AGROCIENCIA URUGUAY

Agrociencia Uruguay 2019 23(2):1-13
ISSN electrónico 2301-1548

Rhizobia Inoculants for Alfalfa in Acid Soils: a Proposal for Uruguay

Tabares-da Rosa S[1], Signorelli S[1], Del Papa M[2], Sabatini O[1], Reyno R[3], Lattanzi F[3], Rebuffo M[3], Sanjuán J[4], Monza J[1]

[1] Laboratorio de Bioquímica, Depto. Biología Vegetal, Facultad de Agronomía, Universidad de la República, Uruguay. [2] Instituto de Biotecnología y Biología Molecular, Facultad de Ciencias Exactas, Universidad Nacional de la Plata, Argentina. [3] Instituto Nacional de Investigación Agropecuaria, INIA La Estanzuela e INIA Tacuarembó, Uruguay. [4] Depto. de Microbiología del Suelo y Sistemas Simbióticos, Estación Experimental del Zaidín, Consejo Superior de Investigaciones Científicas (CSIC), Granada, España.

Recibido: 28/08/19 - Aceptado: 13/09/19

Summary

Ensifer meliloti establishes symbiosis with *Medicago sativa* (alfalfa) and other perennial species of *Medicago* that grow in soils with neutral to alkaline pH, whereas *Ensifer medicae* makes symbiosis with annual medics adapted to moderately acid soils. The new species *Rhizobium favelukesii*, whose strain is LPU83, belongs to an alfalfa group of inefficient rhizobia, known as the Oregon type, initially represented by *Rhizobium* sp. strain Or191. *R. favelukesii* is considered a potential risk in the acid soils where alfalfa is grown, and could explain the inefficient nodulation observed in different countries. In acidic soils from the «Dairy Basin» of Uruguay, producers inoculate alfalfa with *E. melliloti* U143 strain. This edaphic condition is often marginal because the maximum potential of rhizobia-alfalfa symbiosis is not achieved at acid pH. Although Uruguay has an outstanding position in the production and use of rhizobial inoculants, the commercial strains currently used in *Trifolium, Lotus* and alfalfa were selected about 50 years ago in different conditions that the present ones as a consequence of: i) the displacement of cultivated pastures to other sites, ii) the sowing method, and iii) the use of new cultivars. In this review, alfalfa inoculation is analyzed in some countries and a strategy for the development of an inoculant suitable for Uruguayan acid soils is proposed. This strategy is based on the selection of efficient and competitive strains, as the first selection criteria, and persistency in soil as the second one.

Keywords: *Ensifer meliloti,* Oregon strains, available aluminium

Inoculantes rizobianos para alfalfa en suelos ácidos: una propuesta para Uruguay

Resumen

Ensifer meliloti establece simbiosis con *Medicago sativa* (alfalfa) y otras especies perennes de *Medicago* que crecen en suelos con pH neutro a alcalino, mientras que *Ensifer medicae* lo hace con especies anuales adaptadas a suelos moderadamente ácidos. La nueva especie *Rhizobium favelukesii*, cuya cepa tipo es LPU83, pertenece a un grupo de rizobios ineficientes en alfalfa conocidos como tipo Oregon, representados inicialmente por *Rhizobium* sp. cepa Or191. *R. favelukesii*; se considera un riesgo potencial en suelos ácidos en los que se cultiva alfalfa, y podría explicar la nodulación ineficiente en diferentes países. En suelos ácidos de la «Cuenca lechera» de Uruguay los productores inoculan alfalfa con *E. meliloti* cepa U143. Esa condición edáfica a veces resulta marginal para la simbiosis rizobio-alfalfa porque a pH ácido no se logra su máximo potencial. Si bien Uruguay tiene una posición destacada en la producción y uso de inoculantes rizobianos, las cepas comerciales usadas actualmente en especies de *Trifolium, Lotus* y alfalfa se seleccionaron hace unos 50 años en condiciones diferentes a las actuales, consecuencia de: i) el desplazamiento de pasturas cultivadas a otros sitios, ii) el tipo de siembra y iii) el uso de nuevos cultivares. En esta revisión se analiza la inoculación de la alfalfa en algunos países y se propone una estrategia para el desarrollo de un inoculante apto para suelos ácidos en Uruguay. Esta estrategia se basa en la selección de cepas eficientes y competitivas como primer criterio y persistencia en suelos, como segundo.

Palabras clave: *Ensifer meliloti*, cepas Oregon, aluminio disponible

Alfalfa-nodulating rhizobia

The rhizobia that fix nitrogen in legumes of the genus *Medicago* belong to two closely related species, *Ensifer meliloti* and *Ensifer medicae* (syn. *Sinorhizobium meliloti* and *Sinorhizobium medicae*, respectively). While both rhizobia species displayed a nitrogen-fixing phenotype with the model *Medicago truncatula*, *E. meliloti* establishes symbiosis with *Medicago sativa, M. littoralis,* and *M. tornata*, annual alfalfa that grows naturally in neutral to alkaline pH soils, *E. medicae* is associated with *M. polymorpha, M. arabica* and *M. murex*, annual legumes adapted to moderately acid soils[1][2]. Biondi and others[3] suggested a preferential relationship between *E. meliloti* and tetraploid *Medicago* spp., and between *E. medicae* and diploid species such as many annual medics. Based on a genotypic and biochemical characterization, Garau and others[1] proposed that *E. meliloti* and *E. medicae* were adapted to different species of the genus *Medicago* according to the niches that these legumes occupy in their natural habitat, although more studies are necessary to confirm this association.

In addition, the characterization of the populations of alfalfa-nodulating rhizobia from acid soils showed the presence of another lineage of *Rhizobium sp.* that forms ineffective nodules in alfalfa. This group of poorly characterized rhizobia known generically as the Oregon type was initially represented by the Or191 strain[4]. These rhizobia were isolated from *M. sativa* nodules in Oregon (1981-82) from a field having moderately acid soil conditions (pH 5.5 to 5.7), where alfalfa had not been cultivated for at least 10 years. Unlike *E. meliloti* and *E. medicae*, the rhizobia strains of this group generated small colonies, did not acidify and did not grow at 39 °C in YEM[4]. This type of persistent, highly competitive, and inefficient rhizobia in alfalfa was also identified in acid and moderately acid soils from various locations in United State of America (USA), Australia and Canada[4].

In Argentina and Uruguay, populations of alfalfa-nodulating rizhiobia from acid soils were also characterized[5]. A collection of 466 strains were studied and distributed in two groups, the main group consisted of efficient nitrogen-fixing rhizobia and a minor group of inefficient and acid-tolerant rhizobia formed by isolates similar to strain Or191. The sensitivity of *E. meliloti* to acidity was observed to be in the range of pH between 5.6 and 6.0[6], depending on the strain and the level of Ca^{+2} in the culture medium. Conversely, the group of the inefficient alfalfa-nodulating rhizobia grew at pH 5.0 and showed similar phenotypic characteristics among all inefficient isolates, as well as similar to those of strain Or191. For example, these inefficient strains, unlike to *E. meliloti* and *E. medicae*, showed inability to grow in LB media at 28 °C and TY media at 37 °C, shared the same plasmid patterns, lipopolysacharide profiles, insertion-sequence fingerprints and ERIC, MBOREP1 and BOXC1PCR-fingerprinting patterns, nodulated *Phaseolus vulgaris* and *Leucaena leucocephala*[7], and different species of the *Trigonella* and *Melilotus* genera[8]. These characteristics were shared with the strain Or191 isolated from acid soils of Oregon, USA. Some phenotypic, genotypic and symbiotic characteristics of alfalfa-nodulating rhizobia are summarized in Figure 1.

The genetic analysis of the symbiotically inefficient rhizobia demonstrated a very homogeneous genetic background among all isolates and strain Or191[8]. Among the inefficient strains characterized by Del Papa and others[5], and later by Wegener and others[8], the LPU83 strain isolated from Argentina was selected as a representative strain of the acid-tolerant alfalfa-nodulating rhizobia. The LPU83 strain, for which the genome sequence is available[9], and *Rhizobium* sp. Or191 belongs to a novel species named *R. favelukesii*. The type strain of this species is LPU83[10].

Three out of five isolated strains from Uruguayan soils characterized by Del Papa and others[5] corresponded to *E. meliloti*, and were identified in soils from Colonia, Paysandú, and Soriano. The remaining two strains, CE20 and CE26, were collected in soils from Colonia with pH 5.9 and, like Or191 and LPU83 strains, were able to growth in LB and TY media at 28 °C and 37 °C respectively[5]. Thus, the initial characterization of CE20 and CE26 strains indicated that *R. favelukesii* strains are present in Uruguayan soils[5][11].

Although *R. favelukesii* strains are considered a potential risk in acid soils in which alfalfa is cultivated[12], Del Papa and others[5] observed only a low proportion of nodules occupied by them. However, in acid soils they are highly competitive for the nodulation of alfalfa[13], thus, its presence could explain the ineffective nodulation of alfalfa in acid soils around the world[4][13]. In this regard, in a field experiment in Ontario, Canada, *M. sativa* was grown at a single site that had no known history of alfalfa cultivation for two seasons in slightly acid field soil (pH 6.1), and a predominant group of phage-resistant bacteria was isolated from the nodules[14]. From those isolates recovered from

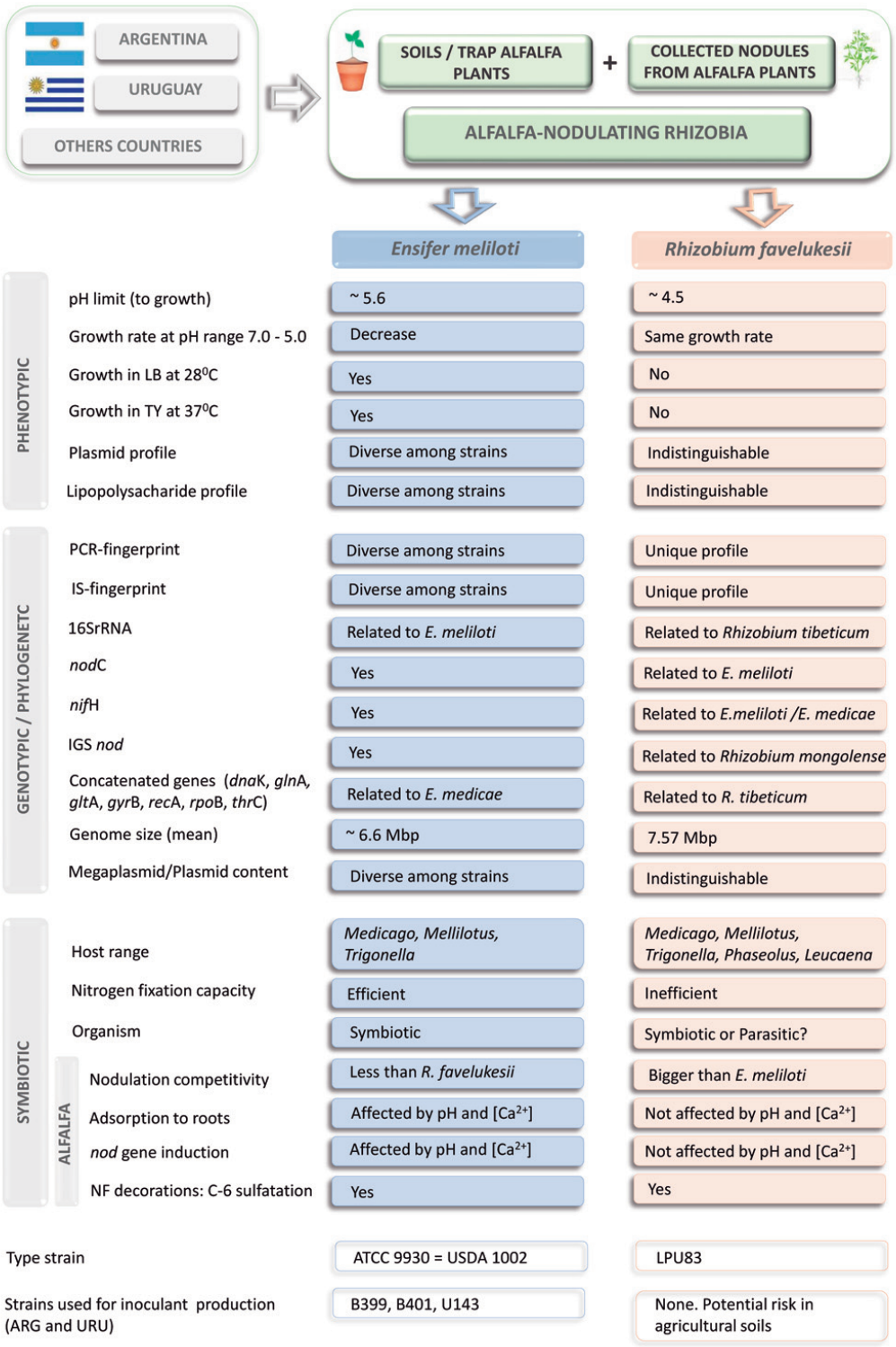

Figure 1. Main phenotypic, genotypic and symbiotic characteristics of alfalfa-nodulating rhizobia isolated from Argentina and Uruguay. References: IS,insertion sequences; IGS, intergenic sequences; NF, Nod Factor.

alfalfa, 69% presented a single genotype that was indistinguishable from strain Or191 by the genetic analysis employed[15]. Consequently, this finding indicates that: i) the genetic uniformity among the *R. favelukesii* isolates is independent of their geographical origin, and ii) soil persistence of *E. meliloti*, and the acid-tolerant *R. favelukesii* isolates in the presence/absence of alfalfa plants is not the same under soil acid stress conditions[16].

When phylogenetic studies for elucidating the genomic relationship of the *R. favelukesii* were performed, the 16S rRNA gene sequences of the *R. favelukesii* LPU83T and Or191 strains were found to be identical (100%), while respective identities of 99.9 and 99.2% were found with *Rhizobium tibeticum* CCBAU 85039T and *Rhizobium grahamii* CCGE 502T[10]. However, different results were obtained from the phylogenetic evaluation of symbiotic genes. For instance, the analysis of the *nod*C gene, encoding the N-acetylglucosaminyltransferase that catalyzes the first reaction in the synthesis of the Nod factor core[17], indicated that the *E. meliloti nod*C gene is the most closely related one and suggested that both *nod*C genes were originated from a common ancestor[18].

A distinctive feature of the rhizobia-alfalfa symbiosis is the marked plant-bacteria specificity and the strict requirement for Nod factors sulfated at its reducing end. In this context, the presence of a functional *nod*H-encoded NF sulfotransferase in *R. favelukesii* LPU83 was reported[16], and phylogenetic analyses based in this gene also pointed to the close relationship of this group with the alfalfa-nodulating rhizobia found in previous studies[18]. The *nif*H genetic tree furthermore demonstrated that *R. favelukesii* strains form a new clade, but the group was also closely related to the tight phylogenetic cluster formed by *E. meliloti* and *E. medicae*. Also, the *nod* cluster of *R. favelukesii* LPU83 has a marked synteny with the clusters of *E. meliloti* and *E. medicae*, but the intergenic region between *nod*E and *nod*G, which has a characteristic length of *Medicago*-nodulating rhizobia, is similar to *Rhizobium mongolense* strains[12]. Furthermore, a robust phylogenetic analysis involving concatenation of seven genes (*dna*K, *gln*A, *glt*A, *gyr*B, *rec*A, *rpo*B, and *thr*C)[19][20] and applying the Maximum-Pairwise, Neighbor-Joining and Maximun-Likehood methods indicated that *R. favelukesii* LPU83 is located close to a clade where *Rhizobium leguminosarum* and *Rhizobium etli* were situated, as had been previously observed for other housekeeping genes.

Restrictions on rhizobia-alfalfa symbiosis imposed by acid pH and Al^{3+}

The problems of implantation and establishment of rhizobia-alfalfa symbiosis are usually due to the acidic pH of the soil or to high concentrations of available aluminum (Al^{3+}), higher than 3 $mg.kg^{-1}$ soil[21]. Both factors negatively affect the rhizobial growth and survival[22][23][24][25][26][27] and interfere with the legume-rhizobia symbiosis by affecting rhizobial attachment to roots and the *nod* gene expression[28][29].

In acid soils, the production of cultivated legumes is lower than in neutral soils, due to the factors that independently or in combination affect: i) the host plant[28][30], ii) the rhizobia population[31], and iii) the interaction between the two[5][32][33][34]. It was estimated that approximately 25% of the world's soils are acidic or are going through a process of acidification[35], and among the nodule-inducing bacteria, *E. meliloti* is the most sensitive to acid pH[34][36]. Because of this, alfalfa infection by its specific rhizobia is reduced in acid and moderately acid soils[2][4].The initial stages of symbiosis are susceptible to pH and Al^{3+} because they negatively affect rhizobia binding to root cells and *nod* gene expression[28][29][37]. Arora and others[38] demonstrated that while RMP5 strain of *E. meliloti* is more tolerant to metal stress than *Bradyrhizobium* sp. strain BMP1, high concentrations of Al^{3+} affected bacterial growth, nitrogenase, nitrate reductase and nitrite reductase activities. Moreover, the growth rate of *E. meliloti* was shown to be lower at acid pH, although this could be improved in the presence of millimolar calcium concentrations[28]. Soto and others[29], studied the effect of pH and Ca^{2+} on diverse aspects of alfalfa nodulation with *E. meliloti* and the acid-tolerant and inefficient *R. favelukesii* LPU83, and observed that the addition of 6 mM Ca^{2+} at pH 5.6 increased the number of nodules per plant elicited by *E. meliloti* 2011 but not by *R. favelukesii* LPU83. Unlike *E. meliloti*, the attachment of the acid-tolerant *R. favelukesii* LPU83 to alfalfa roots is not greatly affected by pH or Ca^{2+} concentration. In addition, media acidification weakens *nod* gene induction in *E. meliloti* strains but not in *R. favelukesii* LPU83. Moreover, the addition of Ca^{2+} at low pH does not affect either *nod* gene expression in alfalfa-nodulating rhizobia (*R. favelukesii* or *E. meliloti*) nor equality of *nod* gene inducers exuded by alfalfa plants. Therefore, Soto and others[29] suggested that, in divergence to other symbiotic systems, the most limiting factor in the establishment of the *E. meliloti*-alfalfa symbiosis at low pH is the attachment of bacterial cells to plant roots.

Consequently, any approach to improve the symbiotic performance of *E. meliloti* in acid soils must be focused on solving the rhizobial attachment to alfalfa roots at low pH. Concerning *R. favelukesii*, neither the root binding nor the *nod* gene expression was affected by acid pH or Ca^{2+}, indicating that the genetic background of *R. favelukesii* LPU83 may be useful for improving the performance of *E. meliloti* in acid pH soils.

In addition to the microsymbiont, plants are also negatively affected by acid pH and high Al^{3+} concentrations[21][28]. At toxic concentrations, Al^{3+} inhibits root growth and therefore decreases the absorption of nutrients by plants. In alfalfa, it was shown that Al^{3+} inhibits the synthesis of indol acetic acid (IAA) in apical buds and its transport, and stimulates the synthesis of callose that prevents symplastic translocation. This leads to an imbalance in the distribution of IAA in the roots, responsible for their defective growth[39].

The low pH and a high content of soluble Al^{3+} in soils disturb several physiological and biochemical processes, including nitrogen fixation, which significantly reduces the productivity and quality of alfalfa under field conditions[40]. Although conventional and transgenic varieties of alfalfa with variable grades of stress tolerance were developed[41][42], none of them were shown to reach optimum levels of nitrogen content or give good forage yield under low pH and high Al^{3+} conditions. This fact can be partially attributed to the inability of these stress-tolerant germplasms to preserve the beneficial plant-microbe interactions under stressful environments. While there is a robust legal framework for the regulation of transgenic plants and there are hundreds of commercial transgenic crops worldwide released for commercial agriculture production, practically no country allows the release of genetically modified microorganisms into agricultural ecosystems, and so, there is no genetically modified acid pH-resistant rhizobia inoculant in the market. In this context, commercial transgenic plants should be associated with beneficial microorganisms isolated from nature and free of genetic manipulations, at least in the near future.

The development of rhizobial inoculants is discussed below in the text, and in terms of alfalfa cultivars suitable in low pH - higth Al^{3+} soils have not been generated yet[21].

Response of alfalfa to inoculation

The presence of efficient rhizobia populations in the soils where legumes are cultivated hinders the observation of the response to inoculation. In Uruguay, this phenomenon has been observed for clovers[43][44] and lotus[45], but not alfalfa, for which the practice of inoculation represents a clear advantage[46]. Studies carried out in different regions of Europe showed a high native-naturalized microbial diversity that efficiently nodulate alfalfa[34][47][48][49], probably the result of the history of this crop introduced about 3,000 years ago[50]. In Spain, it is common to cultivate alfalfa without inoculation, although farmers from the North of the peninsula have sporadically used the strain GRO15 (= ISM-16; conversation with Rodríguez-Navarro; unreferenced), supplied by IFAPA (ex INIA) Seville[51]. However, Ramírez-Bahena and others[34] showed that under controlled conditions, inoculation of alfalfa with selected acid-tolerant strain improved plant biomass production. In Serbia, where the use of commercial inoculants for alfalfa is not a common practice[52], Stajkoviæ-Srbinoviæ and others[47] found that in soils with pH between 5.1 and 8.1 most of the nodules of *M. sativa* were occupied by *E. meliloti*. Additionally, Deliæ and others[52] identified effective native alfalfa strains in acid soils, which represented an interesting finding because 50% of Serbia's arable soils are acid. The effectiveness of these Serbian strains, present in soils with and without a history of alfalfa cultivation, was not influenced by the soil nor the host genotype[52]. In France, populations of rhizobia that nodulate alfalfa were identified in neutral soils (pH 6.8), even after 10 years without cultivating this legume[53]. In Germany, the presence of rhizobia in soils with pH 5.9 to 6.5 was undetectable after 8 years without alfalfa, but after inoculating with strain L33 and growing alfalfa, at least 48% of the nodules were occupied by native strains[54]. In soils from 10 sites of United Kingdom with moderately acid to alkaline soils (pH 5.8 to 8.2), Roberts and others[48] showed that not all of them had strains of *E. meliloti* and in most of them the inoculation significantly increased the number of nodules and biomass production.

In Oceania and America, where alfalfa was introduced 300-500 years ago[50], a clear response to inoculation is commonly observed[46][55][56]. For instance, in New Zealand alfalfa was observed to be highly dependent on the inoculation because it failed to grow in soils where alfalfa-nodulating rhizobia were absent or ineffective[57]. Likewise, in Australia, alfalfa inoculation is also necessary and extensive inoculant development was carried out as documented by Bullard and others[58].

In soils from tropical areas of Brazil, Ferreira and others[59] showed that there was no native population of *E.*

meliloti in the soil and the use of two commercial inoculants, under controlled conditions, increased the nodulation and productivity of three alfalfa cultivars. Oliveira and others[60] also observed a positive response to inoculation with the strain SEMIA-116, which make unnecessary the use of nitrogen fertilizers for alfalfa in the field.

In Argentina, Chile and Uruguay, different responses to inoculation, in relation to the pH and the population of *E. meliloti* present in the soil before cultivating, were observed by Racca and others[46]. In soils from Argentina with pH 6.2 with a high rhizobial population ($5.8x10^3$ rhizobia per gram of soil) a 13% higher yield was recorded in the treatment without inoculation than in the treatment inoculated with the commercial strain, indicating the presence of efficient naturalized populations. In contrast, a marked response to inoculation was observed in soils of Argentina and Uruguay with pH between 5.4 and 6.1, in which no rhizobia populations were detected, reaching increases of 109 % to 199% of biomass. Moreover, in Chilean soils with pH 5.7 and a moderate rhizobial population ($1x10^3$ rhizobia per gram of soil), a high response to inoculation was also observed (98% of biomass increase). Of note, the persistence of *E. meliloti* after harvesting the crop is low[46][61], particularly in acid soils, making the practice of inoculation in those cases necessary.

In addition to edaphic conditions, the alfalfa cultivar used can also determine a different response upon strain used as inoculant. In controlled conditions, Blair[57] observed different symbiotic efficiency in cultivars with different origins (subsp. *sativa* or subsp. *falcata*), whereas in field conditions the author identified some effective strains in a wide range of cultivars. On the other hand, in soils of Brazil containing lime, Oliveira and others[60] and Ferreira and others[59] did not observe different responses of the alfalfa 'Crioula' non-dormant cultivar when inoculated with different strains. Nevertheless, Hartel and Bouton[62] demonstrated that in acid soils the performance of alfalfa genotypes selected for acidity tolerance was enhanced by inoculant strains of rhizobia selected also for tolerance to acid pH. Of note, most alfalfa cultivars selected and used in the Southern Cone have not been accompanied by development rhizobia inoculants, or by studies considering the interaction cultivate alfalfa x rhizobia strain. In this sense, recommended inoculant strains should be evaluated with the commercial cultivars in the intended environment where they are going to be grown.

Liming has been a solution to improve alfalfa production in many countries with acid soils, such as in Brazil[63], Chile[64], Argentina[65], whereas this practice has not become widespread in Uruguay[66]. In any case, liming has economic and practical restrictions[21], so the development of inoculants that could establish efficient symbiosis with the cultivars used in acid soils is a strategy that must be strengthened.

Selection of rhizobia strains for development of alfalfa inoculants

Biological nitrogen fixation in agriculture can be improved with the use of rhizobial inoculants developed with strains selected by their high performance in target cultivars grown in specific soils and environments. Among the criteria for selecting strains as alfalfa inoculants, their tolerance to acid pH and persistence in acid soils should be considered, and in some cases their tolerance to Al^{3+}[13][16][21][29][64]. The tolerance to both acid pH and high Al^{3+} concentrations is rare, therefore these bacterial genotypes would be a minority within populations present in the soils[67]. Thus, developing a rhizobial inoculant suitable for such condition is a challenge, but the needing to promote a sustainable agriculture is leading several countries to make an effort towards this aim.

In Australia, a lot of work was done to select suitable inoculants for alfalfa and other legumes. For instance, different commercial inoculants were developed for perennial and annual *Medicago* species[21][30][58] in order to increase the production in acid and alkaline soils[1]. Acid soils with high levels of Al^{+3} constitute a problem that affects alfalfa cultivation in large areas of Australia and also of New Zealand. In these countries, *E. meliloti* strain RRI128 is used as a commercial inoculant[21]. This strain, which establishes symbiosis with perennial and annual *Medicago* species, was isolated from a nodule from the roots of barrel medic (*M. truncatula*) grown in a greenhouse in Victoria soil of Australia[68]. However, its origin is unclear and it is believed that it was isolated for the first time in 1995 in New Zealand[69]. While the RRI128 strain has been used in Australia since 2000[58], Wigley and others[21] recently showed that two strains evaluated at acidic conditions and at different concentrations of Al^{3+} were more effective than RRI128 in alfalfa, making them promising inoculants.

In Argentina, Chile and Uruguay the selection of rhizobia for alfalfa inoculants also focused on obtaining strains suitable for establishing efficient symbiosis in acid soils,

and in some cases to high concentrations of Al^{3+}. In Chile, alfalfa cultivation is an alternative for soils with pH 7[70][71], and soils of the south with pH 5.5 and high content of Al^{3+}, where strain AG-06 was identified as promising when evaluated in greenhouse conditions[64]. In Argentina, moderate acid-tolerant and efficient strains were obtained, among them the strain LPU63, which was evaluated under controlled conditions[13][16]. In addition, an evaluation of strains from different cultivated areas, concluded with the recommendation of *E. meliloti* strain B399 (= *R. meliloti* 102F34), which is currently the used inoculant for alfalfa in Argentina[72]. This strain is, almost genetically, equal to strain 1026 of *E. meliloti*, although it has a different symbiotic phenotype[73].

Regarding the inoculation of alfalfa in Uruguay, between 1964 and 1990 the strain U45 was used as a commercial inoculant[74]. This strain was isolated in Uruguay but its geographical origin is unclear. Between 1991 and 2003 an inoculant based on U137 and U143 strains was used, both isolated from Uruguayan soils, and since 2004 to date the strain U143, which is more stable than strain U45[75], has been used as a commercial inoculant. The strain U143 is used in soils with a pH between 5.0 and 7.7 in the dairy region, where 63 % of soils have pH <6.0 and 37% pH <5.7[76]. This level of acidity is critical for alfalfa nodulation and for the survival of *E. meliloti*. It should be noted that the intensification of milk production increased the use of short rotations based on grasses and nitrogen fertilizers[77]. This practice enhances the acidification of the soil.

The symbiotic efficiency of U143 strain, which does not persist in acid soils, was lower than the symbiotic efficiency of CE21, CE41 and CE47 strains under controlled conditions[11]. Currently, a project that aims to develop an inoculant for alfalfa suitable for soils in the dairy region of Uruguay (Faculty of Agronomy - INIA, 2018 - 2021) identified 3 strains, among 250, with symbiotic efficiency equal to or greater than the U143 strain, under controlled conditions at pH 6.5 and pH 5.6. Strains S8, E9 and L14 are promising for their efficiency at pH 5.6, which deserves further attention and evaluation under field conditions, as described in the following section.

Interestingly, in Australia ten different commercial inoculants for alfalfa have been used between 1953 and 2003, roughly one per decade[58]. This implies that inoculant selection should be seen as a continuous process, coupled to the selection and implementation of novel plant cultivars, as well as to changes in the cropping areas and in soil/environmental conditions. Moreover, the genetic stability of the bacterial strains should also be considered, because long-term storage could involve genotypic and phenotypic changes[78]. Bloem and others[79] showed differences in the phenotypes of the U45 strain from successive agar subcultures and the lyophilized parental strain, stored for 15 years. Among the differences they found altered ability to fix nitrogen, which reinforces the necessity to check inoculants periodically and make passages through the host under field conditions[80].

As mentioned above, acid tolerance was a major criterion to select for alfalfa inoculants. Additionally, the selection of strains that exhibit the so-called adaptive acid-tolerance response (ATR) must be considered. The ATR is defined as the resistance of cells to an acid shock when they have been previously grown at a moderately low pH. However, validation experiments with soil microcosms and on-field have not been carried out yet. It will be important to determine the possibility to preserve the physiology of acid-adapted rhizobia (ATR^+) in inoculants formulations — based on ATR^+ strains. Draghi and others[81] demonstrated that the ATR^+ can be induced in *E. meliloti*, as shown previously for *E. medicae*, and that the entrance of *E. meliloti* into the adaptive ATR occurs under batch cultivation conditions at moderately acid pH. In marked contrast, no increased tolerance to hydrogen ions was obtained if rhizobia are grown in a chemostat under continuous cultivation at same pH. In addition, they showed that ATR^+ significantly increased (30%) the competitiveness for nodule occupancy at low pH. In *E. medicae*, the two-component sensor-regulator system, *act*SR, was shown to be important for the induction of the ATR^+ phenotype[82]. In other organisms the ATR^+ phenotype confers cross-resistance to other stresses as well, such as heat, ethanol, and sodium chloride[83][84].The basic aspects of the ATR^+ phenotype have not been extensively characterized, and further research is needed to increase our knowledge on the bacterial mechanisms involved in this adaptive response. Clearly, the rational manipulation of the rhizobial ATR will require a detailed physiological and functional characterization of the processes. Meanwhile, the ATR phenotype can be an additional criterion to consider when selecting for acid-tolerant rhizobia.

Strategy for the development of alfalfa inoculants in Uruguay

The maximum potential of rhizobia-alfalfa symbiosis is not achieved at acid pH, so worldwide efforts are

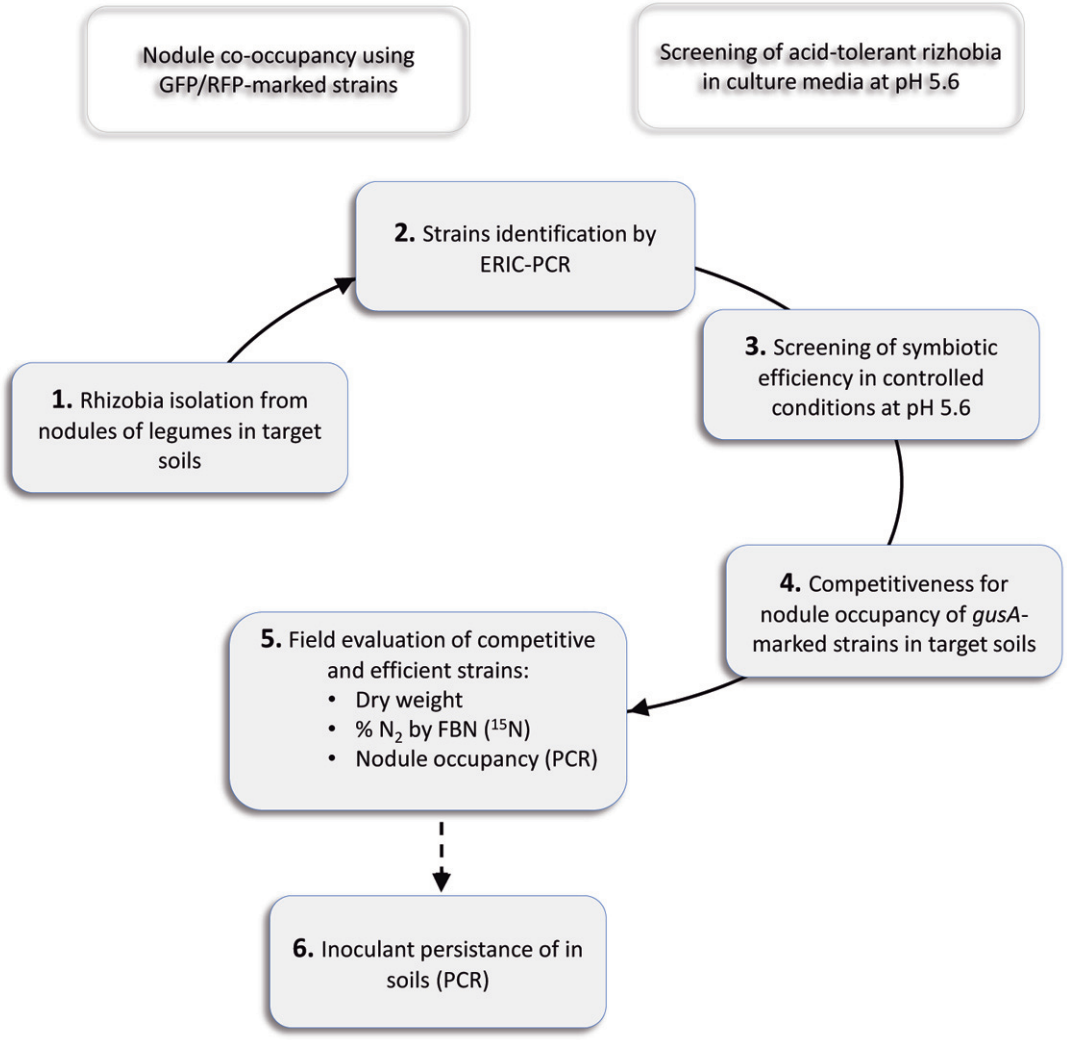

Figure 2. Scheme of the different stages followed for the selection of alfalfa inoculants in acid soils.

made in order to have suitable inoculants for that situation[16][27][46][52][62][85].

In Uruguay, the Biochemical and Microbiology Laboratories of the Faculty of Agronomy and the INIA Pastoral and Forage Improvement Group developed an efficient and competitive rhizobial inoculant for clover[43][44]. The current aim is to develop an alfalfa inoculant following the strategy used for clover (Figure 2).

In this sense, 69 strains were identified by their ERIC profiles, among 250 isolates of plants grown in soils with pH between 5.3 and 6.0, from 9 different sites belonging to the location known as «Dairy Basin». Symbiotic efficiency of these strains, and others from available collections, was evaluated and compared with the commercial inoculant under controlled conditions in pots with vermiculite:sand. Based on this criterion, in the Chaná cultivar, 16 strains were pre-selected in trials at pH 6.5 according to the biomass production accumulated in two cuts (Figure 3). The strains were then evaluated in plant trials at pH 5.6 in sand with irrigation solution buffered with MES[13]. The promising strains in that situation were S8, E9 and L14 isolated from La Estanzuela, Colonia (pH between 5.5 and 6.0), and SJ2 isolated from Juan Soler, San José (pH 5.7). Additionally, to the criteria used in the selection of clover strains, it is considered appropriate to incorporate for the development of an alfalfa inoculant, the evaluation of the acid tolerance of the strains and the inoculant

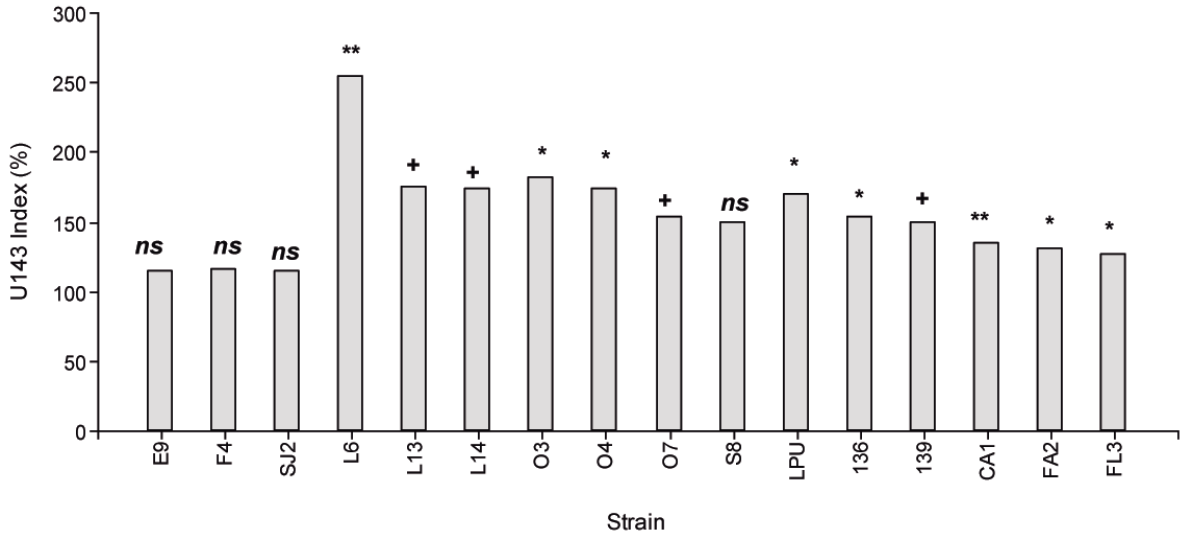

Figure 3. Cumulative production of aerial biomass in 45 days after cultivated under controlled conditions, expressed as % of strain U143. E9, L6, L13, L14, 03, 04, 07 and S8 strains isolated from Colonia, SJ2 from San José, and F4, FA2 and FL3 from Florida. LPU63 strains isolated in Argentina (Collection of the University of La Plata), U136 and U139 Uruguay (National Collection of strains from Ministry of Livestock, Agriculture and Fisheries). The data were analyzed using Mixed Generalized Linear Models (Gamma Family and Log link) InfoStat® [86] and the comparison of means was performed using the post-hoc DGC test of each strain with U143 [87]. The strains were higher than U143 at p <0.01 (**), p <0.05 (*), p <0.1 (+), ns corresponds to strains similar to U143. The N treatment (not shown) produced 109% more biomass than the commercial inoculant (p <0.001).

persistence in the field. Although acid tolerance in culture media is considered a favorable characteristic when selecting a rhizobia strain, this tolerance does not necessarily correlate with the persistence in acid soils or with the ability to express its symbiotic phenotype[16]. Evaluating the persistence of inoculants in the soils where it is used is interesting since the commercial inoculant currently used in Uruguay is not persistent, particularly when used in acid soils. As this characteristic is only known after several years of evaluation, the evaluation of persistence is included as the final stage of the selection strategy (Figure 2).

Although Uruguay has an outstanding position in the production and use of rhizobia inoculants, the commercial strains currently used for clovers, lotus and alfalfa were selected about 50 years ago in agronomic environments that have changed. These changes are a consequence of the displacement of cultivated pastures to other sites, the type of planting (conventional tillage versus non-tillage) and the use of new cultivars. For this reason, it is advisable to consider development of rhizobia inoculants as a continuous process that improves the competitiveness and persistence of strains in soils. In the case of alfalfa in particular, this will contribute to increasing the stability of annual and summer forage production and will lead to an expansion of the cultivation area.

Acknowledgments

Authors are grateful to the funding of grants from *Proyecto de investigación aplicada Fondo María Viñas ANII* (FMV 1-2017-1-135695); *Programa de Desarrollo de Ciencias Básicas* (PEDECIBA) and *Sistema Nacional de Investigadores* (SNI). We would like to thank Ing. Agr., MSc., Roberto Díaz-Rossello for his help.

Author's contribution

These authors contribute equally to this work.

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