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EFFECTS OF INTRODUCED AND INDIGENOUS VA MYCORRHIZAL FUNGI ON NODULATION, GROWTH AND NUTRITION OF *MEDICAGO SATIVA* IN PHOSPHATE-FIXING SOILS AS AFFECTED BY P FERTILIZERS

by J. M. BAREA, J. L. ESCUDERO* and C. AZCON-G. DE AGUILAR

Microbiology Department, Estación Experimental del Zaidín, C.S.I.C., Granada, Spain

KEY WORDS

Endomycorrhiza *Glomus mosseae* Legume nodulation *Medicago sativa* Microbial fertilizers Phosphate-fixing soils *Rhizobium meliloti*

SUMMARY

The legume *Medicago sativa* was grown in two phosphate-fixing soils which received soluble or rock phosphate. The effects of the inoculation with *Glomus mosseae* on plant nutrition and nodulation were studied. The introduced VA fungi became successfully established and improved the degree of infection over level achieved by native endophytes. In all experimental conditions tested, plant dry weight, the total uptake of N and P and nodulation by *Rhizobium meliloti* were increased by mycorrhizal inoculation. The size of the increase was inversely correlated with soluble P content in the soil. Mycorrhization, enhanced by introduction of suitable VA fungi, had similar effects to that of the dose of soluble phosphate tested. Indigenous and native endophytes cooperated in these effects. Results are discussed in terms of reducing the input of soluble P fertilizer to phosphate-fixing soils and the possibility of restoring the phosphate stock using a more rational supply of soluble P, that allows cooperation with VA fungi, or by the use of less soluble and expensive forms of P fertilizers.

INTRODUCTION

Most of the soils contain insufficient available phosphate for maintaining suitable plant growth, furthermore, many soils are also phosphate retentive and large dressings of P fertilizers are required and used to obtain adequate crop yields. In the case of legumes the scarcity of soluble phosphorus is a critical limiting factor because it affects not only plant growth but also nodulation and symbiotic nitrogen fixation[7,30].

Research in recent years has established that vesicular-arbuscular (VA) mycor-

* From Facultad de Agronomía, Montevideo, Uruguay.

rhiza greatly stimulated phosphate uptake and plant growth[9,10,15,23,24,31], therefore, mycorrhization is a promising way to help plants, particularly in phosphate deficient soils. The effects of VA mycorrhiza in improving nodulation, nitrogen fixation and growth of legumes is now well established (see[6,14,22,27]).

It is also well known that application of soluble phosphate fertilizers can greatly reduce the benefit the plants obtain from mycorrhizal infection[18]. In addition, it can also reduce the number of Endogonaceae propagules in soil[13]. Consequently, studies on a more rational use of P fertilizers and its compatibility with VA mycorrhiza will be useful not only from the economic point of view but also from the ecological one.

The above statements are particularly important for us since agricultural soils in Southern Spain are phosphate-fixing and great amounts of P fertilizers are often supplied. Several reports describe the benefits of VA fungi inoculation in these soils, both to legumes[5,6] and non-legumes[2,3,4], and the feasibility of field inoculation is now being investigated. A preliminary field experiment[6] proved to be successful, but to expand these findings more experimental and ecological work is being carried out. In this respect, the present paper shows the results of interactions between natural and introduced VA and Rhizobium endophytes and phosphate fertilizers on growth, mycorrhization, nodulation and nutrition of the legume *Medicago sativa*. The phosphate fixing capacity of the soil will be first demonstrated and then recommendations to reduce the phosphate fertilizers input, using endomicorrhizal fungi as 'biological fertilizers' will be discussed.

MATERIALS AND METHODS

Soils

The soils were collected from Granada Province, Spain. Table 1 gives a summary of the analytical details of the two test soils. Both soils have been intensively cultivated for centuries.

Table 1. Analysis of the test soils

Soils n°	Sand (%)	Loam (%)	Clay (%)	pH (water)	Organic Matter (%)	$CaCO_3$ equiv. (%)	Total N (ppm)	Total P (ppm)	Total K (ppm)	0.5 *M* $NaHCO_3$ soluble P (ppm)
8	18.20	39.8	42.0	7.4	1.23	33.2	910	1072	562	18.2
9	26.0	30.0	44.0	7.5	1.28	55.2	1176	848	385	15.8

Phosphate fertilizers and other treatments

Half of each soil was steam-steralized at 100°C for 1 h during 3 days to destroy its indigenous endophytes. The sterile and unsterile parts of the two soils were, in turn, divided into three batches. These were: C = Control (no P fertilizers added); SP = Soluble phosphate added (1 g KH_2PO_4/kg soil; this is equivalent to doses usually applied by the farmers); and RP = amended with rock phosphate (1.33 g/kg soil). Thus, twelve experimental soils were tested (soils nos. 8 and 9; sterile and unsterile × three P treatments). These experimental soils were incubated in a glasshouse at 19–25°C, with suitable watering, for 3 weeks.

After the incubation period the plant available P was estimated in all the 12 experimental soils. The extraction method of Olsen *et al.*[25] was used. Table 2 shows the results of these analyses. The phosphate-fixing capacity can be assessed by comparing the amount of soluble P added (227.6 ppm) and the resulting P content in the corresponding treatment (SP) after incubation.

At the end of the incubation period, the soils were diluted with nutrient-free sand in the ratio 3 : 1 (v/v) and 10 replicate pots of each one of the 12 soils were prepared. Five of the replicate pots were given a VA mycorrhizal inoculum.

Plants, microbial inocula and growth conditions

Medicago sativa L. cv. Aragón was the test plant. Two-day-old seedling were transplanted into pots containing 250 g of the corresponding experimental soil diluted with sand. Seven plants per pot were grown for 7 weeks in a glasshouse at 19–25°C, watered from below and fed with nutrient solution[16] lacking N and P.

At transplanting, all the plants received 10 ml of rhizobial inoculum prepared by growing the strain 203 of *Rhizobium meliloti* in the medium of Allen[1] in a shake culture for 2 days at 28°C.

The mycorrhizal inoculum was applied to the planting hole in the corresponding pot and it consisted of spores, hyphae and infected root fragments thoroughly homogenized and divided into similar aliquots. The VA micorrhizal endophyte was the yellow vacuolate spore type (YV)[17] *Glomus mosseae*[8].

Table 2. Available phosphate content of the experimental soils at transplanting, after 2 weeks incubation with different phosphate fertilizers

Treatment	0.5 *M* $NaHCO_3$ soluble P (ppm)	
	Soil 8	Soil 9
Unsterile soil		
Control (C)	20.8	16.6
Rock P (RP)	21.4	15.6
Soluble P (SP)	76.0	92.8
Sterile soil		
Control (C)	17.0	16.6
Rock P (RP)	18.8	18.8
Soluble P (SP)	68.2	94.0

Measurements

At harvest, fresh and dry weights of roots and shoots were recorded and the shoots analysed for P and N. After carefully washing the roots, the number of nodules and sporocarps was assessed visually. Mycorrhizal infection was also estimated by examining microscopically stained samples[26], more than 100/pot, of feeder rootlets. Two parameters of the mycorrhizal infection were recorded: Total percent infection and 'mg of root infected'[5].

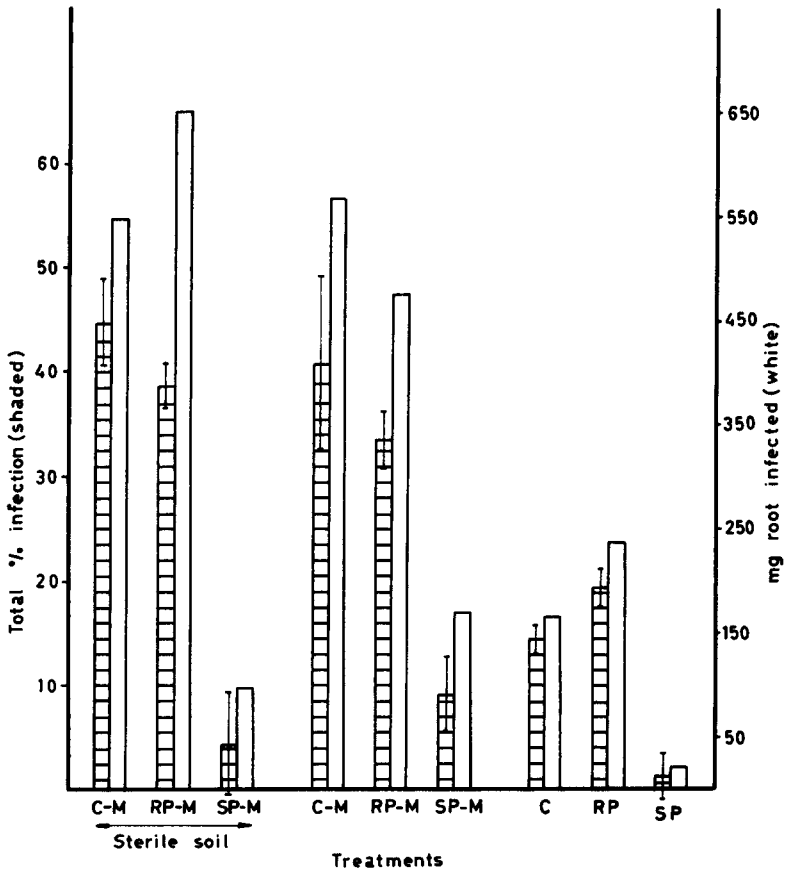

Fig. 1. Effects of introduced and native VA endophytes on mycorrhizal infection in *Medicago sativa* grown in the phosphate-fixing soil n° 8 affected by P fertilizers. C = control (no phosphate added), RP = rock phosphate added, SP = soluble phosphate added, M = mycorrhiza (YV) inoculated.

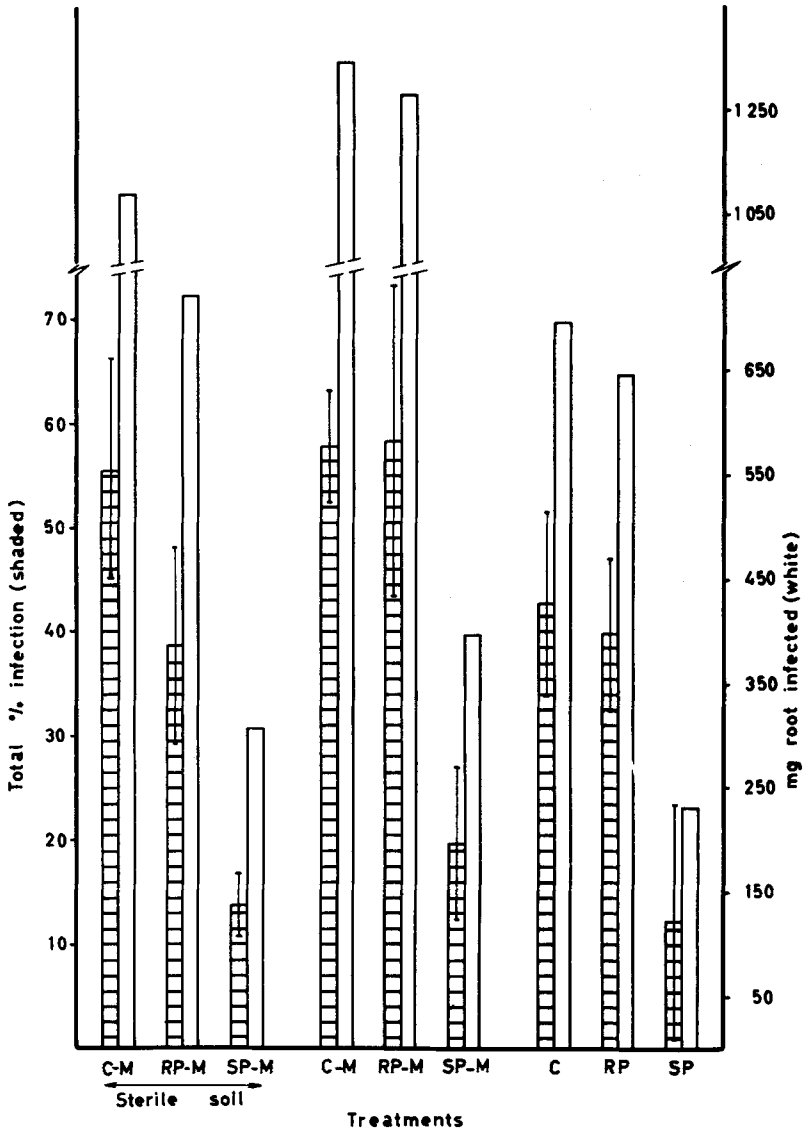

Fig. 2. Effects of introduced and native VA endophytes on mycorrhizal infection in *Medicago sativa* grown in the phosphate-fixing soil n° 9 affected by P fertilizers. Conventions as in Fig. 1.

RESULTS

The soils used in the present experiments are able to fix considerable amounts of the soluble P fertilizer added. As Table 2 clearly shows, about 70 and 61 percent of the soluble phosphate given was fixed by soils nos 8 and 9, respectively, in 3 weeks incubation at 19–25°C. In these neutral-alkaline soils rock phosphate did not significatively increase the available phosphate content (Table 2).

Figures 1 and 2 show the development of mycorrhizal infection in soils 8 and 9 as affected by P fertilizers and the interactions between introduced and native endophytes. Mycorrhizal infection was hardly affected by the added rock phosphate, but soluble phosphate significantly depressed the degree of infection by native or introduced VA endophytes in all the experimental situations tested. The intensity of the VA infection by indogenous fungi in unsterile soil n° 9 (figure 2) is remarkable. In spite of this, the introduced VA fungi improved the degree of infection over the level achieved by native ones. The inoculated endophytes became, therefore, established in unsterile conditions.

Tables 3 and 4 summarize the effect of the inoculation with VA endophytes on growth and nutrition of *Medicago sativa* in the several experimental conditions

Table 3. Dry weight and N and P uptake of alfalfa (shoots) grown 7 weeks in soil n° 8 given different treatments (7 plants/pot, 5 replicate pots/treatment)

Treatment*	Dry weight (mg)	N uptake**		P uptake**	
		C	T	C	T
Unsterile soil					
Control	145 ± 22.3	3.28	4.76	0.16	0.23
Mycorrhiza (M)	432 + 162.0	4.09	17.67	0.19	0.82
Rock P (RP)	203 ± 25.9	4.05	8.22	0.22	0.45
RP + M	374 ± 31.0	4.06	15.18	0.20	0.75
Soluble P (SP)	483 ± 34.8	3.46	16.71	0.28	1.35
SP + M	567 ± 141.5	3.57	20.24	0.34	1.93
Sterile soil					
Control	109 ± 13.4	4.84	5.27	0.08	0.09
M	385 ± 32.3	3.79	14.59	0.16	0.62
RP	182 ± 14.3	4.76	8.66	0.08	0.14
RP + M	452 ± 17.4	3.66	16.54	0.16	0.72
SP	573 ± 75.7	3.23	18.51	0.34	1.95
SP + M	615 ± 52.1	3.34	20.54	0.39	2.40

* All plants received a *Rhizobium meliloti* inoculum.
** C = Content (% dry matter); T = Total uptake (mg).

Table 4. Dry weight and N and P uptake of alfalfa (shoots) grown 7 weeks in soil n° 9 given different treatments (7 plants/pot, 5 replicate pots/treatment)

Treatment*	Dry weight (mg)	N uptake**		P uptake**	
		C	T	C	T
Unsterile soil					
Control	398 ± 21.3	3.53	14.05	0.29	1.15
Mycorrhiza (M)	667 ± 35.8	3.31	22.08	0.31	2.07
Rock P (RP)	409 ± 20.1	3.59	14.68	0.28	1.14
RP + M	577 ± 44.5	3.57	20.60	0.30	1.73
Soluble P (SP)	659 ± 45.4	2.86	18.85	0.31	2.04
SP + M	669 ± 51.0	3.72	24.89	0.39	2.61
Sterile soil					
Control	248 ± 27.9	3.40	8.43	0.12	0.30
M	493 ± 25.4	3.20	15.78	0.26	1.28
RP	158 ± 16.1	4.76	7.52	0.11	0.17
RP + M	470 ± 18.6	2.94	13.82	0.19	0.89
SP	625 ± 25.2	2.71	16.94	0.45	2.81
SP + M	661 ± 10.2	2.96	19.56	0.47	3.11

* All plants received a *Rhizobium meliloti* inoculum.
** C = Content (% dry matter); T = Total uptake (mg).

tested. In all the experimental soils, dry weight and the total uptake of N and P, and, in general, the plant P concentration, were increased by mycorrhizal inoculation. In general, mycorrhization did not increase the availability of rock phosphate. The size of the growth increase obtained in alfalfa by inoculation with VA endophytes was inversely correlated with the level of available soil phosphate.

However, the most noticeable result, because of its practical significance, is that in unsterile soils n° 8 and 9 the effects of the inoculation of VA endophytes on plant growth and nutrition were similar to those achieved, by the dose of soluble P fertilizers used (M *vs.* SP treatments, Tables 3 and 4, unsterile soil). This effect was not reproduced under sterile conditions and the possible influence of natural microbiota including native Endogonaceae propagules, will be discussed.

Two parameters of nodulation are given in Tables 5 and 6: the total number of nodules and the number of the largest ones.

In general, the inoculation with VA fungi increased the number of nodules comparatively with the corresponding non-inoculated controls (Table 5 and 6) but in unsterile soil n° 9 (Table 6) all the plants satisfactorily nodulated since non-inoculated control showed adequate mycorrhization by natural VA endophytes.

Table 5. Macroscopic observations of microbial symbiosis with alfalfa grown 7 weeks in soil n° 8 given different treatments (7 plants/pot, 5 replicate pots/treatment)

Treatment*	Number of nodules		Sporocarps of*** *Glomus mosseae*
	Total	Size 3**	
Unsterile soil			
Control	58 ± 2.8	1.4 ± 0.6	0
Mycorrhiza (M)	90 ± 9.1	4.8 ± 1.6	1
Rock P (RP)	64 ± 6.7	1.2 ± 2.2	0
RP + M	112 ± 6.4	4.8 ± 0.9	1
Soluble P (SP)	169 ± 12.5	16.2 ± 2.5	0
SP + M	143 ± 14.9	19.8 ± 3.6	0
Sterile soil			
Control	10 ± 1.9	0	0
M	105 ± 2.3	1.8 ± 0.5	5
RP	9 ± 1.6	0	0
RP + M	121 ± 20.2	2.0 ± 1.3	4
SP	165 ± 7.9	12.0 ± 1.5	0
SP + M	176 ± 7.8	17.4 ± 3.6	0

* All plants received a *Rhizobium meliloti* inoculum.
** Estimated on a 1 (the smallest) to 3 (the largest) scale.
*** Estimated on a scale from zero (no sporocarps) to 5 (abundant sporocarps).

In summary it can be stated that mycorrhization stimulated nodulation of alfalfa by *Rhizobium meliloti* and the size of the increase was inversely correlated with the soluble P content of the soil. This effect is better shown in Figure 3.

The formation of sporocarps of *Glomus mosseae* associated with the roots, as assessed visually, was only apparent in treatments inoculated with such mycorrhizal fungi. Soluble P inhibited, and sterile conditions improved, the formation of these structures of the life-cycle of *Glomus mosseae*.

DISCUSSION

The known fact that VA mycorrhiza assist their host plants to extract phosphate from P-deficient soils, has a striking practical importance in the situations reported in this paper. The inoculation with VA fungi improved growth, nutrition and nodulation of *Medicago sativa* in all cases, but these results are more relevant in unsterile natural soils.

The main conclusion that can be deduced is that mycorrhization, enhanced by inoculation with suitable VA fungi, had a similar effect to that of the doses of soluble phosphate tested in unsterile soils.

Table 6. Macroscopic observations of microbial symbiosis with alfalfa grown 7 weeks in soil n° 9 given different treatments (7 plants/pot, 5 replicate pots/treatment)

Treatment*	Number of nodules		Sporocarps of*** *Glomus mosseae*
	Total	Size 3**	
Unsterile soil			
Control	112.2 ± 11.2	19.6 ± 2.9	0
Mycorrhiza (M)	131.5 ± 8.7	21.2 ± 2.7	1
Rock P (RP)	113.6 ± 5.9	18.0 ± 1.9	0
RP + M	124.5 ± 9.2	19.5 ± 2.1	1
Soluble P (SP)	121.6 ± 4.9	17.4 ± 1.4	0
SP + M	123.0 ± 5.6	20.0 ± 1.9	0
Sterile soil			
Control	11.2 ± 1.9	0	0
M	152.8 ± 6.4	19.0 ± 1.9	3
RP	1.6 ± 1.1	0	0
RP + M	103.6 ± 7.2	7.6 ± 2.1	1
SP	138.2 ± 4.2	10.0 ± 1.5	0
SP + M	142.8 ± 5.1	16.8 ± 2.9	0

* All plants received a *Rhizobium meliloti* inoculum.
** Estimated on a 1 (the smallest) to 3 (tha largest) scale.
*** Estimated on a scale from zero (no sporocarps) to 5 (abundant sporocarps).

The soils used in the present study are under intensive cultivation and are receiving large supplies of soluble P fertilizers to maintain their level of fertility. However, probably because of their high calcium and clay content, the phosphate became fixed very rapidily[12]. Consequently, the total P is high and the available P relatively low. In spite of the fixing-capacity of the soils, the plant-available P content in SP treatments was high at transplanting (Table 2). This level of soluble P was able to depress mycorrhization, but shoot dry weight and nodulation were not inhibited. These responses can be explained in terms of the 'critical P concentration'[19], since, as has been described, there are plant P concentrations that are supraoptimal for VA infection but not for plant growth[3]. Although in SP treatments the P content was high, it probably was not supraoptimal for growth since mycorrhization (SP + M) improved yield. It is, clear, therefore, that the similar effects of M and SP treatments in unsterile soils were not the result of depressed plant growth in the SP treatment induced by supraoptimal plant P concentrations.

These results were not reproduced in sterile soils. This, is due, probably, to the effect of the native endophytes in unsterile soils, as can be deduced from calcu-

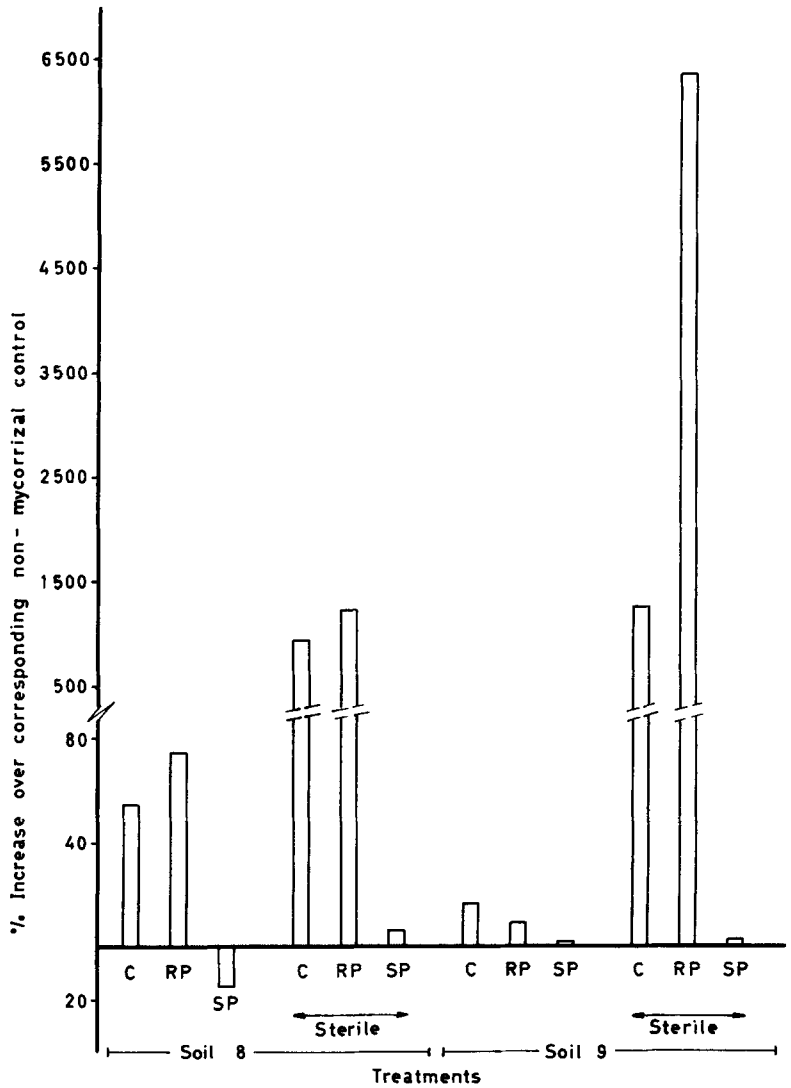

Fig. 3. Effect of introduced and native VA endophytes on number of nodules developped on *Medicago sativa* grown in phosphate-fixing soils affected by P fertilizers. All plants received a *Rhizobium meliloti* inoculum. Conventions as in Fig. 1.

lations of growth increases summarized in Table 7. These data suggest that indigenous and introduced endophytes cooperated.

Thus, the establishment of the introduced endophytes was good, and they coexisted with the indigenous ones forming mixed infections that, sometimes, were easily distinguished in the same root. It is remarkable that plants responded to introduced endophytes even in the presence of infective and active native VA fungi (Figure 2 and Table 4).

It is obvious that the introduction of efficient endophytes that cooperated with indigenous VA fungi, might lead to the impoverishment of soils after several harvests, unless phosphate fertilizers were added. One way of restoring the phosphate stock is the rational supply of soluble P at such doses and stages of the cultivation that allows cooperation with VA endophytes, but this needs further study. Nevertheless, this alternative is uneconomical in phosphate-fixing soils like ours, because a great part of the soluble P added becomes fixed rapidly. Another option to maintain the benefit of inoculation and to keep up soil fertility, would be the use of less soluble forms of phosphate fertilizers. The effect of rock phosphate was assayed here, but there was little indication that VA inoculation improved its availability in these neutral-alkaline soils. This is not unexpected for soils with such a pH[20,21,22,28,29,31], but this kind of phosphate fertilizer might be suitable to maintain the stock of phosphate in soil; in addition, it does not reduce the level of mycorrhizal infections as soluble P does. Since VA mycorrhizal plants also take up their P from the plant available phosphate fraction, and this pool of labile P is restored by chemical dissociation of phosphate ions, it might be speculated that even rock phosphate could be a useful substrate for that, even in high pH soils.

It is clear that nodulation by Rhizobium depends on an adequate mycorrhization or available P supply. Our results corroborate other studies (see[6,14,20,22,26]). Mosse *et al.*[20] suggested that nodulation was negligible when plant P concentration was below 0.2 per cent. This agrees with the results reported here, particularly with the number of size 3 nodules (the largest).

Hence, it can be concluded that legume inoculation with VA endomycorrhizal fungi in phosphate-fixing soils was successful, since it not only improved plant growth and nutrition, but also enhanced the activity of Rhizobium applied as inoculant.

Field inoculation with VA mycorrhiza may, therefore, be worth trying, particularly in legumes, since as it was stated by Harley[11] plants with dual symbiotic associations possess both nutritional and ecological advantages to compensate nutrient-deficient situations, and the establishment of these association can be

Table 7. Growth increases in *Medicago sativa* produced by native or inoculated VA endophytes. (refer to Tables 3 and 4)

Effect of VA endophytes	Percent growth (shoot dry weight) increase	
	Soil 8	Soil 9
1) Native (C unst. *vs* C st.)	33.0	60.5
2) Inoculated (M st. *vs* C st.)	253.2	98.8
Native + Inoculated		
Calculated (1 + 2)	286.2	159.3
Experimental (M unst. *vs* C st.)	296.3	168.9

C = uninoculated control; M = Mycorrhiza inoculated; unst. = unsterile soil; st. = sterile soil.

improved by inoculation with its mutualistic partners Rhizobium and mycorrhizal fungi.

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