SYMBIOTIC EFFECTIVENESS AND ECOLOGICAL CHARACTERIZATION OF INDIGENOUS *RHIZOBIUM LOTI* POPULATIONS IN URUGUAY[1]

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ABSTRACT - The objectives of this work were to describe the distribution, density and seasonal variation of the indigenous populations of *Rhizobium loti* in different Uruguayan soils and to determine the symbiotic effectiveness and stress tolerance factors of different isolates, both with the aim of obtaining selected strains to re-introduce as inoculants in *Lotus* pastures. *R. loti* was present in ten soils studied and their densities varied from year to year and within each soil. All the isolates nodulated *Lotus corniculatus* effectively. The nodules in *Lotus pedunculatus* and *Lotus subbiflorus* were small, red on the surface and ineffective in nitrogen fixation. The study of 50 isolates from the ten soils showed high variability in their symbiotic efficiency and tolerance to pH. The indigenous population was acid tolerant in culture medium (pH 4.5), 83% of them could grow at pH 4.5 in 3 days. This work showed that there was a great diversity between the strains of *R. loti* isolated from Uruguayan soils and supports the importance of selecting among them the most efficient and resistant strains to be included in the inoculants.

Index terms: *Lotus corniculatus*, *Lotus pedunculatus*, rhizobial pH tolerance.

EFICIÊNCIA SIMBIÓTICA E CARACTERIZAÇÃO ECOLÓGICA DE UMA POPULAÇÃO NATIVA DE *RHIZOBIUM LOTI* NO URUGUAI

RESUMO - Os objetivos deste trabalho foram descrever a distribuição, densidade e variação sazonal de populações nativas de *Rhizobium loti* em diferentes solos uruguaios, e determinar a eficiência simbiótica e os fatores de tolerância a estresse de diversos isolados, tendo em vista a obtenção de cepas a serem reintroduzidas como inoculantes em pastagens de *Lotus*. *Rhizobium loti* estava presente em dez solos estudados, e suas densidades variavam de ano para ano e em cada solo. Todos os isolados nodularam efetivamente o *Lotus corniculatus*. Os nódulos no *Lotus pedunculatus* e no *Lotus subbiflorus* eram pequenos, vermelhos na superfície, e ineficazes na fixação de N. O estudo de 50 isolados dos dez solos mostraram alta variabilidade na sua eficiência simbiótica, e alta tolerância ao pH. A população nativa era tolerante à acidez em meio de cultura (pH 4,5); 83% dela pode crescer em pH 4,5 em três dias. O presente trabalho mostrou que há grande diversidade entre as cepas de *R. loti* isoladas de solos uruguaios, e acentua a importância de se selecionar entre elas as mais eficientes e resistentes, para serem incluídas como inoculantes.

Termos para indexação: *Lotus corniculatus, Lotus pedunculatus,* tolerância do rizóbio ao pH.

INTRODUCTION

The activity of native *Rhizobium* population is of great importance, either when a new strain is introduced from an inoculant or as a factor conditioning the persistence of the legume year by year. Inability of the inoculant strains to successfully compete with established *Rhizobium* populations in soil has been frequently observed (Van Rensburg & Strijdom, 1985; Triplett & Sadowsky, 1992). As concluded by Roughley et al. (1976) and Vance & Graham (1995), the success of an inoculant decreases with increase of native rhizobia population in soil. Furthermore native rhizobia form the highest number of nodules (Rupela & Sudarshana, 1990).

Lotus corniculatus was introduced in Uruguay many decades ago, especially for production on soils

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of low and medium fertility (Carambula, 1977). In farming *Lotus* is inoculated with strain U-226. *L. pedunculatus* and *L. subbiflorus* are especially adapted to acid soils (Lowther et al., 1987). *L. corniculatus* and *L. tenuis* are nodulated by fast growing *Rhizobium loti* (Jarvis et al., 1982) and *L. pedunculatus* and *L. subbiflorus* form nodules with *Bradyrhizobium* sp. (*Lotus*). Nevertheless specificity is not yet well defined in the genus *Lotus.*

Bromfield et al. (1986) and Vance & Graham (1995) emphasize the importance of understanding the ecology and characteristics of indigenous rhizobia populations as a prerequisite for inoculant establishment and persistence. Characteristics such as: antibiotic resistance (Mueller et al., 1988), fungicide resistance (Curley & Burton, 1975), low pH tolerance (Munns & Keyser, 1981; Wood et al., 1988), drought and high temperatures tolerance (Munevar & Wollum II, 1981; Graham, 1992), bacteriocin production (Schwinghamer & Brockwell, 1978) are considered in the selection of rhizobial strains with high adaptability in regions with adverse conditions. These properties could explain their saprophytic persistence (Frioni, 1990, 1999).

The objectives of this paper are to describe the distribution, density and seasonal variations of the indigenous populations of *Rhizobium loti* in different soils; to determine the specifity and level of efficiency of these populations and to obtain selected strains with ecological adaptation in order to introduce them as inoculant into soils with less effective populations.

MATERIAL AND METHODS

MPN of rhizobia and efficiency of indigenous populations

Ten soils with different physical and chemical properties were selected in areas with no previous history of legume cultivation (Table 1). The samples were taken from the upper 10 cm surface soil without plant cover. The MPN of *R. loti* was determined with seedlings of *L. corniculatus* growing in tubes with Jensen's medium (Vincent, 1970), incubated at 23ºC and with a photoperiod of l6 hours. Besides the soils, three other treatments were included: (C) control without inoculation or fertilizer, (N) with 70 mg/kg of N as KNO_3 and (I) inoculated with an efficient strain, U-226 , which is the original B-8l6 from the Department of Microbiology, Kensington, Australia, and commercially used in inoculant production.

During the spring and fall samples, *R. loti* densities were compared with *R. leguminosarum* bv. *trifolii* forming nodules in *Trifolium repens* seedlings.

After 6 weeks the MPN per gram of soil was determinated and after 8 weeks information on the efficiency of the isolates of indigenous population was obtained by determining seedling biomass in tubes with a similar *Rhizobium* density.

Rhizobia were isolated from the biggest nodules using yeast extract mannitol agar medium (YEM) and maintained in the same medium with 20% glycerol at -25ºC (Somasegaran & Hoben, 1985).

Symbiotic efficiency

Tubes with Jensen's medium and seedlings of *L. corniculatus*, *L. pedunculatus* and *L. subbiflorus* were inoculated with 10^3 cells /tube with isolates from the soils and strain U-226 in order to define the range of specificity. Data of dry weight of shoots, number and weight of nodules per plant were analyzed by the F test and the averages by the Tukey test (Snedecor & Cochran, 1977). The relative efficiency index (Brockwell et al., 1966) measure the accumulation of N fixed in relation to the controls, and defined as REI = (T - C/ N-C) X 100, where T, C and N = dry weight of shoots in each treatment, in control and in nitrogen treatment, respectively.

Tolerance to low pH

The Wood & Cooper (1985) technique using liquid medium and recording the days until the appearance of net turbidity was employed. The work was done in the range of pH 3.5 to 6.8.

Bacteriocin production

The production of these intraspecific inhibitor substances was evaluated by measuring the inhibition zones (cm) after 7 days of growth in a double layer system. Each strain was sown with a multiple replicator in solid YEM medium and test strain was inoculated in the botton layer and treated with choloform after growth (Pugsley & Oudeg, 1987).

RESULTS AND DISCUSSION

Indigenous population of *Rhizobium loti*

All the ten soils chosen to be representative of the Uruguayan soils (Table 1) contained *R. loti.* They

TABLE 1. Characteristics of soils[1].

Soils and classification	pH(H_2O) (1/2.5)	pH (KCl 1N) (1/2.5)	OM	OrgN	P	Al^{3+}	Clay
Algorta (A) Distric Argisol	5.7	4.8	1.78	0.12	2.2	0	9.6
Estación Experim. del Este (E) Distric Planosol	5.9	4.3	1.98	0.11	1.2	0	19.0
Glencoe (G) Haplic Vertisol	5.9	4.8	7.91	0.33	2.7	0	42.7
Bañado de Medina (M1) Ruptic Vertisol	5.2	4.2	6.27	0.37	9.2	0	50.3
Bañado de Medina (M2) Sub-EutricBrunosol	5.5	4.4	4.74	0.37	6.0	0	28.1
Estación Experim. SUL (S) Distric Brunosol	5.3	4.1	3.16	0.20	1.2	0.40	23.1
Seroterápico (Se) Ruptic Vertisol	6.1	5.1	8.07	0.40	2.9	0	36.1
Suarez (Su) Sub Eutric Brunosol	5.7	4.4	4.33	0.20	0.8	0	26.1
Tacuarembó (T) Ocric Luvisol	5.2	4.2	2.68	0.14	5.4	0.46	10.1
Young (Y) Eutric Brunosol	5.9	4.9	5.77	0.34	5.0	0	36.4

[1] Methods: OM (%) - Walker & Black; N (%) - Kjeldahal; P (mg/kg) - Olsen; Al (mg/kg) - Black; Clay (%) - Bouyoucus.

nodulated *L. corniculatus* effectively, but in assays with *L. pedunculatus* and *L. subbiflorus*, the nodules formed were small, ineffective and red at the exterior as described by Pankhurst et al. (1987) and seems to be related to the flavolan content of nodulated roots of several *Lotus* species, proved to be toxic to many *R. loti* strains.

Table 2 shows the density of *R. loti* and *R. leguminosarum* bv. *trifolii* using the MPN technique. In general a higher *R. loti* population in relation to *R. leguminosarum* bv. *trifolii* was noticed. In the winter samples *R. loti* was not detected except in T soil (log =2.23). The average for the autumn season was 3.08 log units, with values from 1.57 to 5.35. The average for the spring-summer samples was 3.3 log units. The eutric Brunosol Y showed the highest *Rhizobium* densities related to the nutritional levels and textural properties.

TABLE 2. *Rhizobium loti* and *R. leguminosarum* bv. *trifolii* in soils (log_{10} Nº/g dry soil)[1].

Soils[2]	*R. loti*	*R. loti*		*R. leguminosarum*	
	Winter	Spring	Autumn	Spring	Autumn
A	0	0	3.51	2.76	2.46
E	0	0	2.01	ND	0
G	0	0	2.76	2.33	2.92
M1	0	0	3.51	0	3.92
M2	ND	0	1.57	0	ND
S	0	2.76	3.28	0	1.48
Se	ND	3.26	2.33	0	0
Su	0	2.49	3.54	0	ND
T	2.33	3.76	2.98	2.76	1.48
Y	0	4.23	5.35	4.23	4.46

[1] ND not detected; 0 not determinated.
[2] See Table 1.

Only for the *R. leguminosarum* bv. *trifolii* there was a positive correlation between their densities and organic N (%) (r = 0,65) and clay content of soils (r = 0,69). The *R. loti* densities did not correlate with soil parameters presented in Table 1. However, Woomer et al. (1988) related total rhizobial populations to many soil parameters as: temperature, soil pH and phosphorus content.

The result of the symbiotic efficiency in the indigenous population of *R. loti* in the ten soils, in the treatment with U-226 and N supply are presented in Table 3. The shoot dry weight was lower for all of the soils populations compared to the N or U-226 treatment. Only native *Rhizobium* population of soil S neither differ statistically from U-226 nor from the nitrogen treatment. Although the soil Y presented the highest rhizobia density related to their nutritional levels and textural properties it was not the most efficient (Table 3). The REI of the indigenous populations of *R. loti*, except soil S, was lower than 50%.

TABLE 3. Symbiotic efficiency of indigenous population of *R. loti*.

Soils[1]	Dry weight of shoots (mg/plant)	REI[2]	Nº nodules/ plant[3]
A	8.83b	34.10	10.5bc
E	5.80c	16.88	5.8e
G	10.45abc	48.54	8.7d
M1	9.91abc	44.86	8.9d
M2	6.08c	18.79	5.1e
S	14.95ab	79.17	13.7a
Se	7.91bc	31.24	6.5e
Su	8.60bc	35.94	9.1cd
T	8.86bc	37.71	10.9bc
Y	9.76bc	43.84	10.1cd
U 226[4]	17.79a	82.50	12.3ab
N[5]	20.95a		
F[6]	++		+
CV (%)	20.53		10.3

[1] See Table 1.
[2] Relative efficiency index.
[3] Data normalized.
[4] Commercial strain.
[5] N treatment.
[6] + and ++ = significant differences at 5% and 1% respectively.

Rhizobium loti isolates

In relation to REI of the 50 isolates of *R. loti,* 6% was between 100-119% and another 6% were located in the 0-40% range (Fig. 1). The mean of the REI of the isolates from each soil compared with the efficiency determined from the indigenous populations of the soils was shown in Fig. 2.

Isolates from five soils showed REI superior to 70% and the mean of the REI of the other five soils was 60%. REI values of the isolates in relation to REI of the indigenous population is visibly higher in all cases, except in the soils G and S. In two cases the REI of isolates was up to four times higher than that of the respective soil *Rhizobium* population.

A great variation in the REI of the isolates of the same soil was observed. REI of strains from the

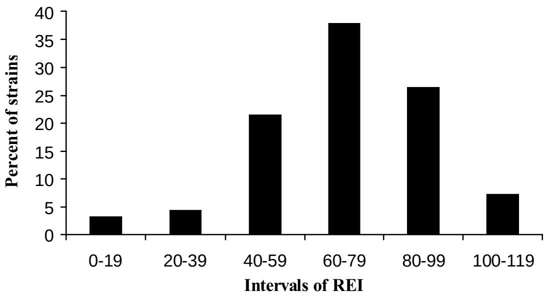

FIG. 1. Symbiotic efficiency of 50 isolates of *R. loti* as intervals of relative efficiency index (REI).

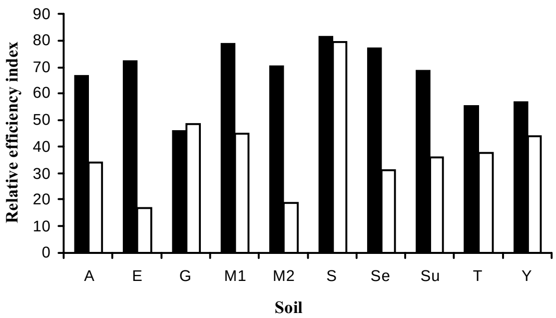

FIG. 2. Symbiotic efficiency in each soil expresed as relative efficiency index (REI) in isolates of strains (■) and indigenous populations (□) of *R. loti*.

Brunosol varied from less than 40% to 90%. Soil M1 is characterised by a high level of organic matter and phosphorus (Table 1), with a high indigenous population of *R. loti* (log = 3.51) (Table 2). The sandy soil T with low levels of organic matter and pH showed a great dispersion in REI of their isolates (15 to 93%).

The diversity in symbiotic performance of native population of *R. loti* emphasised the importance of selection of an inoculant strain for the successful establishment in soils (Bonish & Mac Farlane, 1987).

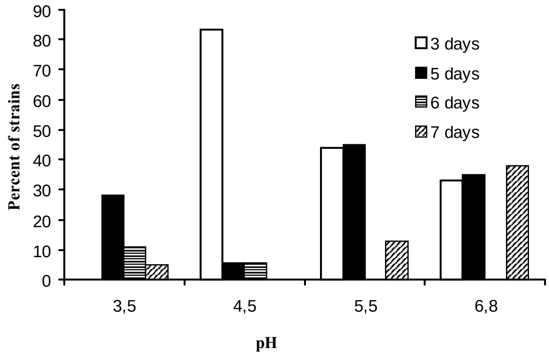

FIG. 3. Growth of *R. loti* at diferent pH.

Nodulation

In general the levels of nodulation with the isolates were of the same order as the U-226 (Table 3). The correlation indexes between nodulation and shoots dry weight were: nodules dry weight/nodule number (r = 0.48), shoots dry weigth/nodule number (r = 0.32) and shoot dry weight/nodule dry weight (r = 0.31).

Tolerance to low pH

All 50 isolates of *R. loti* grew at pH 4.5, 5.5 and 6.8 and most of them during the three days of incubation (Fig. 3). At pH 3.5, 44% of the strains grew and 28% of them did it in five days of incubation. At pH 4.5, 83% of the isolates grew in three days, at 5.5, 44%, and at pH 6.8, 33%. The U-226 strain grew at pH 4.5 and 5.5 in five days and in pH 6.8 in seven days.

Jarvis et al. (1982) stated a minimum pH for this species of 4.0. Cooper (1982) found that none of 20 strains of *Bradyrhizobium* sp. (*Lotus*) developed at pH 4.6, while three out of seven strains of *R. loti* did grow at this pH. We found a growth optimum of the population to be near pH 4.5 (83% grew in three days). These seem to be acidophilic strains. Changes in metabolic activity as a consequence of external pH could explain the growth in acid medium. Ayanaba et al. (1983) noted significant ammonia production in acid-tolerant cowpea rhizobia in medium of pH 4.5.

In more neutral pH, 73% of the strains needed more than five days for giving a net turbidity. The neutral pH revealed different behaviour between *R. loti* strains. Valdes & Martinez-Bravo (1986) found that 28% of *R. loti* strains isolated from *Leucaena* were acidophilic (pH 5.0). Laboratory studies have been effective in identifying sensitive strains to the environmental factors but field studies are important to verify the results (Blanchar & Lipton, 1986). Vargas & Graham (1989) found good correlations between growth in acidic medium and nodulation capacity in acidic soils.

Bacteriocines

Only 4% of the tested strains presented antagonistic activity: G4 against U226 with bacteriocin inhibition zone diameter of 1.5 cm and A12 against E7, 2.5 cm inhibition zone. The bacteriocin producing strains, could increase nitrogen fixation by means of the suppression of competition for nodulation sites in the native population (Hodgson et al., 1985).

CONCLUSIONS

1. The ten soils analyzed present *R. loti* populations with logarithmic densities ranged from 1.48 to 5.35 per gram. In general, a higher *R. loti* population in relation to *R. leguminosarum* bv. *trifolii* was found in the analized soils.

2. The symbiotic efficiency of 50 isolates show a great diversity in *Lotus corniculatus* and 22% of the strains accumulate significantly more dry matter than U-226 used as a inoculant.

3. Eighty three percent of the isolates can grow at pH 4.5 in three days in liquid medium and sup-

ported lower pH in relation to U-226 strain used in the inoculants production.

4. There is a great diversity between the isolates of *R. loti* from Uruguayan soils.

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