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FIELD INOCULATION OF *MEDICAGO* WITH V–A MYCORRHIZA AND *RHIZOBIUM* IN PHOSPHATE-FIXING AGRICULTURAL SOIL

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Summary—Field inoculation of *Medicago sativa* with its symbiotic partners *Rhizobium meliloti* and the endomycorrhizal fungus *Glomus mosseae* was assayed under standard agricultural conditions in untreated arable phosphate-fixing soil. *Glomus mosseae* was successfully introduced and efficiently stimulated plant growth, N incorporation and P uptake. In contrast with a previous filed experiment, *Rhizobium meliloti* was also effective when inoculated alone. The dual inoculation of *Rhizobium* + *Glomus* more than doubled yield compared to an uninoculated control.

INTRODUCTION

Inoculation of plant rhizospheres with soil microorganisms to improve crop yields has been a subject of much controversy. In this respect, free-living microorganisms were assayed, but in general, with a lack of success (Brown, 1974). But, field inoculation with microorganisms able to form symbiotic associations with plants seems to be feasible in specific circumstances. This is the case with *Rhizobium*, the use of which is recognized world wide, and endomycorrhizal fungi, which have received less attention under field conditions.

In a previous report (Azcón-G. de Aguilar *et al.*, 1979) we described field inoculation of *Medicago sativa* with its symbiotic partners *Rhizobium meliloti* and the endomycorrhizal fungus *Glomus mosseae* under agricultural conditions in an untreated arable soil. We showed that inoculation with *Glomus* was always effective in promoting plant growth and nutrition, but *Rhizobium* was only able to enhance growth of *Glomus*-inoculated plants. As is well known, an adequate phosphate supply is needed for growth, nodulation and N_2 fixation in legumes (van Schreven, 1958; Gates and Wilson, 1974) and probably P was the limiting factor for *Rhiziobium* activity because of the scarcity of available phosphate and the low number of propagules of the Endogonaceae in our test soil. Hence, plants did not respond to *Rhizobium* inoculation unless they were also inoculated with VA fungi. This agrees with other reports that describe the role of endomycorrhizal endophytes in improving growth and nodulation of legumes (Crush, 1974; Daft and El-Giahmi, 1974, 1976; Mosse *et al.*, 1976; Powell, 1977a,b; Smith and Daft, 1977; Abbott and Robson, 1977; Mosse, 1977a,b; Hayman, 1977; Carling *et al.*, 1978; Daft, 1978; Sparling and Tinker, 1978; Powell, 1979; Waidyanatha *et al.*, 1979; Smith *et al.*, 1979; Azcón-G. de Aguilar *et al.*, 1979).

A new field inoculation experiment was designed with the aim of extending our previous work. It was done in other plots of the same soil which differed from the ones used before in factors known to influence plant responses to microbial inoculation: for example, number and effectivity of Endogonaceae propagules and its phosphate status (Mosse, 1977b; Powell and Daniels, 1978; Powell, 1977b).

Positive responses to mycorrhizal inoculation are to be expected mainly when plants are pre-inoculated before transplanting (Sanders and Hayman, 1977), but, for same crops, it would be more realistic, from the agricultural point of view, to apply the inoculum directly with the seeds at sowing so we tested this procedure.

As alfalfa plants are usually harvested by serial cutting, and because they regrow after cutting, the persistence of the effect of endosymbionts in a second harvest was also studied.

MATERIALS AND METHODS

Soil

The experiment was done in the irrigated calcareous field soil used before (Azcón-G. de Aguilar *et al.*, 1979), but in plots that had been amended with farmyard manure and then being used to grow vegetable crops for 3 months. Once these were harvested, the soil was rotavated to initiate the microbial inoculation experiment. The analytical characteristics of the experimental plots which differed from those of the ones previously used are shown in Table 1.

The infection potential of the field soil in the experimental plots was estimated in two different ways: the density of Endogonaceae spores, and the level of infection reached by the test plant grown in the glasshouse in soil cores taken from the plots. Both tests were carried out since the number of Endogonaceae spores gives only partial information of the potential infectivity of the soil (Mosse, 1978).

The Endogonaceae spore population

Three bulked samples, each consisting of several sub-samples obtained at random from the top 15 cm

Table 1. Analytical characteristics of the test soil

pH (water)	Organic matter (%)	Total P (ppm)	Soluble P* (ppm)
7.4 (7.8)	1.94 (1.74)	1164 (611)	17.6 (9.2)

In parenthesis analytical data of the test soil used in the field experiment of Azcón-G. de Aguilar *et al.* (1979).
* 0.5 M NaH CO_3 soluble P (Olsen *et al.*, 1954).

of soil, were examined. Spores were recovered from 100 g portion of each thoroughly mixed-sample by wet sieving and decanting (Gerdemann and Nicolson, 1963). The fractions retained on 100 μm and 250 μm mesh were resuspended in water, spread on a counting dish and the spores and sporocarps were identified (Mosse and Bowen, 1968) and counted under a dissecting binocular microscope.

Pot experiment using soil cores

To determine the natural infectivity of the test soil and the advantage of mycorrhizal inoculation, a glasshouse experiment was carried out with *Medicago sativa*, the test plant to be used for the field trial. The plants were grown for 8 weeks in pots containing the test soil, collected from the experimental plots in the field as described by Bell and Nutman (1971). An inoculum of *Glomus mosseae* was applied to the seedlings in a series of these pots, to compare its effects with those of the natural endophytes. Two measures of mycorrhizal infection were recorded, per cent infection and "mg of root infected" (Azcón-G. de Aguilar and Barea, 1978), in samples (more than 100 pieces of root per replicate) of the stained root system (Phillips and Hayman, 1970). Nodules were counted and plant growth and the N and P uptake estimated (Barea *et al.*, 1975).

Field trial: host plant and endophytes

Medicago sativa L. cv. Aragon, was the test plant in the field experiment and the endophytes used were endomycorrhizal fungi with yellow vacuolate-spores (Mosse and Bowen, 1968), probably synonymous with *G. mosseae* (Gerdemann and Trappe, 1974) and *Rhizobium meliloti* 203, isolated in this laboratory.

There were four inoculation treatments: Uninoculated control (*C*), *Rhizobium* (*R*), *Glomus* (*G*) and *Rhizobium* + *Glomus* (*R* + *G*). The rhizobial inoculum was a suspension of *R. meliloti* (10^6 cells ml^{-1}) and the mycorrhizal one consisted of a mixture of spores, hyphae and infected root segments of the VA endophyte.

Design of the experimental field plots

The experimental field was divided into four general plots: *C*, *R*, *G* and *R* + *G*, each one consisted of five replicate 1 m^2 microplots, designed for statistical analysis. Each microplot received 40 groups of seeds (10 to 12 seeds per group).

Seeds in the *G* and *R* + *G* treatments were sown above a pad of mycorrhizal inoculum. Ten ml of the rhizobial culture were applied to the seeds in the *R* and *R* + *G* treatments.

Table 2. Population of Endogonaceae spores in the test field soil

Spore types	Number of spores per 100 g soil
Yellow vacuolate*, *Glomus mosseae*†	56 ± 4.5 (14)
Laminate* but probably synonymous with *Glomus macrocarpus var. geospora*†	10 ± 3.1 (2)
Unidentified	9 ± 6.0 (8)
Total Endogonaceae propagules	75 ± 9.3 (24)

* Mosse and Bowen (1968).
† Gerdemann and Trappe (1974).
In parenthesis spores counted in the field experiment of Azcón-G. de Aguilar *et al.* (1979).

Plants were irrigated by field staff in the usual way, and no fertilizers were applied during the experiment.

Harvest and measurements

Seeds were sown on 9 October and plants were harvested for the first time after 25 weeks of winter growth. The plants were harvested by cutting shoots 1 cm above soil level. Upon regrowth the second harvest was made after 10 weeks of spring growth.

The dry weight of the plant shoots from each of the replicate microplots were recorded separately and the shoots analyzed for N, P and K (Barea *et al.*, 1975).

RESULTS

The population of Endogonaceae spores found in the experimental soil is shown in Table 2. It is clear that the yellow vacuolate-spore type predominated. In spite of the infectivity of the test soil the introduction of *G. mosseae* enhanced the degree of infection and improved plant growth (Table 3).

Tables 4 and 5 record the effects of the inoculation treatment on plant growth and nutrition in the field.

In the first harvest (Table 4) all inoculation treatments significantly increased plant growth. Although

Table 3. Infectivity of the test soil and additional effects of inoculation with *Glomus mosseae* on the formation of and responses to microbial symbiosis with *Medicago sativa* plants grown in soil cores in the glasshouse for 8 weeks

Parameter	Infective test soil Control*	Infective test soil *Glomus*-inoculated†
% VA infection	39 ± 5	61 ± 9
"mg root VA infected" $plant^{-1}$	107 ± 5	187 ± 12
No. nodules $plant^{-1}$‡	10 ± 1	16 ± 2
Shoot dry weight (mg) $plant^{-1}$	53 ± 3	96 ± 6
% Shoot P	0.28	0.31
% Shoot N	3.4	3.3

* Effects of the indigenous endophytes.
† Effects of the indigenous + introduced endophytes.
‡ *Rhizobium* was not added.

Table 4. Dry weight and N, P and K content of *Medicago sativa* plants given different microbial treatments. Field experiment, 1st harvest

Inoculation treatments	Shoot dry weight (g)*	Content (% dry matter) N	P	K
Control (*C*)	74.9 ± 4.5	3.88 ± 0.25	0.32 ± 0.03	2.22 ± 0.17
Rhizobium (*R*)	171.5 ± 12.7	3.54 ± 0.36	0.32 ± 0.04	2.21 ± 0.18
Glomus (*G*)	119.1 ± 13.8	4.63 ± 0.36	0.35 ± 0.01	2.86 ± 0.16
R + *G*	199.9 ± 15.4	4.57 ± 0.31	0.36 ± 0.03	2.98 ± 0.18

* Each replicate consisted of 400 plants.

Table 5. Dry weight and N, P and K content of *Medicago sativa* plants given different microbial treatments. Field experiment, 2nd harvest

Inoculation treatments	Shoot dry weight (g)*	Content (% dry matter) N	P	K
Control (*C*)	325.7 ± 42.7	2.52 ± 0.16	0.16 ± 0.01	2.44 ± 0.06
Rhizobium (*R*)	611.8 ± 48.3	2.74 ± 0.10	0.18 ± 0.01	1.91 ± 0.05
Glomus (*G*)	495.9 ± 31.3	2.88 ± 0.17	0.22 ± 0.02	2.17 ± 0.17
R + *G*	725.7 ± 51.8	3.13 ± 0.22	0.21 ± 0.01	2.33 ± 0.10

* Each replicate consisted of 400 plants.

the best treatment was *R* + *G*, there were no significant differences between *R* + *G* and *R*. It is noteworthy that plants inoculated with *Glomus* (*G* and *R* + *G* treatments) possessed higher N, P and K content than uninoculated control or *R* treatments.

At the second harvest (Table 5) it is obvious that the effects of the introduced endophytes persisted. The dual inoculum (*R* + *G*) was significantly more effective than any other treatment.

DISCUSSION

Our results support the feasibility of field inoculation with efficient mycorrhizal fungi and *Rhizobium*.

It is accepted that the introduction of VA endophytes seems more likely to be successful where the indigenous ones are sparse or inefficient (Mosse, 1977b; Sanders and Hayman, 1977). In this respect, the number of Endogonaceae spores in the field plots was low, but fall in the range of 10 to 500 per 100 g soil recovered in most cases (Hayman, 1975; Hayman *et al.*, 1975). However, the soil can be said to possess "high infectivity" (Mosse, 1977b). In spite of this, *G. mosseae* was successfully introduced as indicated by the results from the glasshouse experiment; the introduced VA endophyte being also more efficient than the indigenous ones. The effectiveness of *G. mosseae* was also corroborated in the field trial, where these fungi swiftly became established, survived and infected the alfalfa plants growing in the field. This can be deduced from the increase in shoot growth and nutrition that the introduced fungus caused when inoculated below oversown seeds.

The efficiency of indigenous VA fungi, together with the concentration of available P in the soil, could cause plants to respond to *Rhizobium* inoculated alone. This effect was not found in our previous field experiment (Azcón-G. de Aguilar *et al.*, 1979) in which plants did not respond to *Rhizobium* inoculation unless they were also inoculated with *Glomus*. Results recorded in Tables 1 and 2 support the differing behaviour of *Rhizobium* when applied to plants grown in these soils.

The agricultural field soil used is receiving large amounts of P fertilizers, but because of its high pH and clay content, it fixes the phosphate at a high rate (Barea *et al.*, 1980). These facts could condition the establishment and response to introduced endophytes, since: (1) Fertilizer application can depress the number of spores in soil (Hayman, 1975), so lowering the natural infectivity potential; (ii) the introduced endophytes seem to be more tolerant of added fertilizers, as has been suggested by Mosse (1977b); (iii) because of the phosphate-fixing capacity of the soil, its available P content, in spite of the phosphate supplies, was not high enough to interfere with mycorrhiza formation (Azcón *et al.*, 1978). Finally, the spore type used as inoculant (yellow vacuolate of Mosse and Bowen, 1968) was probably ecologically adapted to the habitat (Table 2).

The effect of *Glomus* in increasing the % N in the plants (Table 4) is striking. Whether *Glomus* acted by enhancing nodulation, or nitrogenase activity in legume nodules (Azcón-G. de Aguilar *et al.*, 1979) or by stimulating the number and activity of free-living N_2-fixers (Bagyaraj and Menge, 1978) was not tested, but all of these hypothesis could account for the above-mentioned effects.

In conclusion, the results of these field trials show that *Glomus mosseae* + *Rhizobium* is an efficient "biological fertilizer" that helps legumes obtain their nutrients when grown in phosphate-fixing soils.

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