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EFFECTIVENESS OF *RHIZOBIUM* AND VA *MYCORRHIZA* IN THE INTRODUCTION OF *HEDYSARUM CORONARIUM* IN A NEW HABITAT

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ABSTRACT

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We have conducted some experiments aimed to help the introduction in new habitats of *Hedysarum coronarium* L., a legume of high potential for animal nutrition. A survey was first undertaken to examine whether *H. coronarium* is nodulated and mycorrhizal in its natural growing area. Pot experiments were then set up to assess the feasibility of field inoculation in soils (new habitats) previously studied for some of their physical, chemical and biological (microbial) characteristics. Finally, a field experiment was carried out in a selected soil.

Rhizobium and the VA mycorrhizal fungus were both successfully established in the rhizosphere of *H. coronarium* growing in the field. They stimulated the yield of plant material and the nutrient uptake by the plants beyond that achieved by adding a standard dose of a compound N-P-K fertilizer.

INTRODUCTION

Microbe—plant symbiosis as with the Vesicular-Arbuscular (VA) mycorrhiza, which greatly enhance plant uptake of phosphorus, and the legume root nodules, where an efficient nitrogen fixation takes place, play a major role in contributing to plant productivity. Therefore, the potential of manipulating these associations to save energy (fertilizers) and environmental costs is being explored.

Legumes are, in general, nodulated and mycorrhizal in nature (Mosse, 1977), but the responsible *Rhizobium* and VA fungi are not always the most suitable for each given ecological situation (Bowen, 1980). Inoculation with the host-specific and effective rhizobial strains is often required, being a "sine qua non" condition in the introduction of legumes in new habitats (Postgate and Hill, 1979). Naturally occurring strains of VA mycorrhizal fungi can also be replaced with more competitive and effective microorganisms by means of inoculation (Mosse, 1977).

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Methods for selecting suitable *Rhizobium* strains and for preparing inoculant for the corresponding legume have been developed (Thompson, 1980), but the inoculation of VA mycorrhizal fungi has received less attention because of difficulties in obtaining suitable inocula (Hayman, 1980).

Legumes are known to have high phosphorus requirements (Postgate and Hill, 1979), and mycorrhizal inoculation has been demonstrated to improve growth, nodulation and nitrogen fixation. A great number of papers (see Mosse, 1977; Smith and Daft, 1977; Asimi et al., 1980; Azcón-Aguilar and Barea, 1981, for references) describe these effects as deduced from glasshouse experiments carried out by growing legumes in pots, but few field assays have been developed (Azcón-G. de Aguilar et al., 1979; Powell, 1979; Hayman and Mosse, 1979; Azcón-Aguilar and Barea, 1981).

The present paper deals with field inoculation work to study the introduction in new habitats of a legume having a high nutritional potential for animal feeding. *Hedysarum coronarium* ("sulla") grows in limited areas of the mediterranean region (Pascual, 1978) and it seems to form a symbiosis with a *Rhizobium* sp. (Castelli, 1964) that is unable to nodulate other legumes; conversely, all the known species of the genus *Rhizobium* cannot nodulate sulla (Cabrera et al., 1979). We decided to examine whether mycorrhizal inoculation can aid the establishment of this legume and its nodulation by the specific *Rhizobium* sp.

MATERIALS AND METHODS

Survey of the natural growing area of Hedysarum coronarium

A survey was undertaken to assess if *Hedysarum coronarium* L. ("sulla") was nodulated and mycorrhizal under natural conditions in the Cadiz district of Southern Spain where this legume grows. Four sites were selected representing hill pastures, undisturbed areas populated by long-established vegetation and eroded banks. Four or five samples of sulla roots were randomly collected from each of the selected locations.

Experimental test soils (new habitats)

Two soils in Granada province, Spain, were tested for the introduction of the new legume *Hedysarum coronarium*. Table I gives a summary of the analytical characteristics of these test soils. The mycorrhizal endophyte content was estimated by the technique of wet sieving and decanting. For each soil three bulked samples consisting of several subsamples obtained at random from the top 15 cm of soil, were examined. Roots retained on the 700 μm sieve were removed, cleared and stained for mycorrhizal infection (Phillips and Hayman, 1970) and the percent root length occupied by the fungus was measured by the gridline intersect method (Giovannetti and Mosse, 1980). The fractions retained on the 100 and 250 μm sieves were resuspended

in water and spores counted and identified (Mosse and Bowen, 1968) under a dissecting binocular microscope.

Pot experiment to assess the feasibility of field inoculation

Pot trials with *Hedysarum coronarium*, the test plant to be introduced, were set up to determine the natural infectivity of the two test soils (new habitats) and to check if appreciable responses could be obtained by inoculation with selected mycorrhizal fungi.

Half of each soil was steam-sterilized at 100°C for 1 h during 3 days to destroy its indigenous endophytes. The sterile and nonsterile parts of the two soils were diluted with sterile nutrient-free sand in the ratio 3 : 1 (*v/v*) and 8 replicate plots of each one of the four experimental soils were prepared. Four of the replicates were given a VA mycorrhizal inoculum which consisted of spores, hyphae and infected root segments, collected from a stock plant culture. The mycorrhizal endophyte assayed was the yellow-vacuolate spore type (YV) (Mosse and Bowen, 1968), a form of *Glomus mosseae* (Gerdemann and Trappe, 1974). This inoculum was applied to the planting hole in the corresponding pot. All pots received a standard inoculum of the specific *Rhizobium* sp.

Two-day-old seedlings of *Hedysarum coronarium* were transplanted into the pot holding 250 g of the appropriate experimental soil and the corresponding microbial inocula. Three plants per pot were grown for 10 weeks in a glasshouse at 19—25°C, watered from below and fed with Long Ashton nutrient solution (Hewitt, 1952) lacking N and P.

At the end of the experiment, fresh weights of roots and shoots were recorded and, after carefully washing the roots, the number of nodules was assessed visually and the mycorrhizal infection estimated in samples of the stained root systems as before.

Field trial in the selected soil

The assay was carried out in soil number 10 (Table I). There were two types of treatments (fertilizers) and an untreated control, to study the effect of the natural fertility of the soil. One treatment (NPK) consisted of the application of a compound fertilizer supplying 112, 75 and 75 kg/ha, respectively, of N, soluble P and K. The other treatment (RM) was the "biological fertilizer" consisting of a mixture of the mycorrhizal and rhizobial inocula as assayed before. The mycorrhizal inoculum consisted of 5 g of soil and root debris from the stock cultures and the rhizobial one was 1 g of a standard peat inoculum containing about 10^7 cells g^{-1}. This mixture was placed below seeds in the furrow.

There were five 2 m^2 replicates for each of the three general treatments: Nil, NPK and RM, representing, respectively, the control, the chemical and the biological treatments. Each replicate received 52 groups of *Hedysarum*

TABLE I

Analysis of the test soils (new habitats)

Soil no.	Sand (%)	Loam (%)	Clay (%)	pH (water)	Organic matter (%)	$CaCO_3$ equiv. (%)	$CaCO_3$ activ. (%)	Total N (ppm)	Total P (ppm)	Total K (ppm)	Soluble P (ppm)[a]
10	21.2	34.7	44.1	7.4	1.39	22.8	12.5	1197	618	280	5.6
11	31.6	42.8	25.2	7.2	2.17	22.2	2.1	1645	1557	311	33.0

[a]0.5 *M* $NaHCO_3$ soluble P (Olsen et al., 1954).

TABLE II

Mycorrhizal infectivity of the test soils and additional effects of inoculation with *Glomus mosseae* in the formation of and responses to microbial symbiosis with *Hedysarum coronarium* plants grown in the glasshouse

	Soil no. 10				Soil no. 11			
	Sterile		Nonsterile		Sterile		Nonsterile	
	Control	Mycorrhiza	Control	Mycorrhiza	Control	Mycorrhiza	Control	Mycorrhiza
% VA infection[a]	0	57 ± 7	20 ± 5	74 ± 11	0	3 ± 2	52 ± 10	60 ± 6
Nodulation[b]	2	4	2	4	3	4	3	4
Shoot weight (g)[a]	2.1 ± 0.2	3.8 ± 0.1	2.5± 0.2	4.1± 0.3	4.1 ± 0.3	4.2 ± 0.7	5.0 ± 1.0	4.9 ± 1.0
Root weight (g)[a]	2.7 ± 0.2	3.5 ± 0.7	2.4± 0.5	3.3± 0.4	2.1 ± 0.4	2.1 ± 0.2	2.3 ± 0.4	2.3 ± 0.8

[a]Mean value ± confidence limit at 5% level of significance.
[b]Estimated on a scale from 0 (no nodules) to 4 (abundant nodulation).

coronarium seeds (about 20 seeds per group). Plants were irrigated by the farmers in their usual way.

The experiment was set up on 1 April and after 12 weeks of growth, plants were sampled for leaf analysis to evaluate their nutritional status. The usual precautions to obtain representative samples for plant tissue analysis (Ulrich, 1978) were observed and young mature leaves taken from 10 to 20 plants per replicate were selected according to this author. After a further 3 weeks of growth, plants were harvested by cutting shoots 1 cm above soil level. The dry weight of the plant shoots from each of the replicates was recorded separately and the shoots were analyzed for N and P (Barea et al., 1975).

At harvest, the amount of VA mycorrhizal infection and the nodulation of the plants grown in natural conditions in the new habitat were estimated as before, as a measure of the establishment of the introduced endophytes.

RESULTS AND DISCUSSION

The survey of the natural growing area of *Hedysarum coronarium* showed that all the root samples were mycorrhizal and possessed rhizobial nodules. The indigenous VA mycorrhizal infection occupied about 50—75% of the feeder roots. The typical VA fungal elements (coarse intercellular aseptate hyphae, hyphal coils, vesicles, arbuscules, clumps of external mycelium bearing spores) were easily shown in most samples of root systems.

The soils used in the present study to investigate their possible use as new habitats for *Hedysarum coronarium* have been under intensive cultivation and had received large doses of fertilizers. This is reflected in the soil analysis data (Table I). Although the total P is high, the available P is quite low in soil no. 10, probably because the P fertilizer added became fixed as a consequence of the high pH, Ca and clay content of this test soil (Hayman, 1975a). The soils also differ in their native VA endophyte population. Soil no. 10 had a higher number of spores (80 vs. 35 per 100 g soil) and mycorrhizal infection (50% vs 17%) than soil no. 11. This could be related to the lower available phosphate content in soil no. 10 (Hayman, 1975a). However, both soils possess a fairly low endophyte population and this is probably due to the fertilizer application these soils received in the past, treatments that, as is known, can depress the number of spores in soil (Hayman, 1975b). *Glomus mosseae* was the dominant spore species and it was chosen as the mycorrhizal inoculant.

The results of the glasshouse experiment to assess the feasibility of the inoculation are summarized in Table II. It is obvious that *Glomus mosseae* was successfully introduced into *Hedysarum coronarium* rhizosphere in soil no. 10, increasing, through its activity there, plant growth and nodulation under either sterile or nonsterile conditions. These results, therefore, show that field inoculation with the selected endophytes may be worth trying in soil no. 10. In contrast, *Glomus mosseae* was not successfully introduced in soil no. 11, probably because of the level of available phosphate (Table I). Con-

sequently there are no growth increases as a response to *Glomus* inoculation. However, the indigenous endophytes possess high infectivity, being most adapted to the environmental conditions of the soil, mainly to its available P content, but these did not produce significant growth increases. This corroborated the statement by Bowen (1980), "the trend of evolution has been for survival, not high productivity. . .". Hence, soil no. 11 was not selected to investigate field inoculation.

The typical modified roots of sulla, the "palette" as described by Castelli (1964), were observed. These are mycorrhizal but do not bear rhizobial nodules.

Tables III and IV record the effect of the chemical and biological treatments on growth and nutrition of *Hedysarum coronarium* in the field (soil no. 10). Table III shows the results of the leaf analysis carried out for evaluating the nutritional status of growing plants and Table IV shows the yield data after harvest.

Results in Table III indicate that plants inoculated with *Rhizobium* + mycorrhizal fungus *Glomus mosseae* possessed in their leaves the highest N, P and K content. In addition, the leaf mineral composition of these plants was the best balanced. The effect of *Rhizobium* on the leaf concentration of nitrogen is striking.

A growth response to microbial inoculation was also clearly evident at harvest (Table IV). In fact, inoculated plants were significantly the heaviest, having the highest N and P content in their shoots. That the introduced endophytes established themselves inside plant roots in the field was deduced from the data of Total % VA infection after harvest, which were: Control = 39%; NPK = 20% and RM = 61%, on average. There was no nodulation unless the specific *Rhizobium* was inoculated. The natural mycorrhizal infection was depressed by the application of chemical fertilizer, corroborating previous statements deduced from field observations (Hayman, 1975b).

The results of the field trial show that the "biological fertilizer" used was

TABLE III

Effect of chemical and biological fertilizers on mineral composition of leaves of *Hedysarum coronarium* growing in the field soil no. 10

Nutrient	Leaf content (% dry matter)[a]		
	Control (Nil)	Chemical (NPK)	Biological (RM)
N	1.81 ± 0.32	2.67 ± 0.29	3.30 ± 0.16
P	0.20 ± 0.03	0.25 ± 0.02	0.26 ± 0.01
K	1.00 ± 0.07	1.27 ± 0.17	1.37 ± 0.16
Ca	2.28 ± 0.22	2.35 ± 0.25	2.23 ± 0.16
Mg	0.70 ± 0.05	0.64 ± 0.05	0.68 ± 0.07

[a]Mean value ± confidence limit at 5% level of significance.

TABLE IV

Effect of chemical and biological fertilizers on the yield and nutrition of *Hedysarum coronarium* grown in the field soil no. 10

Treatments	Shoot dry weight[a]	Shoot content (% dry matter)[a]	
(fertilizers)	(g)	N	P
Control (Nil)	805 ± 30	2.40 ± 0.26	0.23 ± 0.03
Chemical (NPK)	903 ± 44	2.58 ± 0.12	0.23 ± 0.02
Biological (RM)	1151 ± 102	3.64 ± 0.13	0.30 ± 0.01

[a]Mean value ± confidence limit at 5% level of significance.

quite efficient at helping the legume *Hedysarum coronarium* to obtain its nutrients when grown in phosphate-fixing agricultural soils.

Thus, such microbial inoculation can reduce the chemical fertilizers input to this legume crop, thereby saving energy and environmental costs.

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