indicating plant growth enhancement by VA mycorrhizas infection under nutrient-deficient environmental conditions (Hayman, 1982; Tinker, 1982). In most plants, growth stimulus is attributed mainly to improved phosphate nutrition in the plant (Mosse et al., 1973; Daft and El Giahmi, 1974; Smith and Daft, 1977; Barea and Azcon-Aguilar, 1983). It is well established that legumes require a high level of phosphate for growth and nodule establishment (Mosse et al., 1976), as well as for maintaining efficient N_2-fixation activity in the nodule (Abbott and Robson, 1977). It was therefore, not unexpected when earlier studies showed that tripartite symbiosis among legume, *Rhizobium* and mycorrhizal fungi results in a considerable increase in plant growth, nitrogenase activity, nodule dry weight, nodule size and phosphorus content (Smith and Daft, 1977; Smith et al., 1979; Asimi et al., 1980). Concomitantly, Smith and Daft (1977) reported that the mycorrhizal effect on the above occurs before any positive growth response to VAM is observed. It was concluded that this effect could be attributed to other factors rather than the mycorrhizal improvement of phosphate nutrition (Smith et al., 1979; Barea and Azcon-Aguilar, 1984).

The successful infection and subsequent establishment of a nodule on legume roots could be regarded as a process influenced by certain factors as in the rhizosphere (Vincent, 1974; Sprent, 1979). Some studies have established an increase in nodule number as a result of combined inoculation (Smith et al., 1979; Asimi et al., 1980), however, Smith et al. (1979) pointed out that this trend is variable and seems to be related to the experimental system used and to plant age. Since no competition between the fungi and the bacteria on infection sites was recognized, other factor(s) seem(s) to be involved in the process of nodule formation in dual-inoculated plants. Although successful infection and nodule formation were recognized as important aspects of mycorrhiza-infected legumes, the early events which lead to these processes appear to have received little attention.

It is still not clear whether the two microsymbionts (*Rhizobium* and VAM) interact directly outside the host or indirectly via some physiological changes in the host plant following VAM fungi infection. In the present study, we

investigated the effect of VAM infection and inoculum localization on nodule initiation and nodule distribution along the root system, as well as their activity and contribution to the host's productivity.

2. Materials and Methods

Experimental systems

Seeds of *Medicago sativa* L. and *Trifolium alexandrinum* L. were surface-sterilized with 70% (v/v) ethanol for 7 min, rinsed in ten changes of sterile distilled water, and germinated on water agar (1% Difco, Bacto-agar) in the dark for 20 hr. Experiments were conducted using autoclaved soil in 1.5 L tin-coated metal containers previously mixed with a small amount of phosphorus (15 mg superphosphate/kg soil) and adjusted to water field capacity with modified N-free nutrient solution (Johnson et al., 1957). The standard N-free nutrient solution was modified by elimination of K_2HPO_4. Eight seedlings were sown in each pot and plants were thinned out to six after emergence of the first trifoliate leaves. After 10 days of plant growth, each pot was watered to water field capacity by adjusting the weight every other day with the modified N-free nutrient solution. Plants were allowed to grow under greenhouse conditions (14/10 hr day/night cycle, and 28/18°C day/night temperature), with natural illumination supplemented by fluorescent light.

A local isolate of *Glomus macrocarpum* Tul. and Tul. (kindly provided by Dr. J.H. Haas, Dept. of Plant Pathology, ARO, Volcani Center) and maintained under sterile conditions in the greenhouse in pot cultures of melon and corn as host, was used as the mycorrhizal inoculant. Each seedling of alfalfa and *Trifolium* was inoculated at sowing time with 1 ml of culture suspension containing 10^8 colony forming units of local isolate of *Rhizobium* alone, respectively, with *Glomus macrocarpum* alone, or both. Mycorrhiza inoculum was applied by using 10 g of a mixture of spores and infected roots pot culture. The VAM fungi inoculum concentration used in all experimens described in this study contained not less than 2.5 viable propagules per gram of the mixture as measured by the MPN method (Haas and Krikun, 1985). Routinely, experiments conducted with lower viable propagules were discarded.

Data collection

At each harvest, the root system was separated from the shoot and dry weight was determined after drying for 36 hr at 70°C. Nitrogen fixation activity of detached root systems was determined by acetylene reduction assay (Sprent, 1971) using "Varian" gas chromatography model 3700 with a flame

ionization detector. Results were expressed as μmol acetylene reduced per mg nodule dry weight per hour. Total nitrogen content of dried, ground plant material was determined by micro-Kjeldahl assay, and phosphorus content by the ammonium molybdate method (Olsen and Dean, 1965).

Visual observation of mycorrhizal infection was made by clearing washed roots in 10% KOH and staining with 0.05% trypan blue in lactophenol (v/v) according to Phillips and Hayman (1970). Mycorrhiza colonization in the infected roots and its infection intensity was estimated by determining colorimetrically the conversion of fungal chitin to glucosamine (Hepper, 1976). The number of nodules on the main and lateral roots were counted by direct observation using a magnifying glass. Root lengths of *Rhizobium* inoculated alone, and a combination of *Rhizobium*- and mycorrhiza-inoculated plants were cut into 6 cm-long pieces each and the nodules were counted to describe the distribution of nodules on that length of root as affected by inoculation with and without mycorrhiza fungus. Data were analysed by standard statistical methods (Steel and Torrie, 1960).

3. Results

Significantly higher dry weight was recorded in dual infected alfalfa and *Trifolium* plants than in plants inoculated by VAM or *Rhizobium* treatments alone (Table 1). Mycorrhizal establishment was associated with an increase in alfalfa nodule mass (Table 2). Dual-inoculated plants had fewer nodules than did *Rhizobium* inoculated alone treatments, but nodules were much bigger (size and weight) than on non-mycorrhizal plants (11.8 mg dw/plant *vs.* 2.7 mg). Mycorrhizal plants were not only larger but had higher P and N contents (Table 1). Both *Trifolium* and alfalfa plants had higher N concentrations in dual-infected plants than in other treatments (Table 1), but P concentration was affected significantly only in alfalfa (Table 1).

Nodule number, which expresses the number of successful *Rhizobium* infection sites along plant roots, showed a specific change as a result of VAM inoculation (Figs. 1a,b). In both legume hosts, a significantly higher percentage of nodules was clustered on the roots at the first 6 cm below the soil surface (B.S.S.) in mycorrhizal than in non-mycorrhizal plants (65% and 35% respectively). On assessment of a lower root fraction (6–12 cm B.S.S.), it was observed that the number of nodules on mycorrhizal plants was reduced significantly as compared with non-mycorrhizal plants (Fig. 1a,b). The number of nodules continued to decline significantly in mycorrhizal *Trifolium* plants as compared to non-mycorrhizal plants at the lowest region assessed (12–18 cm

Table 1. Effect of mycorrhizal inoculation on plant dry weight, nitrogen and phosphorus concentration and contents. Uninoculated control, C; mycorrhizal inoculated, M; *Rhizobium* inoculated; R; and *Rhizobium* plus mycorrhiza inoculated, R+M. Values given are the means ± standard errors of six replicate pots (six plants per pot).

	Treatment	Plant dry wt.	N	Nitrogen	P	Phosphorus content
		(mg/plant)	(%)	(mg/plant)	(%)	(mg/plant)
Alfalfa	C	138.5±16.8	1.03±0.02	1.4±0.2	0.15±0.01	1.9±0.2
	M	205.0±10.0	1.00±0.04	2.0±0.1	0.21±0.01	4.2±0.3
	R	264.0±15.8	2.45±0.08	6.7±0.4	0.16±0.01	4.3±0.4
	R+M	458.7±23.4	2.40±0.06	11.4±0.7	0.31±0.02	14.1±1.2
	LSD (0.05)	50.0	0.16	1.2	0.04	1.9
Trifolium	C	542.0±47.2	1.32±0.17	5.6±1.2	0.49±0.09	1.8±0.2
	M	476.0±37.7	1.28±0.08	6.7±0.7	0.61±0.07	3.0±0.4
	R	590.2±33.1	2.50±0.02	14.8±1.3	0.48±0.05	2.9±0.4
	R+M	754.2±17.47	2.50±0.04	19.0±0.6	0.60±0.04	4.4±0.3
	LSD (0.05)	41.5	0.28	2.9	0.04	1.1

LSD, least significant difference

Table 2. The effect of mycorrhiza inoculation and depth of application on colonization intensity in alfalfa (*Medicago sativa* L.) roots after 3 and 7 weeks of growth respectively. *Rhizobium*, R: *Rhizobium* plus Mycorrhiza, R + M. Colonization intensity was measured by the glucosamine assay (see M&M) in the upper (0–7 cm; UP) or lower (7–18 cm; DOWN) root parts. Values are means (±SE) of six replicate pots, each of which contained six plants.

Treatment	Root segments analyzed (cm)	Glucosamine concentration (μg/g root dw)	
		3rd week	7th week
R + M	0–7	28	160
(4 cm)	7–18	14*	94*
R + M	0–7	16	120
(10 cm)	7–18	30*	136

*Significant at P (0.05)

B.S.S.), indicating a specific influence (inhibition) of VAM inoculation on the location of nodule formation along both alfalfa and *Trifolium* root systems.

Infection rates of *Rhizobium* and mycorrhiza on alfalfa plants were ascertained. Fifty percent of *Rhizobium* nodules were observed after 18 days of plant growth, whereas 50% of the VAM colonization occurred after 35 days (Fig. 3). Microsymbionts establishment and infection rates did not differ when analysed in dual-inoculated plants, as when compared to a separate VAM or *Rhizobium*-inoculated treatment (data not shown).

The percent infection and glucosamine concentration in mycorrhizal roots which indicate the intensity of infection were correlated (Fig. 2). The extent of mycorrhizal infection and its colonization intensity on alfalfa roots was measured by the glucosamine assay (Table 2). At the 3rd week, when no significant differences in plant development could be observed, the glucosamine concentration was higher at the exact position of mycorrhizal inoculum placement. These differences remained significant after 7 weeks of growth for the 4 cm B.B.S. application, but not for the 10 cm B.B.S. treatment. This indicates a spread-up of VAM colonization in the root system between the 3rd and 7th weeks of growth. When VAM inoculation was applied at 4 cm B.S.S., the total number of nodules per plant declined significantly but, the reduction was much more pronounced on the lower part of the root system (Table 3). This contrasts the observation in the 10 cm B.S.S. inoculum placement where nodules were evenly distributed on the upper and subterranean parts with no significant influence on nodule number (Table 3). Nodule weight was significantly

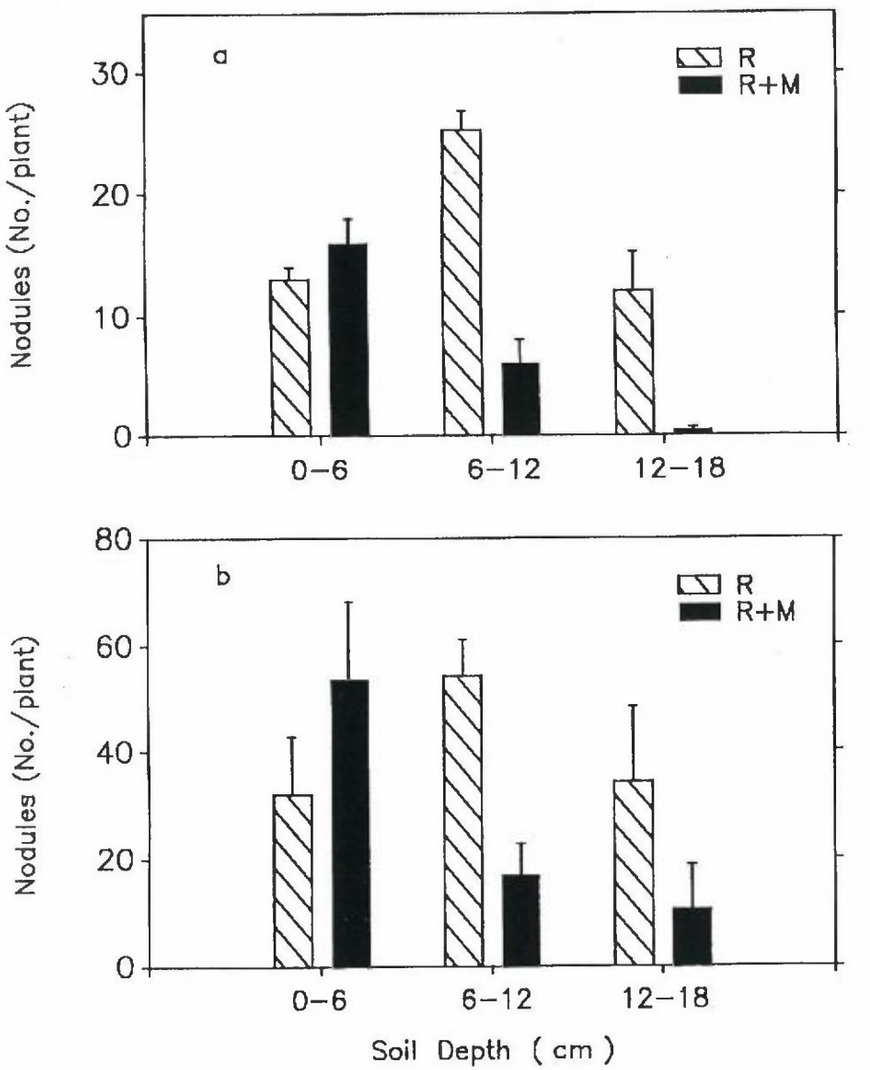

Figure 1. The effect of *Rhizobium* (R) and *Rhizobium* plus mycorrhiza (R+M) inoculation on nodule distribution along the root system of (a) Alfalfa (*Medicago sativa* L.) and (b) *Trifolium* (*Trifolium alexandrinum* L.) plants. Values plotted are means of six replicate pots (6 plants per pot). Vertical bars represent the SE.

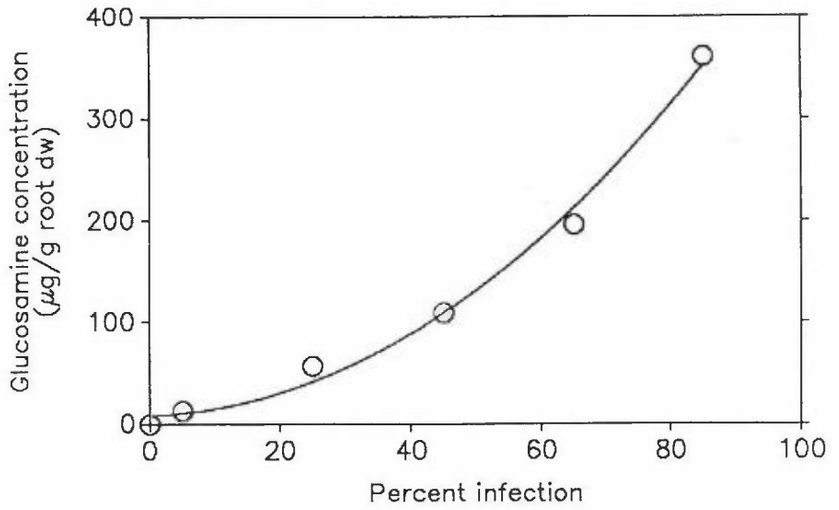

Figure 2. Glucosamine concentration in relation to percent infection.

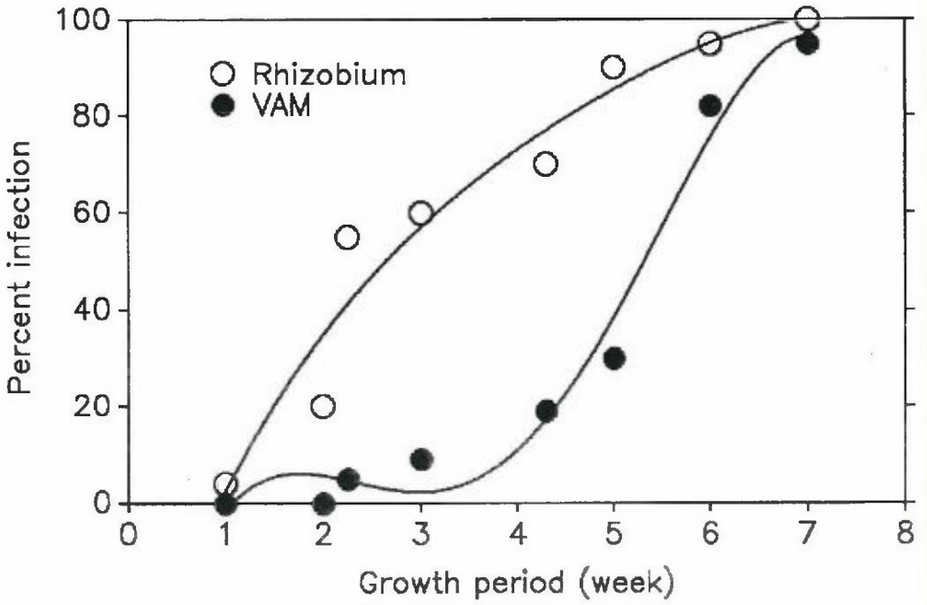

Figure 3. The rate of mycorrhizal infection and *Rhizobium* nodule initiation on alfalfa (*Medicago sativa* L.) roots. Mycorrhiza infection rate was estimated by the glucosamine assay (additional details in the text). Plotted values are means of six replicate pots, each containing six plants.

Table 3. The effect of mycorrhiza inoculation and depth of application on nodule number, weight and specific activity of alfalfa (*Medicago sativa* L.) root. *Rhizobium*, R; *Rhizobium* plus Mycorrhiza, R + M. Plants were grown under controlled conditions for 7 weeks. Values are means (±SE) of six replicate pots, each of which contained six plants).

Treatment	Root segment analyzed	Number of nodule	Nodule dry weight	F_2 fixation
	(cm)	(no/plant)	(mg/nodule)	μmole C_2H_2 (h mg. nod. dw)
R	0–7	39±2	1.3±0.1	0.25±0.04
	7–18	29±7	1.4±0.4	0.23±0.01
R + M	0–7	29±2	10.0±0.9	5.92±1.20
(4 cm)	7–18	13±1*	1.8±0.3**	0.35±0.09**
R + M	0–7	34±2	3.6±0.6	0.62±0.20
(10 cm)	7–18	32±3	4.2±0.7	1.73±0.57**
LSD (0.05)		12	0.5	0.35

* Significant at 0.05
** Significant at 0.01

affected by VAM inoculation (4 and 10 cm B.S.S. treatments) and inoculum placement (4 cm B.S.S. treatment only) (Table 3).

Nitrogenase specific activity (as measured by acetylene reduction method) was correlated with VAM inoculum placement (Table 3). In both inoculation treatments (4 and 10 cm B.S.S.), highest activity was recorded for nodules located adjacent to the site of VAM inoculation.

4. Discussion

A local isolate of *G. macrocarpum* inoculated with *Rhizobium* sp. was found to have a significant positive effect on alfalfa and *Trifolium* plant growth, N and P content and nitrogenase activity (demonstrated for alfalfa only). Similar results were reported for dual inoculated treatments in the rhizosphere of *Medicago sativa* L., *Trifolium subterraneum* L. and *Glycine max* inoculated with different VAM fungi (Smith and Daft, 1977; Smith et al., 1979; Asimi et al., 1980, respectively).

Fewer and bigger nodules were formed on mycorrhizal as compared to non-mycorrhizal plants. This observation contrasts other works (Smith et al., 1979;

Asimi et al., 1983) where more nodules were formed on mycorrhizal than non-mycorhizal plants. These nodules, however, had higher dry weight, possessed greater nitrogenase activity and were specifically patterned along the roots, than nodules of non-mycorrhizal plants (Table 3).

This specific distribution of nodules could be attributed to inhibition of nodule formation on lower parts of the root system (Fig. 1), and this in turn could be linked to a direct or an indirect response of mycorrhizal fungi inoculation. A possible mechanism by which VAM fungi can influence nodule formation is by competition for infection sites along the root. However, earlier reports ruled out such a possibility (Smith and Bowen, 1979). In our experimental system the significant nodule onset pattern induced by mycorrhiza inoculation does not seem to be related directly to the extent of mycorrhiza colonization. This conclusion could be drawn from the fact that *Rhizobium* infection and nodule formation were found much earlier than any significant VAM establishment was detected (Fig. 3). Measurement of rate and intensity of VAM fungi colonization by the glucosamine method substantiates this fact (Fig. 3). The method proved advantageous since it enabled such estimation for a longer period of plant growth, but it failed to differentiate between active and inactive fungal structures. The events that led to a similar nodulation pattern in both legume hosts (alfalfa and *Trifolium*), and the fact that there was a significant increase in P concentration in alfalfa plants only, suggest that P nutrition might not be implicated in this distribution phenomenon. The distribution of nodules could also be related to the time and rate of VAM fungi infection in the roots. In the present study, by using low-fertility autoclaved-soil, introduced with mycorrhizal inoculum consisting of spores and infected root segments, mycorrhizal establishment was delayed as previously demonstrated by Smith et al. (1979) in a similar experimental system. But the fact that VAM infection was delayed could not be the sole reason as in our experimental system, a further delay of VAM infection in the 10-cm-depth inoculation treatment could not alter such a nodule pattern. In a recent study, it was demonstrated that the time of endophyte introduction and establishment affected the development of other symbiotic partners but, the mechanism for this phenomenon was attributed to competition for root carbohydrates between the symbiotic partners (Bethlenfalvay et al., 1985). It could therefore be that the early establishment of *Rhizobium* in our experimental system caused a further delay in the VAM fungus establishment.

In effect, the presence of mycorrhiza inoculum applied at a depth of 4 cm not only significantly changed the normal pattern of nodules along the entire root system, but also led to higher specific nitrogenase activity. This effect on nodule activity was localized at the site adjacent to VAM fungi inoculation

in both application treatments (Table 3). It can therefore be inferred that the specific distribution and subsequent patterning of nodules, coupled with the formation of fewer and bigger nodules, was a direct consequence of the very high nodule-specific activity (Table 3). The establishment of mycorhizal infection in legume plants and its influence on specific activity is in agreement with previous observations (Daft and El Giahmi, 1974; Gates, 1974; Smith et al., 1979). It is too early to speculate if this nodule partitioning on legume root is an direct or an indirect response to nodule-specific activity. Smith et al. (1979), however, contended that a steady supply of P to root cells, and to adjacent nodules, in conjunction with other factors, could be stimulating to the development of nodule efficiency. It can therefore be argued further that when some of these factors are met, partially by the presence of mycorrhizal fungi, the need to construct more nodules is curtailed as the plant seems content with the fewer, bigger, and more efficient nodules established. Additionally, it can be speculated that lower efficiency in bacteroid activity could influence this unique nodule pattern, however, this should be demonstrated experimentally. Although no effort has been made to engage the suggested mechanism to different strains of VAM fungi, this unique experimental system has been employed to investigate and understand VAM fungi infection and its contribution to nodule initiation and activity.

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