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Nodulation, N_2 Fixation (^{15}N) and N Nutrition Relationships in Mycorrhizal or Phosphate-Amended Alfalfa Plants

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Abstract

The legume *Medicago sativa* L. (alfalfa) (+ *Rhizobium meliloti*) was grown under controlled conditions in two soils amended with increasing amounts of assimilable phosphate (P_1, P_2 and P_3), or with a vesicular-arbuscular mycorrhizal (VAM) inoculum. A small quantity of ^{15}N-labelled ammonium sulphate was added to each pot for a qualitative estimate of N_2 fixation, allowing to compare the effects of VAM and P treatments on nodule activity. Plant response (dry matter) to VAM inoculation was equal to the response to P addition at the highest level (P_3). The P and N concentrations were higher in the VAM than in the P_3 treatment. However, the effects of VAM inoculation on the levels of nodulation and N_2 fixation (^{15}N) were equivalent to those of the P_2-treated plants, while the P_3 treatment still increased both nodulation and N_2 fixation rates over the P_2 and VAM treatments. These observations suggest, for the tested plant cultivar/VAM fungus combination, a limitation for VAM plants to reach their full potential for nodulation and N_2 fixation under the prevalent ecophysiological conditions of this experiment. In spite of this limitation, the results indicate that VAM fungi can enhance N acquisition by plants by mechanisms additional to P-mediated improvement of nodulation and N_2 fixation. These mechanisms could be based on a capability of VAM fungi to use soil N which is less available to non VAM plants.

Keywords: N_2 fixation, VA mycorrhiza, Nodulation, ^{15}N-labelled fertilizers, *Rhizobium*-legume symbiosis, N uptake

1. Introduction

The network of extraradical mycelium of the fungal symbiont in VA mycorrhizas is recognized as an extension of the absorbing surface of the root (Mosse, 1973; Krikun, 1991). Nutrient uptake by VAM fungi are mainly expressed in the case of ions of low mobility, like phosphate. The VAM symbiosis has developed physiological and biochemical mechanisms to improve the P nutrition of the plant (Smith and Gianinazzi-Pearson, 1988). Therefore, nodulation and N_2 fixation in the legume-*Rhizobium* symbiosis, being dependent on P supply, are usually enhanced by VAM fungi (Barea and Azcón-Aguilar, 1983; Hayman, 1986). Mechanisms in addition to those mediated by P uptake have been also suggested for some of the interactions between *Rhizobium*, VAM fungi, and legumes concerning the efficiency of the N_2 fixation process (Harris et al., 1985; Bayne and Bethlenfalvay, 1987; Brown and Bethlenfalvay, 1988; Brown et al., 1988; Patterson et al., 1990). In contrast, competition for root carbohydrates between the symbiotic partners can give way to antagonistic effects of VAM on nodulation (Bethlenfalvay et al., 1985).

However, VAM fungi may improve N nutrition of the host legume not only by direct or indirect effects on the enhancement of N_2 fixation, but also from increased N uptake from the soil. The source of the N in legume tissues can be estimated by using a ^{15}N isotope dilution method, which allows assessing the relative contribution of atmosphere, soil or fertilizers to plant N nutrition (Danso, 1988). The procedure requires a reference crop which does not fix N_2, but this might introduce errors for quantitative determinations. Appropriate reference plants can be selected, and Barea et al. (1987) and (1989) suggested that VAM fungi are able to improve both N_2 fixation and N uptake from soils. These findings need to be extended to other situations to establish the actual mechanisms of contribution of VAM to the N yield in a legume. In this context, two methodological topics are important to be considered: (1) simplification of the ^{15}N isotope dilution procedure, and (2) use of appropriate non mycorrhizal, P amended control treatments to match the VAM effects on dry matter, and on N- and P- biomass yield.

The ^{15}N-isotope dilution technique could be simplified when qualitative estimates can yield the required information. In these cases a reference crop is not needed, thus avoiding possible errors and saving ^{15}N-labelled fertilizer. A small amount of this material is added to each pot for all treatments. The assessment of the $^{15}N/^{14}N$ ratio in plant tissue permits ranking the effect of these treatments on N_2 fixation (Danso, 1988). The lower this ratio is in the plant, the better is the treatment in enhancing N_2 fixation, since this biological process increases the proportion of ^{14}N in the plant. Obviously, appropriate

non VAM controls can be obtained by preparing a series of pots receiving increasing doses of soluble phosphate fertilizer, and selecting for comparison, that matching the VAM effects (Abbott and Robson, 1984). The objectives of this study were to consider these two methodological approaches in comparing the effect of VAM inoculation on N yield, nodulation and N_2 fixation by alfalfa grown in two P-fixing agricultural soils under controlled conditions.

2. Materials and Methods

Experimental design

The experiment utilized two soils, four levels of soluble phosphate, and a VAM inoculation treatment, giving ten treatments that were replicated five times for a total of 50 pots. A completely-random arrangement was followed.

Host plant and test soils

Alfalfa (*Medicago sativa* L. cv. Aragón) was the test plant. Five-day-old uniform seedlings obtained from surface-sterilized seeds as described by Patterson et al. (1990) were transplanted (four plants/pot) into pots containing 500 g of the corresponding experimental soil, as previously supplied with the appropriate amount of soluble phosphate (P_1, P_2 and P_3)) or a VAM inoculum (M). An unamended (P_0), non VAM control was also used. At transplanting, all the plants received an inoculum of a local *Rhizobium meliloti* strain. This inoculum was obtained in Allen medium (Allen, 1957) and contained 10^8 cells/ml. The test soils were collected from Granada Province, Spain. The characteristics of these test soils are given in Table 1. The soils were sieved (2 mm), diluted with sand (5:2, v:v), steam-sterilized (100° C for 1 hr during 3 consecutive days) and then reinoculated with a soil filtrate containing their own microbial population except VAM propagules (Barea et al., 1989). Each pot received 2 ml of such filtrate.

Mycorrhizal inoculation and phosphate treatments

Each one of the experimental soils was divided into five batches: P_0 (untreated control), P_1, P_2, P_3, which represent levels of H_2KPO_4 at 150, 200 and 250 mg/kg soil respectively, and M (mycorrhizal inoculum, applied at transplanting). These were left to equilibrate for 2 weeks and then analyzed for plant available P (Olsen). The results (Table 2) indicate high P-fixing capacity of the test soils. A VAM inoculum (3 g) was applied to each one of the corresponding pots. The inoculum was from a thoroughly homogenized soil sample of

Table 1. Analytical characteristics of the test soil

Parameter	Soil	
	"Zaidin"	"Arenales"
pH (water)	7.30	7.60
Clay (%)	42.50	33.50
Silt (%)	34.00	37.80
Sand (%)	23.50	28.70
Organic matter (%)	1.18	1.53
Total N (%)	0.18	0.12
Extractable P (mg/kg)	6.10	4.50
Extractable K (mg/kg)	0.68	0.57

Table 2. Dry matter yield, nitrogen and phosphorous concentration in the shoots of nodulated alfalfa as a function of P amendment or VAM inoculation

Soil	P	Treatment (mg/kg KH_2PO_4)	Extractable P (mg/kg of soil)	Plant dry wt. (g/pot)	N (mg/g)	P (mg/g)
Zaidín	P_0	0	6.1	1.04^a	2.22^a	0.10^a
	P_1	150	12.3	1.36^b	2.71^b	0.12^b
	P_3	200	17.4	1.46^c	3.13^c	0.13^b
	P_3	250	25.8	1.68^d	3.04^d	0.14^b
	M	VAM	6.1	1.56^d	3.93^e	0.16^c
Arenales	P_0	0	4.5	0.48^a	2.04^a	$0.09a$
	P_1	150	9.1	1.56^b	1.66^b	0.08^a
	P_2	200	19.2	1.76^c	1.80^a	0.12^{ab}
	P_3	250	28.2	1.91^d	2.72^b	0.13^b
	P_1	VAM	4.5	1.85^d	3.09^c	0.15^c

For each soil and parameter, mean values (five replicates) not sharing a letter differ significantly ($P < 0.05$).

the VAM fungus *Glomus mosseae* (Nicol. & Gerd.) Gerd. and Trappe grown in stock culture with *Allium cepa* L. The isolate of *G. mosseae* was derived originally from Rothamsted U.K. The inoculum consisted of spores, mycelium, and mycorrhizal root fragments. The inoculum potential, as determined by the MPN method (Haas and Krikun, 1985), was 3.2 viable propagules per gram.

Growth conditions

The plants were grown in a greenhouse under a day/night cycle of 16/8 hr, 21/15° C, 50% relative humidity. A photosynthetic photon flux density of 600–700 μmol $m^{-2}sec^{-1}$ was applied as supplementary light. During the assay plants were fertilized (10 ml/week/pot) with Long Ashton nutrient solution (Hewitt, 1952) lacking N and P. Throughout the experiment, the pots were weighed every day and water loss to reach field capacity was replaced by top watering. After 10 days of plant growth, each pot was given a solution of $(NH_4)_2SO_4$ with 10% ^{15}N of atomic excess. Two mg N/kg soil (1 mg/pot) was given, which is equivalent to 5 kg N/ha.

Measurements

After a growth period of 77 days the plants were harvested. Shoot dry weight was recorded after drying at 70° C. Shoot P and N concentrations were measured after Kjeldahl digestion (for N) or by the molybdenum blue (for P) procedures (Lachica et al., 1973). Shoot N isotope composition was determined by mass spectrometry, as described by Fiedler and Proksch (1975) at the FAO/IAEA Agricultural Biotechnology Laboratory, Seibersdorf, Austria. Since lower enrichment in ^{15}N means higher input of ^{14}N by N_2-fixation, values of % ^{15}N atomic excess (a.e.) are inverse to the N_2-fixing potential induced by the treatment. Therefore, to facilitate the study of a graphic representation, these values are given as 1/ % ^{15}N atomic excess. The roots were carefully washed and the number of nodules was assessed visually. The percentage of mycorrhizal root length was estimated by microscopic examination of stained samples (Phillips and Hayman, 1970), using the grid-line intersect method of Giovannetti and Mosse (1980).

3. Results

The dry matter yield and the concentration of N and P in alfalfa increased with the level of assimilable phosphate in soil (Table 2). Mycorrhizal inoculation produced the same ($P > 0.05$) plant biomass as the highest P treatment. The concentrations of N and P in the shoots were higher in VAM plants than

in P-amended non VAM plants (Table 2). VAM colonization of root length was $73 \pm 8\%$ in "Arenales" and $65 \pm 3\%$ in "Zaidin" soils.

Nutrient content comparisons show that non VAM plants provided with the highest amount of additional P and VAM plants accumulated equivalent amounts of N and P (Fig. 1). However, the effects of VAM inoculation on

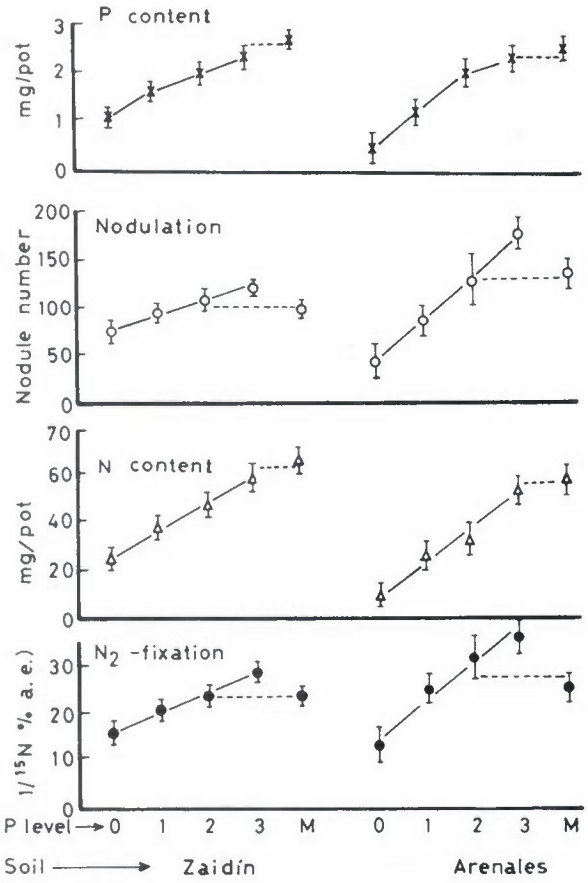

Figure 1. Nodulation, N isotopic composition, and N and P contents of alfalfa shoots under different phosphate treatments, (see Table 2). Dashed lines relate plant response to VAM or P-fertilization effects. Vertical bars represent confidence limits ($P > 0.05$).

nodulation and N_2 fixation were equivalent to those of the P_2 treatments, while the P_3 treatment produced higher rates of nodulation and N_2 fixation than the P_2 and VAM treatments (Fig. 1).

4. Discussion

Comparisons of dry matter and N and P contents of VAM and P-amended non VAM legumes have demonstrated that VAM fungi can still improve nodulation and N_2 fixation over the non VAM counterparts (Barea and Azcón-Aguilar, 1983; Hayman, 1986). However, in the present study nodulation was reduced in VAM alfalfa, relative to the non VAM P treatment (P_3). Competition for root carbohydrates can lower nodule number in VAM plants (Bethlenfalvay et al., 1985). However, if such competition actually took place in the present experiment, it was rather low.

Results reported by Brown et al. (1988) also showed that, in spite of improved nodulation, nodule specific mass and activity (C_2H_2 reduction) in VAM plants were lower in comparison to P-sufficient, non VAM controls. This report, like others also based on the C_2H_2 reduction technique (Pacovsky et al., 1984), reached the same conclusion as that of the present experiment: nodulated VAM legumes had lower nodule activity than P-amended control plants. The use of ^{15}N techniques, as in the present study, permits the most direct determination of the contribution of N_2 fixation to plant the N uptake. The results show that VAM, which was similar to the best P treatment (P_3) to improve N content, was not the best in enhancing N_2 fixation. This indicates that the VAM fungus helps the plant to acquire N from sources other than N_2. These findings support those by Barea et al. (1987) and (1989) reporting that the network of VAM hyphae was able to increase N uptake from soil in two particular situations, in which there was no limitation in nodulation nor in N_2 fixation. Our data (unpublished) indicate that the amount and rate of N uptake (^{15}N) are much too low to affect N_2 fixation, but appear sufficient to satisfy for the N requirements of the plant. This is even more important in the case of non nodulated VAM plants. Barea et al. (1991) found that VAM fungi increase the size of the apparent soil N pool (A_N value), suggesting that VAM plants possibly use available N forms more efficiently, or can derive N from sources less available to non VAM plants.

This report, therefore, confirms previous findings that VAM plants possess alternative mechanisms to satisfy their N requirements in case of competition between the VAM fungus and *Rhizobium*, when growth conditions limit either nodulation or nodule activity.

The fact that nutrient concentrations of VAM plants were higher than those of the P_3, even though dry mass and nutrient contents were almost the same, appears of interest. If the relationships between biomass production nutrient concentration and nutrient content, as found in the present study, are discussed on the basis of the statements by Jarrel and Beverley (1981), it can be

concluded that VAM plants were accumulating nutrients relative to those under the P_3 treatment. Obviously the nutrient dilution/concentration balance is dependent on the harvest times.

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