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Occurrence, Diversity and Effectiveness of Mid-Acid Tolerant Alfalfa Nodulating Rhizobia in Uruguay

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Abstract

The occurrence, diversity and symbiotic properties of mid-acid tolerant (MAT) alfalfa nodulating strains (isolates able to grow at pH 5.5 but not at pH 5.0) in Uruguay were analysed. Uruguay has an indigenous alfalfa-nodulating rhizobial population, 10% of which is tolerant to acidic conditions. Although some genotypes were found repeatedly, PCR analysis of the MAT isolates suggests that considerable diversity is present. In addition, we provide evidence that strains from acid soils are not necessarily acid-tolerant. Effectiveness performance studies showed that *Medicago* species differ not only in their ability to achieve nodulation when growing on acidic soil, but also that distinct genotypes of alfalfa have different abilities to achieve nodulation. Symbiotic analysis of the isolates carried out in pots confirms that they have the potential to improve the growth of alfalfa in acidic and neutral soils. We suggest that a population of native alfalfa-nodulating strains found in Uruguayan soils is able to establish alfalfa in acidic soils.

Keywords: Alfalfa, mid-acid tolerant, sinorhizobia

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1. Introduction

The consumption of meat and milk, and the production of leather has been increasing in the world, and the improvement of pastures to feed cattle for breeding has become an important economic resource for countries with dairy industries. However, the extent of unproductive grasslands compromises cattle production. New sustainable technologies are needed to develop and maintain suitable grassland for animal production. Nitrogen fertilisers can be used to enrich soils; but, this practice is expensive and produce many deleterious consequences for the environment (Smil, 1997).

One alternative to the use of nitrogen fertilisers is to enhance the establishment of the highly effective rhizobia-legume plant nitrogen fixing symbiosis. From an economic viewpoint, the ability of some plant-rhizobia associations to use atmospheric nitrogen, obviating the need for massive inputs of fertiliser, is a desirable trait. However, biotic and abiotic factors affect this symbiosis (Zahran, 1999; Graham, 1992). Plant response to inoculation is determined by the presence and the quality of the native rhizobial population (Singleton and Tavares, 1986), soil nitrogen availability, soil physicochemical (Howieson and Ewing, 1986) and climatic conditions (La Favre and Eaglesham, 1986). For example, soil acidity causes a decrease in rhizobia number, nodulation score, and relative yields of alfalfa in fields and greenhouse experiments. *Sinorhizobium meliloti*, in particular, is sensitive to low pH (Graham and Parker, 1964).

Biological nitrogen fixation is catalysed by nitrogenase, an enzyme composed by two proteins called Fe protein (component II) and MoFe protein (component I) (Peters et al., 1995). The expression of the structural genes for nitrogenase (*nif*HDK operon), and genes encoding accessory functions are regulated at the transcriptional level by the product of *nif*A. The predicted sequences of all NifA proteins are approximately of similar lengths, and indicate that the protein consists of different domains with variable conservation (Fischer, 1994). Increase in alfalfa yield has been demonstrated in recombinant cells of *S. meliloti* with an additional copy of both *nif*A and *dct*ABD (Bosworth et al., 1994). Castillo et al. (1999) have performed similar experiments. However, the potential for horizontal transfer of the introduced DNA material poses additional problems. In that sense, the selection of native rhizobial strains able to increase alfalfa yield in the field and the development of new commercial inoculants has become of economical interest for developing countries such as Uruguay. Thus, the identification of alfalfa cultivars in acidic soils should first concentrate on the selection of acid tolerant rhizobia with high effectiveness and competitiveness.

The identification of selected strains is usually done using biochemical, molecular or serological tests. Different technologies are useful for

distinguishing strains in population studies (Tiedje et al., 1999). However, DNA structure and composition provides the most useful information. Phylogenetic relationships and diversity of rhizobia isolates have been extensively studied by analysis of 16S rRNA (Hernandez-Lucas et al., 1995; Laguerre et al., 1997) and RFLP (Demezas et al., 1991; Bromfield et al., 1998). However, de Bruijn (1992) and Martinez-Romero (1994) proposed PCR analysis as a good technology to be used in diversity studies, because it allows recognition of closely related strains and is easy and fast to perform. In addition, de Bruijn (1992) showed that results from *rep*-PCR techniques are in agreement with phylogenies derived from multilocus enzyme electrophoresis.

The presence of native alfalfa nodulating strains in Uruguay was previously analysed by del Papa et al. (1999). From 166 isolates assayed, 15 were able to grow and effectively nodulate alfalfa at pH 5.5 and were designated as mid-acid tolerant (MAT) and 2 isolates (CE20 and CE26), which were able to grow and nodulate alfalfa at pH below 5.0, designated as acid-tolerant (AT) strains. The AT isolates were classified as *Rhizobium* spp. Oregon type. *S. meliloti* and *S. medicae* are not the only symbionts of alfalfa. Eardly et al. (1985) isolated *Rhizobium* spp. strains from alfalfa, grown in moderately acidic soils in Oregon. These *Rhizobium* strains possessed the unique ability to nodulate both alfalfa and common bean (*Phaseolus vulgaris*) and were closely related to *Rhizobium etli*, a symbiont of common bean (Eardly et al., 1992).

The aim of this work was to contribute to the understanding of the genetic diversity among the mid-acid tolerant (MAT) alfalfa nodulating strains obtained from Uruguayan soil, and the determination of symbiotic effectiveness of these promising isolates in acidic soils.

2. Materials and Methods

Organisms and acid tests

Fifteen MAT and two AT (CE20 and CE26) nodulating-alfalfa isolates used in this work are listed in Table 1. These isolates were selected from 166 alfalfa nodulating strains obtained from 79 different Uruguayan soils, as described by del Papa et al. (1999). *S. meliloti* U137 and U143 (indigenous strains used as inoculants in Uruguay) were provided by the Laboratory of Soil Microbiology, MGAP-Uruguay. *S. meliloti* 2011 was obtained from J. Dénarié and LPU63, described as an efficient nitrogen fixing strain at low pH isolated in Argentina (Segundo et al., 1999) was obtained from del Papa et al. (1999).

The ability of isolates to grow at different pHs, in buffered modified Howieson liquid minimal medium (MM) was determined as described by del Papa et al. (1999).

Table 1. Effectiveness of alfalfa-nodulating strains determined in growth-pouches at high and low pH

Strain	Shoot dry matter (mg per plant)*	
	pH 6.8	pH 5.6
CE15	8.6 cde**	8.5 cd
CE17	8.3 de	5.2 fg
CE20	5.2 g	5.0 fg
CE21	9.4 bcd	9.9 ab
CE26	4.2 g	5.0 fg
CE27	7.7 e	6.5 ef
CE31	11.3 a	8.9 bc
CE32	9.1 bcd	8.3 cd
CE33	9.7 bc	8.9 bc
CE35	9.4 bcd	9.0 abc
CE41	9.6 bc	10.3 a
CE45	7.6 ef	8.4 cd
CE46	6.3 g	7.1 de
CE47	9.7 b	10.1 ab
CE56	10.6 ab	9.0 abc
CE65	8.8 bcd	8.3 cd
CE72	7.5 ef	7.5 de
U137	9.7 b	7.3 de
U143	6.5 fg	6.7 e
Sm2011	10.0 b	9.9 ab
+N control	11.9 a	7.7 cde
–N control	5.7 g	4.5 g

*Shoot dry matter is the average of 8 replicated pouches (containing 5 plants each) per strain and pH. **Letters within the same column indicate significant differences among strains ($P<0.001$).

PCR amplification

DNA was obtained from cell-lysates, and PCR reactions were carried out essentially as described by Versalovic et al. (1994). Each 25 µl PCR reaction contained: 375 pmol each of the opposing REP primers (REPIR-1, 5'-III-ICG-ICG-ICA-TCA-GGC3'; REP2R-1, 5'-ICG-ICT-TAT-CIG-GCC-TAC 3'), 5 µl of cell lysate, 0.2 mM of each dNTP, 3 mM magnesium chloride, 500 µg of bovine serum albumin (BSA), 1 unit of AmpliTaq DNA polymerase in 50 mM TRIS buffer (pH 8.3). The cycles used were: 1 cycle at 95°C for 7 min; 35 cycles at 95°C for 1 min, at 40°C for 1 min, and at 72°C for 8 min; 1 cycle at 72°C for 16 min; and

a final soak at 4°C. Amplification products were separated on 1% agarose gels, stained with ethidium bromide, and photographed with a Polaroid type 667 film. The presence or absence of each PCR product band was recorded and used to produce a two-dimensional data matrix of binary codes for all isolates of interest. Statistical analysis by Ward's Method (Ward, 1963), not seen cited by StatSoft, Inc. (1999) STATISTICA for Windows [Computer program manual], for population genetics to calculate a dendrogram was used.

RFLP and hybridisation analysis

An aliquot (1–2 μg) of each genomic DNA sample was digested with *Eco*RI or *Bam*HI at 37°C overnight using an excess of enzyme. Restriction fragments were separated by electrophoresis in 1% agarose horizontal gels made up in TBE (Sambrook et al., 1989). Electrophoresis was carried out at 35V for 16h. Molecular size markers were included in the gel. DNA restriction fragments were blotted onto a 0.45 μm nitrocellulose filter. The *nif*A (1.3 kb) and *nif*H (6.4 kb) hybridisation probes were prepared from *S. meliloti* 2011 by PCR and labelled by biotinylation. Standard hybridisations at 68°C were done according to the NEBlot Phototope Kit Instruction Manual (Biolabs, Inc.). Chemiluminescent detection was done with Phototope-Star Detection Kit (Biolabs, Inc.) and the emitted light was captured on X-ray film.

Effectiveness tests in pouches

Seeds of *Medicago sativa* cv Crioula, Chaná-Estanzuela and Pioneer 5939 were surface-sterilised (Vincent, 1970), germinated, transferred to plastic growth pouches (at least eight pouches for each treatment; 5 seeds per pouch) filled with Howieson medium (Howieson et al., 1993) at pH 6.8 (10 mM PIPES) or pH 5.6 (10 mM MES), and were inoculated five days later with alfalfa-nodulating strains (10^7 cfu per seed). Uninoculated nitrogen-control plants were grown with and without supplying Howieson medium with KNO_3 at a final concentration of 0.05%. Plants were grown in a controlled environment cabinet (16 h light: 8 h dark; at 20°C) for 30 days. Shoot dry matter for each treatment (plants inoculated with a strain at one pH) was determined after drying the aerial part of the alfalfa plants at 80°C for 24 h. Experiments were replicated twice.

Effectiveness tests in pots

The effectiveness of eight alfalfa-nodulating strains on alfalfa cv Crioula was analysed by employing four MAT isolates and four control rhizobia strains

(Sm2011, U137, U143, LPU63). Seeds were surface sterilised, soaked in water, transferred to twelve-1 kg pots (8 seeds per pot) per treatment filled with a mixture of sterile soil-sand (1: 1) and inoculated with 10^7 cfu of sinorhizobia per seed. After two weeks, five plants from each pot were eliminated and the best three plants were kept. A total of thirty-six plants were employed for each strain and pH. Acidic and neutral soils were dark, loamy, free of aluminium and initial pH values were 5.3 and 6.2, respectively.

Plants were grown in a greenhouse and watered once a week alternatively with distilled water, Howieson medium diluted ten-fold in distilled water, or with calcium hyperphosphate solution (1 g/l). Control plants with and without nitrogen supply also were grown. Alfalfa plants were cut 1 cm above the pot soil at 50 days, followed by a second cut at 90 days after inoculation. Shoot dry matter was determined as described above. Experiments were replicated once, but the data presented are the result of one experiment.

Competitiveness test

Plants growing in pots as described above were inoculated with a mixture (1:1) of two different sinorhizobia (5×10^6 cfu of each strain per seed). Plants were harvested 45 days after planting and nodules were cut, surface sterilised and crushed as described by Beattie and Handelsman (1989). Nodule occupancy was determined by the capacity of isolates to support growth in TY media (Beringer, 1974); to produce melanin (Castro et al., 2000), Mel^+ or Mel^- strains; or to support growth on antibiotics.

Data analysis

Analysis of variance was performed using ANOVA-1 and the least significant difference (LSD) with a confidence level of 99% was determined. Based on the LSD, a ranking system was established.

3. Results

Occurrence of MAT alfalfa nodulating strains

All MAT isolates grew at pH values of 5.5 and 6.8 in liquid culture (data not shown). The doubling time was between 6 and 8 h or between 10 and 15 h when microorganisms were grown at pH 6.8 (final pH 6.6) or 5.5 (final pH 5.6), respectively.

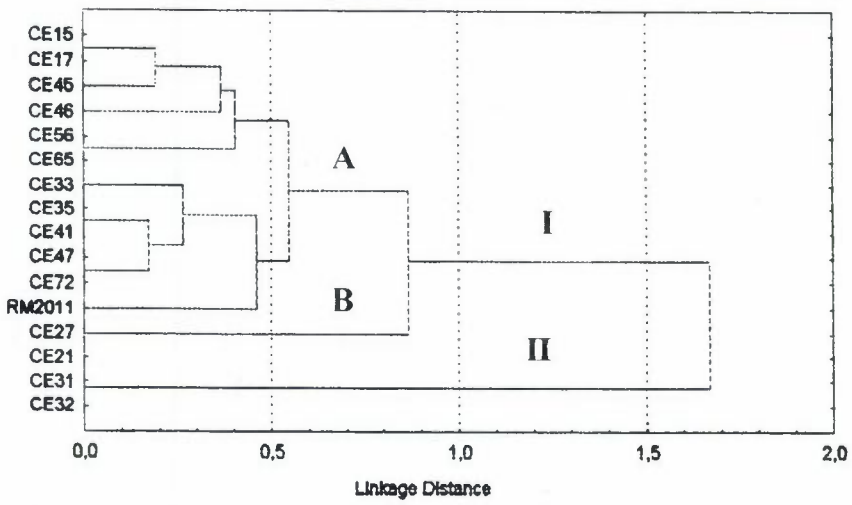

Figure 1. Dendrogram of the genetic structure of a population of MAT strains.

Genetic diversity

Total genomic DNA from the 15 MAT isolates was used as a template for *rep*-PCR with REP primers. The reactions yielded between 10 to 22 bands per strain of sizes from 0.13 to 2.3 Kb. The dendrogram (Fig. 1) confirms that the acid-tolerant strains could be divided in two groups. Group I (80% of tested isolates) is divided into two subgroups: (A), including 11 isolates and Sm 2011; and (B), represented by one isolate. Group II (3 isolates) shares a PCR pattern similar to that of strains U45, U137, and U143, currently used as inoculants in Uruguay (data not shown). Groups I and II are clearly differentiated with a linkage distance of 1.50. Distances ranging from a minimum of 0.18 to a maximum of 1.68 separate all these strains. A total of 10 distinctive multilocus genotypes was identified.

nifA and nifH hybridisation

Of the 18 strains tested (15 MAT, 2 *Rhizobium* spp. Oregon type strains, and the reference strain Sm 2011) only Sm 2011 had two *nif*A-hybridising fragments of 6.9 and 5.0 kb when DNA was restricted with *Eco*RI. Among the MAT isolates two groups were identified: 1) all strains belonging to Group I, the same Group I as PCR analysis showed, presented a single *nif*A-hybridising band of 7.5 kb and; 2) all strains belonging to Group II, as PCR analysis showed, also yielded a single *nif*A-hybridising band, but it had 5.5 kb. *Rhizobium* spp. Oregon type strains (CE20 and CE26) did not show hybridising fragments, and

thus comprise a third group. Both Group I and II isolates showed two *nif*A-hybridising fragments of 1.6 and 3.0 kb when DNA was restricted with *Bam*HI and, no hybridising fragments were detected with restricted DNA from CE20 and CE26 strains. In addition, when *nif*H probe was tested, a hybridising fragment of 4.0 or 3.5 kb was detected for MAT and *Rhizobium* spp. Oregon type strains, respectively (data not shown).

Symbiotic effectiveness in pouches

Since plant cultivars affect phenotype variability relative to symbiotic properties (Gandee et al., 1999; Triplett and Sadowsky, 1992), effectiveness of four randomly chosen MAT isolates (all different strains, based on REP-PCR analysis) was assayed in three different plant genotypes of *Medicago sativa* cv (Crioula, Chaná-Estanzuela and Pioneer 5939). Alfalfa cv Crioula seems to be an appropriate cultivar for effectiveness studies, since significantly higher yields, based on shoot dry matter, were obtained (data not shown).

The effect of MAT isolates on growth of alfalfa cv Crioula, kept in pouches filled with mineral nutrient solution at pH 5.6 or 6.8, is shown in Table 1. All strains led to nodule development and fixed nitrogen at both pH values. At pH 6.8, strains CE31 and CE56 led to a shoot dry matter similar to the one of control plants supplemented with nitrate. However, at pH 5.6 most of the strains produced higher dry matter than plants supplemented with nitrate. The Uruguayan *Rhizobium* spp. Oregon type strains (CE20 and CE26), the MAT isolate CE27, and the inoculant strain U143 produced 50 to 65% less dry matter than the reference Sm2011 strain. The strains CE20 and CE26 had been characterised as ineffective in alfalfa by del Papa et al. (1999), but effective on common bean (*P. vulgaris*). Statistical analysis of variance showed that shoot dry matter differed significantly between strains at both pH values ($P<0.001$). The best results at pH 5.6 were obtained with strains CE21, CE41, and CE47.

Statistical analysis of growth parameters at the two pH values (6.8 and 5.6) identified three groups of isolates, strains that produce shoot dry matter with: 1) a highly significant difference ($P<0.001$) (CE17, CE27, CE31, CE46, CE56); 2) a significant difference ($P<0.05$) (CE45); and 3) no significant difference (CE15, CE 20, CE21, CE32, CE33, CE35, CE41, CE47, CE65 and CE72).

Symbiotic effectiveness and competitiveness in pots

Plant pot experiments were performed with neutral and acidic soil (Fig. 2) and eight MAT strains selected according to the results obtained with the pouch experiments. All induced nodule development and fixed nitrogen in potted plants. Shoot dry matter varied from 400 to 600 mg per plant, but the

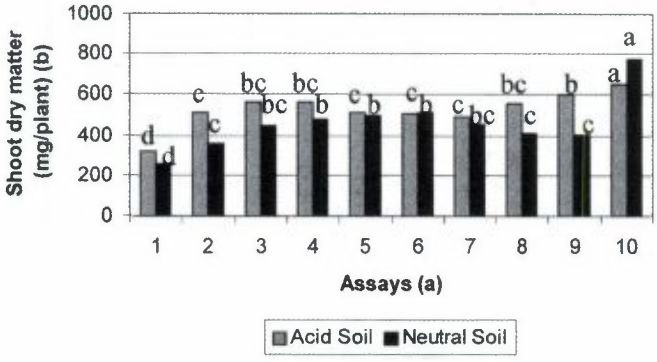

Figure 2. Effectiveness of MAT isolates on soil-grown *M. sativa* cv Criola in pots. (a) assays: 1 – Control without nitrogen; 2 – CE 17; 3 – CE32; 4 – CE41; 5 – CE47; 6 – U143; 7 – Sm2011; 8 – U137; 9 – LPU 63; 10 – Control with nitrogen. (b) Shoot dry matter was the average, for each assay and pH, of 72 alfalfa plants at 50 (first cut) and at 90 (second cut) days after inoculation. Letters within the same column indicate differences among strains.

Table 2. Competitiveness assays of *S. meliloti* U143 and different MAT isolates on acidic and neutral soils

Soil pH	Competitor strain	Selectable marker of competitor	Percentage of occupation (%)		
			Competitor strain	U143*	Co-occupancy
Neutral	CE17	Mel^-	19**	81**	0
Acidic	CE17		10	83	7
Neutral	CE20	TY^-	3	85	12
Acidic	CE20		22	50	28
Neutral	CE41	$Sp50^S$	11	89	ND***
Acidic	CE41		3	97	ND
Neutral	CE47	Mel^-	24	76	0
Acidic	CE47		40	57	3
Neutral	LPU63	$Str100^R$	55	45	ND
Acidic	LPU63		54	46	ND

*Strain U143 is a melanin producer (Mel^+), able to grow on TY medium (TY^+), tolerant to 50 µg of spectomycin per ml ($Sp50^R$) and sensitive to 100 µg of streptomycin per ml ($Str\ 100^S$). **% of nodule occupancy. ***not determined.

differences for both soils were not significant ($P<0.02$; Waller-Duncan test).

From a practical standpoint high competitiveness is as important as effectiveness of symbiotic nitrogen fixation (Triplett, 1990). Therefore, paired competition assays (Table 2) between one strain belonging to Group II (U143, an inoculant strain) and five competitor strains belonging to: Group I (CE17, CE41 and CE47), an *Rhizobium* sp. Oregon type strain (CE20), and LPU63 strain (an efficient nitrogen fixing Argentinean isolate) (Segundo et al., 1999) were performed. Studies were carried out on both neutral and acidic soils. Strain U143 was highly competitive when assayed in pairs with CE41, CE17 or CE20. Only LPU63 was able to compete with U143 at both neutral and acidic soil, and strain CE47 in acidic soil.

In addition, nodule distribution was reasonably affected by pH. For example, percentage of single nodule occupancy varied from 3 to 22 and from 24 to 40 for the competitor strains CE20 and CE47, respectively, in neutral and acidic soils.

4. Discussion

Our results confirm that alfalfa nodulating isolates obtained from acid soils are not necessarily acid-tolerant and that isolates obtained from neutral or moderate acidic soils could be acid-tolerant. We found that from 77 isolates obtained from acidic soils only 6 were capable to grow at pH 5.5. In addition, from the 89 isolates collected from soils with pH 6.0 upward, 9 were able to grow at pH 5.5. Similar results were reported by Richardson and Simpson (1988). The explanation of this discrepancy appears to lie in the different conditions that bacteria are immersed, liquid minimal medium or soil.

Our MAT strains represent 10% of the total isolates, suggesting that a significant percentage of native alfalfa-nodulating rhizobia strains may be tolerant to acidic conditions. Although some genotypes occurred repeatedly, PCR analysis of the MAT strains suggests that considerable diversity exists between the mid-acid tolerant alfalfa nodulating strains. The inoculant strains U137 and U143 had a PCR pattern similar to that of strains CE21, CE31, CE32; however, CE21 and CE31 were isolated from no-tillage soil, suggesting that the strains belonging to this multilocus genotype are widely dispersed in Uruguay, maybe by migration. A similar level of genetic differentiation of *S. meliloti* was also reported by Hartmann and Amarger (1991) and Gandee et al., (1999). In contrast, Bromfield et al. (1998) detected no differentiation between populations of *S. meliloti* sampled from 2 field sites 2 km apart in Ottawa. Here, we report genetic variation of a specific group of alfalfa nodulating strains (mid-acid tolerant) from fields distant up to 400 km.

In agreement with REP-PCR analysis, *nif*A hybridisation patterns showed that MAT strains could be divided into two main groups. However, PCR

analysis showed higher diversity between native isolates than that obtained with *nif*A hybridisation pattern. Interestingly, the observation that *Rhizobium* spp. Oregon type strains did not hybridise with *nif*A probe, obtained from Sm2011, it did not surprise us because these strains are related with *R. etli* and not related with *S. meliloti* (Eardly et al., 1992). In addition, the 1.3kb *nif*A probe from *S. meliloti* has only a region of 240 pb with 79% of homology with *nif*A of *R. etli*. It could explain that no hybridising fragments were detected. Additionally, the sequence of the *nif*H gene of *Rhizobium* Or 191 is substantially different from other published *nif*H sequences (Eardly et al., 1992). The hybridisation pattern of restricted DNA (*Bam*HI) with *nif*H probe from Sm2011 showed a smaller hybridising fragment from Oregon type strains than that obtained with MAT strains.

Effectiveness assays carried out in pouches suggest that our indigenous MAT *M. sativa*-nodulating strains are highly effective for nitrogen fixation at both pH 5.6 and 6.8. Statistical analysis of shoot dry matter confirms that CE41 and CE47 strains were very effective at both acid and neutral pH. These results suggest that CE41 and CE47 isolates have the potential for improving alfalfa growth in acidic and neutral soils and that they could be chosen as potential suitable strains to be used at low pH.

As nodule occupancy is influenced by biological (Evans et al., 1979) as well as by environmental factors (Beattie et al., 1989), effectiveness and competitiveness assays were performed in both neutral and acidic soils. We found that the currently used inoculant strain U143 is not a suitable inoculant for alfalfa in acidic soils. However, based on competitiveness results, it appears that strain U143 has a competitive advantage. In addition, the Oregon type strain CE20 could not compete with strain U143. The low abundance of the Oregon type strains in the tested soils (1.2%; two in 166 total isolates) and their low competitiveness suggests that they would not interfere with nodulation of alfalfa by other more efficient inoculant strains. Besides, nodule distribution was reasonably affected by pH for all competitor strains tested, except for strain LPU63, probably because all the tested strains were mid-acid tolerant.

The soils tested possess a native alfalfa-nodulating sinorhizobia population. Ten percent of the isolated strains are tolerant to acidic conditions, fix nitrogen on alfalfa and show considerable diversity. Strains belonging to one of the genotypes are highly distributed in Uruguayan fields. Strain U143, that belongs to this group, showed increased competitiveness in both acidic and neutral soils. These results are in agreement with those of Bromfield et al. (1986), who proposed that dominance may be due to higher competitiveness for nodulation of a group or to a higher abundance of this group in soil. Finally, as Bromfield et al. (1986) and Gandee et al. (1999) argued, we think that the presence of a local soil population with high competitiveness is the major barrier for the establishment of a new inoculant strain. We suggest the native

populations of alfalfa nodulating strains, as the ones found in Uruguayan soils may be suitable for use as inoculant for acidic soils. These inoculants should be tested in the future in field experiments.

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REFERENCES

Beattie, G.A. and Handelsman, J. 1989. A rapid method for the isolation and identification of *Rhizobium* from root nodules. *Journal of Microbiological Methods* **9**: 29–33.

Beattie, G.A., Clayton, M.K., and Handelsman, J. 1989. Quantitative comparison of the laboratory and field competitiveness of *Rhizobium leguminosarum* biovar *phaseoli*. *Applied and Environmental Microbiology* **55**: 2755–2761.

Beringer, J.E. 1974. R-transfer in *Rhizobium leguminosarum*. *Journal General Microbiology* **84**: 188–198.

Bosworth, A.H., Williams, M.K., Albrecht, K.A., Kwiatkowski, R., Beynon, J., Hankinson, T.R., Ronson, C.W., Cannon, F., Wacek, T.J., and Triplett, E.W. 1994. Alfalfa yield response to inoculation with recombinant strains of *Rhizobium meliloti* with an extra copy of *dct*ABD and/or modified *nif*A expression. *Applied and Environmental Microbiology* **60**: 3815–3832.

Bromfield, E.S.P., Behara, A.M.P., Singh, R.S., and Barran, L.R. 1998. Genetic variation in local population of *Sinorhizobium meliloti*. *Soil Biology & Biochemistry* **30**: 1707–1716.

Bromfield, E.S.P., Sinha, I.B., and Wolynetz, M. S. 1986. Influence of location, host cultivar and inoculation on the composition of naturalised populations of *Rhizobium meliloti* in *Medicago sativa* nodules. *Applied and Environmental Microbiology* **51**: 1077–1084.

Castillo, M., Flores, M., Mavingui, P., Martinez-Romero, E., Palacios, R., and Hernandez, G. 1999. Increase in alfalfa nodulation, nitrogen fixation, and plant growth by specific DNA amplification in *Sinorhizobium meliloti*. *Applied and Environmental Microbiology* **65**: 2716–2722.

Castro, S., Carrera, I., and Martinez-Drets, G. 2000. Methods to evaluate nodulation competitiveness between *Sinorhizobium meliloti* strains using melanin production as a marker. *Journal of Microbiological Methods* **41**: 173–177.

de Bruijn, F.J. 1992. Use of repetitive (repetitive extragenic palindromic and enterobacterial repetitive entergeneric consensus) sequences and the polymerase chain reaction to fingerprint the genomes of *Rhizobium meliloti* isolates and other soil bacteria. *Applied and Environmental Microbiology* **58**: 2180–2187.

del Papa, M.F., Balague, L.J., Castro-Sowinski, S., Wegener, C., Segundo, E., Martínez-Abarca, F., Toro, N., Niehaus, K., Puhler, A., Aguilar, M.O., Martinez-Drets, G., and Lagares, A. 1999. Isolation and characterisation of alfalfa-nodulating rhizobia present in acidic soils of central Argentina and Uruguay. *Applied and Environmental Microbiology* **65**: 1420–1427.

Demezas, D.H., Reardon, T.B., Watson, J.M., and Gibson, A.H. 1991. Genetic diversity among *Rhizobium leguminosarum* bv. *trifolii* strains revealed by allozyme and restriction fragment length polymorphism analyses. *Applied and Environmental Microbiology* **57**: 3489–3495.

Eardly, B.D., Hannaway, D.B., and Bottomley, P.J. 1985. Characterization of rhizobia form ineffective alfalfa nodules: ability to nodulate bean plants [*Phaseolus vulgaris* (L.) Savi.]. *Applied and Environmental Microbiology* **50**: 1422–1427.

Eardly, B.D., Young, P.W., and Sulander, R.K. 1992. Phylogenetic position of *Rhizobium* sp. strain Or 191, a symbiont of both *Medicago sativa* and *Phaseolus vulgaris*, bases on partial sequences of the 16S rRNA and *nif*H genes. *Applied and Environmental Microbiology* **58**: 1809–1815.

Evans, J., Barnet, Y.M., and Vincent, J.M. 1979. Effect of a bacteriophage on colonization and nodulation of clover roots by paired strains of *Rhizobium trifolii*. *Canadian Journal of Microbiology* **25**: 974–978.

Fischer, H.M. 1994. Genetic regulation of nitrogen fixation in rhizobia. *Microbiological Reviews* **58**: 352–386.

Gandee, C.M., Harrison, S.P., and Davies, W.P. 1999. Genetic characterisation of naturally occurring *Rhizobium meliloti* populations and their potential to form effective symbioses with Lucerne. *Letters of Applied Microbiology* **28**: 169–174.

Graham, P.H. 1992. Stress tolerance in *Rhizobium* and *Bradyrhizobium*, and nodulation under adverse soil conditions. *Canadian Journal of Microbiology* **38**: 475–481.

Graham, P.H., and Parker, C.A. 1964. Diagnostic features in the characterisation of the root-nodule bacteria of legumes. *Plant and Soil* **20**: 383–396.

Hartmann, A. and Amarger, N. 1991. Genotypic diversity of an indigenous *Rhizobium meliloti* field population assessed by plasmid profiles, DNA fingerprinting, and insertion sequence typing. *Canadian Journal of Microbiology* **37**: 600–608.

Hernandez-Lucas, I., Segovia, L., Martinez-Romero, E., and Pueppke, S.G. 1995. Phylogenetic relationships and host range of *Rhizobium* spp. that nodulate *Phaseolus vulgaris* L. *Applied and Environmental Microbiology* **61**: 2775–2779.

Howieson, J.G. and Ewing, M.A. 1986. Acid tolerance in the *Rhizobium meliloti-Medicago* symbiosis. *Australian Journal Research* **37**: 55–64.

Howieson, J.G., Robson, A.D., and Ewing, M.A. 1993. External phosphate and calcium concentrations, and pH, but not the products of rhizobial nodulation genes, affect the attachment of *Rhizobium meliloti* to roots of annual medics. *Soil Biology & Biochemistry* **25**: 567–573.

La Favre, A.K. and Eaglesham, A.R. J. 1986. The effect of high temperatures on soybean nodulation and growth with different strains of bradyrhizobia. *Canadian Journal of Microbiology* **33**: 343–345.

Laguerre, G., van Berkum, P., Amarger, N., and Prevost, D. 1997. Genetic diversity of rhizobial symbionts isolated from legume species within the genera *Astragalus, Oxytropis,* and *Onobrychis. Applied and Environmental Microbiology* **63**: 4748–4758.

Martinez-Romero, E. 1994. Recent developments in *Rhizobium* taxonomy. *Plant and Soil* **161**: 11–20.

Peters, J.W., Fisher, K., and Dean, D.R. 1995. Nitrogenase structure and function: a biochemical genetic perspective. *Annual Review of Microbiology* **49**: 335–366.

Richardson, A.E. and Simpson, R.J. 1988. Enumeration and distribution of *Rhizobium tropici* under a subterraneous clover based pasture growing in an acid soil. *Soil Biology & Biochemistry* **20**: 431–438.

Sambrook, J., Fritsch, E.F., and Maniatis, T. 1989. *Molecular Cloning: A Laboratory Manual.* Second Edition. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Segundo, E., Martinez-Abarca, F., van Dillewijn, P., Fernandez-Lopez, M., Lagares, A., Martìnez-Drets, G., Niehaus, K., Puhler, A., and Toro, N. 1999. Characterisation of symbiotically efficient alfalfa-nodulating rhizobia isolated from acid soils of Argentina and Uruguay. *FEMS Microbial Ecology* **28**: 169–176.

Singleton, P.W. and Tavares, J.W. 1986. Inoculation response of legumes in relation to the number and effectiveness of indigenous *Rhizobium* populations. *Applied and Environmental Microbiology* **51**: 1013–1018.

Smil, V. 1997. Global population and the nitrogen cycle. *Scientific American* **277**: 59–63.

Tiedje, J.M., Asuming-Brempong, S., Nusslein, K., Marsh, T.L., and Flynn, S. 1999. Opening the black box of soil microbial diversity. *Applied Soil Ecology* **13**: 109–122.

Triplett, E.W. 1990. The molecular genetics of nodulation competitiveness in *Rhizobium* and *Bradyrhizobium. Molecular Plant-Microbe Interactions* **3**: 199–206.

Triplett, E.W. and Sadowsky, M.J. 1992. Genetic of competition for nodulation of legumes. Annual Review of Microbiology **46**: 399–428.

Versalovic, J., Schneider, M., de Bruijn, F., and Lupski, J.R. 1994. Genomic fingerprinting of bacteria using repetitive sequence-based polymerase chain reaction. *Methods of Molecular Cell Biology* **5**: 25–40.

Vincent, J.M. 1970. *A Manual for the Practical Study of Root – Nodule Bacteria.* IBP Handbook no. 15 Blackwell Scientific Publications, Oxford.

Ward, J.H. 1963. Hierarchical grouping to optimise an objective function. *Journal of the American Statistical Association* **58**: 236.

Zahran, H.H. 1999. *Rhizobium*-legume symbiosis and nitrogen fixation under severe conditions and in an arid climate. *Microbiology and Molecular Biology Reviews* **60**: 968–989.