



REVIEW

# Rhizobia biodiversity in Uruguay: preservation and uses

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## Abstract

This review presents a summary of the work done in Uruguay on the diversity and agricultural use of rhizobia, a group of soil bacteria able to establish symbiotic associations almost exclusively with legumes and fix atmospheric nitrogen. Uruguay has a long tradition regarding the use of microbial inoculants for agriculture. It is worth mentioning that since 1960, Uruguay has formally regulated the use of microorganisms in agriculture, being considered one of the pioneer countries where state policies on microbial inoculants have been implemented. In general, the vast majority of the work carried out in Uruguay has focused on the selection and characterization of promising bacteria to be used as biofertilizers. Therefore, the most studied symbiotic associations were those established with legumes of agricultural interest for the country, such as alfalfa, clover, lotus and soybean. The studies carried out with autochthonous alpha- and beta- rhizobia associated with diverse legume species are also considered, taking into account their ecological importance and the interest in knowing and preserving native germplasm.

**Keywords** Growth-promoting rhizobacteria (PGPR) · Rhizobial inoculants · Native strains · Rhizobia selection strategy

## Introduction

Rhizobia are soil bacteria whose most conspicuous characteristic is the ability to establish symbiotic associations with legumes acquiring the ability to convert atmospheric nitrogen (N<sub>2</sub>) into ammonia by a process called nitrogen fixation. A rhizobial species generally recognizes one or a few legumes as hosts; nonetheless, others have a promiscuous behavior and can nodulate different legume species and vice versa (Andrews and Andrews 2017). The most studied and best-characterized strains of rhizobia belong to the alpha-proteobacteria and mainly to the genera *Mesorhizobium*,

*Ensifer*, *Sinorhizobium*, *Rhizobium*, *Bradyrhizobium* and *Azorhizobium*. Rhizobia belonging to the group of beta-proteobacteria were discovered more recently and, so far, have only been found in the *Paraburkholderia*, *Cupriavidus* and *Trinickia* genera (Andrews and Andrews 2017; Chen et al. 2001; Dall'Agnol et al. 2017; Moulin et al. 2001).

This review will present the work done on the diversity of rhizobia associated with Uruguayan native legumes as well as on rhizobia associated with introduced legumes with agronomic uses. Although biodiversity is generally associated with fauna and flora, microorganisms are the main forms of life in sustaining biodiversity. Microorganisms are

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key participants in the biogeochemical cycling of elements vital for life, and any change in their activities can affect the processes in the soil, air, and water. Understanding the diversity of the native rhizobial germplasm is necessary to preserve and make a sustainable use of this natural resource.

Among the legumes with agronomic use, stand out *Trifolium repens* (white clover), *Trifolium pratense* (red clover), *Medicago sativa* (alfalfa), *Lotus corniculatus*, *Lotus uliginosus* and *Lotus subbiflorus* (lotus), that were introduced in Uruguay as forage legumes, mostly between the 1930s and 1960s. Accompanying the introduction of these legumes, in 1960, the Ministry of Livestock, Agriculture, and Fisheries (MGAP) established the bases for rational management of rhizobial inoculants to recover natural grasslands and facilitate the articulation of an agricultural-livestock production system. (Labandera 2007). Thereafter, the National Inoculant Industry and the legal framework were established to guarantee the quality of the products (Labandera 2005; Real et al. 2005). Most of the strains used as inoculants were evaluated decades ago (Labandera et al. 1985; Labandera 2005; Lindström et al. 2010), and, due to the displacement of the meadows and the incorporation of new cultivars and new agricultural practices, the search and evaluation of novel strains continues until today.

## Rhizobia that nodulate legumes of the Papilionoideae (Faboideae) subfamily

### Clover symbionts

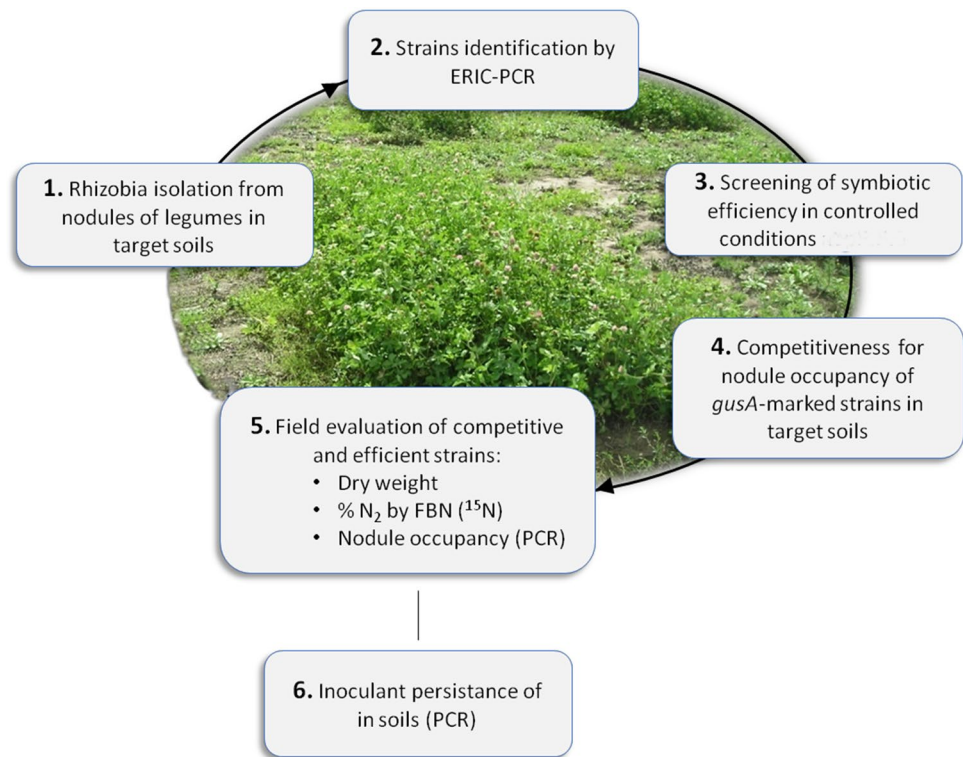
The clovers originally used in Uruguay were subterranean (*Trifolium subterraneum*), white (*T. repens*), and red clover (*T. pratense*). The currently used commercial inoculant for white and red clover was developed in the early 1960s. Between 1964 and 1967, *Trifolium* sp. was inoculated with a mixture of U20 (= TA1, Australia) and U26, then with strain U20 alone. Since 1967, both legumes have been inoculated with *Rhizobium leguminosarum* sv. *trifolii* strain U204 (= U28, = CIAT 2445) introduced from the United States of America (Labandera et al. 1982). Subterranean clover was initially inoculated with the Australian strain WU290 (= U185), and later with the strain U204. Thus, the U204 strain has been used as a commercial inoculant for almost 50 years. Although this strain has high efficiency in symbiosis with clover, a survey showed that about 50% of farmers abandoned the practice of inoculating clover and lotus seeds because they did not always see a benefit in it (DIEA 2010). The competitiveness of the strains is an essential characteristic of inoculants that was difficult to assess until molecular techniques were developed and became accessible (Bourion et al. 2018). Batista et al. (2015) evaluated U204 competitiveness and reported that the strain is rarely found in red

clover nodules under field conditions. Irisarri et al. (2019) showed that most white clover nodules were occupied by native-naturalized rhizobia, which we will generically call autochthonous rhizobia. The lower occupation of nodules by commercial inoculants compared to rhizobia strains already present in the soil has also been observed in other countries and different clover species (Denton et al. 2002; Duodu et al. 2007; Nangul et al. 2013).

Since the ability to fix nitrogen and the competition with other rhizobia to nodulate the host legume are not intrinsically linked, both characteristics must be considered separately to develop elite rhizobial inoculants (Checcucci et al. 2017). The strategy that allows the selection of competitive strains among the efficient ones is summarised in Fig. 1. Thus, Batista et al. (2013) and Irisarri et al. (2019) selected the strains U317 and N2, which are more efficient and competitive than the commercial inoculant for red and white clover (Table 1). Besides, the research on rhizobia competitiveness confirmed that: i) the nodule occupation by the inoculant was more than twice in soils with no history of clover cultivation concerning soils with history, ii) the occupation by native strains of root nodules in general and stolon roots, in particular, was five times greater than the inoculant, iii) in soils with a high number of efficient rhizobia, the response to inoculation may not be evident; otherwise, inoculation is essential.

The presence of parasitic strains constitutes a risk for establishing efficient symbioses (Labandera and Vincent 1975). *T. repens* presents some difficulties for the implantation as cover and reseeded on natural fields in the country's East and North. These failures are attributed to indigenous populations that nodulate *Trifolium polymorphum* and are inefficient-parasitic on Mediterranean clovers (Yates et al. 2005). Rodríguez et al. (2010) evaluated the competitiveness of two indigenous rhizobia strains (T70 and T126) against the commercial inoculant (U204) in pot experiments with *T. repens*, and *T. pratense*, and found that strain T126 showed good levels of nodule occupancy when co-inoculated with the commercial strain. However, T126 also led to a plant growth promotion comparable to that obtained with inoculation of U204. These results also open the possibility of finding new promising strains among autochthonous rhizobia. In a recent study, Morel et al. (2021) evaluated the competitiveness of the parasitic strain P3 on *T. repens*. P3 is scarcely found in red clover nodules when co-inoculated with high concentrations ( $> 1 \times 10^6$  UFC/seed) of inoculant U204. However, most red clover nodules were occupied by the parasitic P3 when co-inoculated with inoculant concentrations lower than  $1 \times 10^4$  UFC/seed. Commercial inoculants' lower occupation of nodules when the parasitic rhizobia are present in the soil is clear evidence of the need for inoculation before pasture sowing.

**Fig. 1** Selection strategy of efficient and competitive strains to improve FBN in grasslands



**Table 1** Rhizobia strains isolated from Uruguayan soils promising for their use as inoculants of forage legumes

Rhizobia	Strains	Isolated from	Reference
<i>Rhizobium anhuiense</i>	U317	<i>Trifolium pratense</i>	Batista et al. (2015)
<i>Mesorhizobium</i> sp.	N2		Tartaglia et al. (2029)
	U125	<i>Lotus corniculatus</i>	Sotelo et al. (2011)
	S948		
	N441		
	N22		
<i>Bradyrhizobium</i> sp.	L30	<i>Lotus uliginosus</i>	Camargo (2012)
	Lul17	<i>Lotus subbiflorus</i>	Irisarri et al. (1996)
	Ls7	<i>Lotus angustissimus</i>	In field evaluation
	GL7		
	GL9		
<i>Bradyrhizobium</i> sp.	Oc8	<i>Crotalaria ochroleuca</i>	Berriel et al. (2021)
<i>Ensifer meliloti</i>	CE41	<i>Medicago sativa</i>	Castro-Sowinski et al. (2002)
	CE47		In field evaluation
	CA1		
	L14		

The phylogenetic relationships between rhizobia with different symbiotic efficiency present in Uruguayan soils were analysed through sequences of housekeeping, 16S rRNA, and symbiotic genes by Tartaglia et al. (2019). The authors found that efficient autochthonous strains and strain U204 grouped in the same cluster separated from parasitic native strains. Furthermore, the efficient strains were grouped into two groups: one with *R. leguminosarum*-related strains and the other were *Rhizobium anhuiense*-related strains. The results clearly show the horizontal gene transfer (HGT) from

the commercial inoculant to autochthonous rhizobia strains with high symbiotic efficiency (Tartaglia et al. 2019). Interestingly, the HGT of symbiotic genes from strain U204 to 1116, a parasitic native strain, is accompanied by a partial improvement in its symbiotic efficiency with white clover (Morel et al. 2021).

## Lotus symbionts

The legumes best adapted to Uruguay's climatic and edaphic conditions belong to the genus *Lotus*, consisting of species of different symbiotic groups. While the *L. corniculatus* complex (*L. corniculatus*, *L. tenuis*, and *L. japonicus*) establishes efficient symbioses with *Mesorhizobium* sp., the *L. uliginosus* complex (*L. uliginosus*, *L. subbiflorus*, and *L. angustissimus*) do so with *Bradyrhizobium* sp.

*L. corniculatus* was inoculated from 1964 to 1971 with U24 strain that originated in the United States and from 1972 until the present with U510 strain (=U226;=B816) introduced from Australia (Labandera et al. 1982). *L. tenuis* is also inoculated with this strain.

On the other hand, there is a high diversity of autochthonous strains that nodulate these legumes in soils, with equal or greater symbiotic efficiency than the commercial inoculant (Baraibar et al. 1988; Baraibar et al. 1999; Monza et al. 1992; Sotelo et al. 2011). These autochthonous strains belong to *Mesorhizobium* species, *M. huakuii*, *M. amorphae*, *M. septentrionale* and *M. caraganae*, according to the partial sequences of 16S rRNA, *atpD*, and ITS16-23S genes analysis. At the same time, they cluster with *M. tarimense* and *M. loti* based on symbiotic *nodC* and *nifH* genes (Sotelo et al. 2011). The discordance between clusters is evidence of probable events of HGT, in this case of symbiotic islands, between *Mesorhizobium* strains (Ramsay and Ronson, 2015). Although the HTG explains the cluster of autochthonous strains with *M. tarimense* and *M. loti* (R7A and NZP 2213), the origin of the symbiotic genes is not necessarily the latter strains, whose use in Uruguay is not documented.

If we consider that the U510 strain has been used as a commercial inoculant for decades, the fact that it was not frequently found among the isolates analysed would indicate a low adaptation to our edaphoclimatic conditions. Some of these promissory strains are presented in Table 1. The acquisition by autochthonous strains of symbiotic genes from introduced strains seems to explain the origin of efficient populations. Also, the response to inoculation is more pronounced in soils without a history of inoculation (Pérez and Labandera, 1998).

*L. uliginosus*, introduced in the 1980s, has been inoculated since 1995 with strain NZP 2309 (=U526,=U1401) isolated in New Zealand (Batista et al. 2013). The ERIC profiles of *L. uliginosus* rhizobia isolated from different regions with contrasting soil conditions (pH and Al<sup>3+</sup> concentration) and different inoculation histories show a high rhizobial diversity (Batista et al. 2013). The commercial inoculant NZP 2309 profile was identified in 9 nodule isolates out of 111 analysed (Batista et al. 2013), showing an adaptation to different edaphoclimatic conditions (Table 1).

Interestingly, the phylogeny of symbiotic genes (*nodC* and *nifH*) showed that the autochthonous strains cluster mostly with NZP 2309, while all of them were *Bradyrhizobium* bv. *genistearum* (Batista et al. 2013). Among these rhizobia, HGT would also have been responsible for the spread of symbiotic genes within the genus *Bradyrhizobium* (Lorite et al. 2018).

Due to *Mesorhizobium* sp. strain NZP2037 (=U261) ability to nodulate both *L. uliginosus* and *L. corniculatus* plants (Gonnet and Diaz, 2000; Irisarri et al. 1996), this strain was used as an inoculant in the 1980s. However, its use was abandoned due to its moderate-low efficiency in both hosts (Irisarri et al. 1996).

*L. subbiflorus* is inoculated with *Bradyrhizobium* sp. strain U531 (Table 1) isolated from Uruguay, although there is no record of the precise site and date of isolation. On the other hand, a study of strains isolated from *L. subbiflorus* nodules from two regions of Uruguay showed that they are fix<sup>+</sup> in *L. uliginosus* (Irisarri et al. 1996).

Other *Lotus* spp. incorporated in grassland are *L. angustissimus* and *L. uliginosus* cv. E-Tanin (2n). None of them has a defined inoculant for their own; instead, they are inoculated with the U531 and NZP 2309 strains, respectively. Other vegetal materials to consider are the interspecific hybrids G1 (*L. uliginosus* x *L. corniculatus*) and G5 (*L. corniculatus* x *L. uliginosus*) (Castillo et al. 2012; Castillo 2012). These hybrids constitute a particular situation because the parental species belong to different inoculation groups. In both hybrids, a "mother effect" was observed, and the most efficient strains are U531 and U526 in G1 and G5, respectively (Mederos 2016).

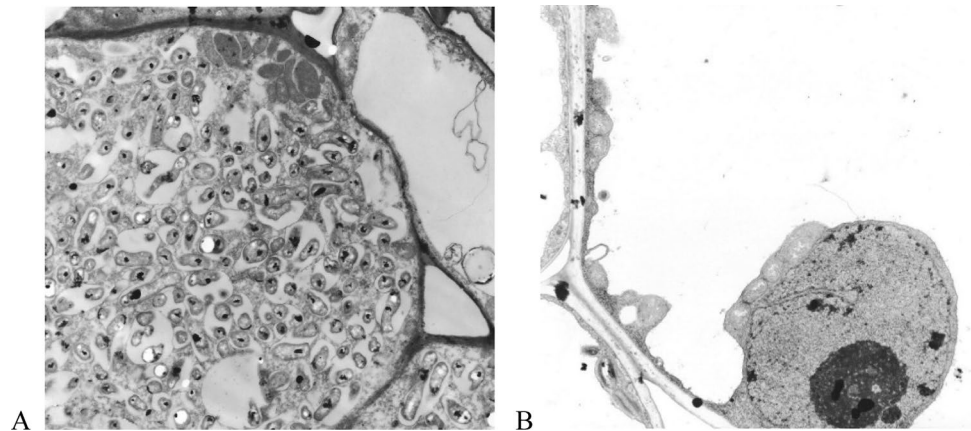
When rhizobia from one lotus symbiotic group nodulate a host from the other group inefficiently, a parasitic relationship is established (Lorite et al. 2018). This occurs when *L. uliginosus* is planted in soils where *L. corniculatus* was previously sown (Irisarri et al. 1996; Monza et al. 1992). Figure 2 shows electron microphotography of nodules induced by *Mesorhizobium* sp. strain BM15 in roots of *L. corniculatus* whose cells are occupied by bacteroids and pseudonodules with cells without bacteroids in roots of *L. subbiflorus*.

## Alfalfa symbionts

Alfalfa (*M. sativa*) is used in pastures, either alone or in mixtures with other legumes and perennial grasses. In opposition to lotus and clover, all the rhizobial strains used as commercial inoculants were isolated from Uruguayan soils and belong to *Ensifer meliloti* species. However, there is no record of the precise site of isolation. Between 1964 and 1990, strain U45 isolated from a *Melilotus* sp. nodule was used as inoculant (Labandera et al. 1982), and a mixture of two strains, U137 and U143, was used afterward (1991–2003). Since U143 (=MCH3) is genetically more



**Fig. 2** Ultrastructure of root nodules induced by *Mesorhizobium* sp. strain BM15. **A** Nodule cell induced in *L. corniculatus* root. Cell with bacteroids within the symbiosomes (X 3.200). **B** Nodule (pseudonodule) induced in *L. subbiflorus*. Cells without bacteroids, with cytoplasm, nucleus and organelles displaced by the vacuole (X 3.900)



stable than the U45 strain, it has been used as a commercial inoculant from 2004 up to now (Altier et al. 2013).

In Uruguay, the soil pH where alfalfa is grown ranges from 5.0 to 7.7 (Morón and Baethgen 1996). About 63% of soils have pH < 6.0, whereas 37% have pH < 5.7, a critical acidity level for alfalfa nodulation and rhizobial survival (Altier et al. 2013). Del Papa et al. (2003) and Glenn and Dilworth (1994) reported that pH below 5.8 restricts *E. meliloti* growth and persistence. However, several researchers in the region have identified rhizobial strains with tolerance toward an acidity of pH 5.5, which have the potential to be used as inoculants (Castro-Sowinski et al. 2002; Del Papa et al. 1999; Segundo et al. 1999). The search for pH-tolerant rhizobia strains continues. Although two strains have performed well in field studies, their symbiotic efficiency and competitiveness did not surpass U143 in the evaluated soils (Table 1).

In addition, in acidic soils, there are parasitic, promiscuous and competitive Oregon-type rhizobial strains that constitute a challenge for establishing an efficient symbiosis (Castro-Sowinski et al. 2002; Torres-Tejerizo et al. 2011). The genome of the ORY1 parasitic strain, isolated from Uruguayan acidic soil, was recently sequenced (Berais et al. 2021). ORY1 belongs to *Rhizobium favelukesii*, such as the strains LPU83 and OR199, isolated from acidic soil in Argentina and the USA, respectively (Torres-Tejerizo et al. 2016). Estimating the competitiveness of the parasitic ORY1 strain with respect to the commercial inoculant U143 showed that the ORY1 strain occupied 20% of the nodules when the inoculum ratio was 1:99 (parasitic:commercial) and 70% when the ratio was 99:1 (Berais et al. 2021). Recently, it has been suggested that the competitiveness of *R. favelukesii* in acidic soils, can represent a resource of genetic information useful to improve *E. meliloti* performance in these soils (Tabáres et al., 2019).

Some non-rhizobial plant growth-promoting rhizobacteria (PGPR) have been tested in co-inoculation experiments

with alfalfa in Uruguay. Examples are bacteria of the genus *Pseudomonas* (De la Fuente et al. 2002; Bagnasco et al. 1998, Quagliotto et al. 2009), and *Delftia* (Braña et al. 2016). Some of these non-rhizobial-bacteria can enhance the *E. meliloti*-alfalfa symbiosis by the production of phytohormones (mainly auxins) (Morel 2010; Morel et al. 2011); qualitative change of flavonoids pattern secreted by plants (Morel et al. 2016a); or solubilization of non-available nutrients, mainly phosphorus (Yanes 2014).

*Delftia* sp. strain JD2 is a native bacterium isolated from heavy metal contaminated soil with plant growth-promoting activities (Morel et al. 2011, 2010). This strain has been studied since 2005 because of its potential use in bioremediation strategies and agriculture (Morel et al. 2016a). JD2 strain can produce auxins that stimulate root growth and siderophores, and acts as a “helper” bacterium, enhancing legume-rhizobia symbiosis, such as alfalfa-*E. meliloti* association. The strain can colonise alfalfa roots and enhances its nodulation and biological nitrogen fixation (BNF) (Morel et al. 2014).

### Soybean symbionts

In Uruguay, the soybean or soya bean (*Glycine max*) has had significant growth in the last two decades, remaining one of the country's main crops during the last years (DIEA 2021). The commercial formulations currently available in Uruguay for soybean contain two strains of *Bradyrhizobium elkanii* of Brazilian origin: U1301 (SEMIA 587) and U1302 (SEMIA 5019), both were recommended in 1984 by MGAP. The use of this mixed inoculant is based on the symbiotic efficiency of different rhizobia. *B. elkanii* U-1301 and U-1302 are also two of Brazil's four bradyrhizobia strains (SEMIA 587, SEMIA 5019, *B. japonicum* 5079, and *B. diazoefficiens* SEMIA 5080) recommended for soybean inoculants (de Souza et al. 2019). The presence of *B. japonicum* SEMIA 5080 strain was verified in Uruguayan soils with a history of soybean crops. This strain has high persistence and great

competitive capacity (Lagurara 2018). In addition, new "naturalized" strains of soybean nodulating bradyrhizobia were reported by Rodríguez et al., (2023). Some of these naturalized strains were better than the commercial mixture of *B. elkanii* U-1301 and U-1302 strains (Rodríguez et al. 2023), possibly due to a better adaptation to the environment and changes in their symbiotic characteristics.

A new national formulation for soybean-based on *B. elkanii* U-1301 and U-1302 grown in a culture medium containing isoflavones was reported by Riviezzi et al. (2020). The authors found an improved plant response (nodulation, plant growth, and seed yield) when seeds were inoculated with the new formulation.

After a decade of research on the tripartite association between soybean, *B. elkanii*, and *Delftia* sp. JD2, several authors reported that the co-inoculation of seeds with the strain JD2 leads to plant growth promotion (Cagide et al. 2018; Riviezzi et al. 2021a, 2021b). Many strains from *Delftia* sp. are known for their plant-growth-promoting properties (Bhat et al. 2022; Braña et al. 2016; Ubalde et al. 2012). The co-inoculation of seeds with bradyrhizobia and *Delftia* sp. JD2 results in increased root biomass and nodulation, early root nodulation, and increased yield. Moreover, under greenhouse conditions, plant growth is promoted in co-inoculated plants in the vegetative stage (fourth node stage, or V4). A significant foliar accumulation of total N and ureides occurs in co-inoculated plants at the reproductive stage of full flowering (R2). Co-inoculation effects are also reflected in a higher grain yield (Cagide et al. 2018). Recently Riviezzi et al. 2021a reported that the seed's co-inoculation changes the flavonoids and other phenolic compounds' composition either in the rhizosphere or the roots. Additionally, this co-inoculation produces changes in the root phytohormones' composition, especially cytokinins and auxins. Riviezzi et al. 2021b suggested that co-inoculation with JD2 prepares the soybean plants to deal with unfriendly environmental conditions. The multi-resistance and safety of *Delftia* sp. JD2 was studied in (eco) toxicological tests and in silico analysis (Morel et al. 2016b).

### Lupine symbionts

*Lupinus* is a legume genus with more than 270 described species, distributed mainly in the American continent (Azani 2017, Drummond 2012). Grains of lupines have a high protein content, which ranges from 30 to 50% of its dry weight, and they are used in both human and animal nutrition, being considered an alternative to soybeans (Abraham et al. 2019). In Uruguay, seven species of native lupines have been identified: *L. albescens*, *L. bracteolaris*, *L. gibertianus*, *L. lanatus*, *L. linearis*, *L. multiflorus* and *L. paraguayensis* (Izaguirre and Beyhaut 2003; Alonso Paz 1991), but none of them is currently used with agronomic purposes. About 4 years ago, *L.*

*angustifolius* was introduced as a complementary winter crop to soybean. Costa et al. (2023) surveyed nodules of native species of lupines in order to isolate and identify their microsymbionts. According to the 16S rRNA gene analysis, the isolates belonged to various species within the *Bradyrhizobium* genus and grouped in at least six well-defined clusters. Representative strains from each cluster were selected, and symbiotic traits such as *nifH* and *nodA* phylogenesis and symbiotic specificity with different lupine species were characterized. Results indicated that symbionts of native lupine species were diverse and constitute a population of rhizobia that should be taken into account when selecting promising strains for commercial use (Costa et al. 2023).

### Symbionts of *Crotalaria* spp. and *Cajanus cajan*

*Crotalaria ochroleuca*, *Crotalaria juncea*, *Crotalaria spectabilis*, and *Cajanus cajan* are legume species used as cover crops (Berriel et al. 2020; Oliveira et al. 2007; Pereira et al. 2016). These tropical forage legumes can be nodulated by a relatively large group of rhizobia (Jorin et al. 2021). Berriel (2022) has identified several rhizobial strains in root nodules of *C. ochroleuca*, *C. juncea*, *C. spectabilis* and *C. cajan*, grown in soils of Uruguay. Recently, a strain isolated from a nodule of *C. ochroleuca* was characterized and its draft genome published. Results obtained suggest that this strain (named as Oc8) may represent a new species of *Bradyrhizobium* (Berriel et al., 2021).

### *Sesbania* symbionts

1. The genus *Sesbania* contains more than 50 species, including some species able to grow well in waterlogged conditions and form N-fixing nodules, both on the roots and stems (de Farias et al. 2019). *Sesbania* is highly promiscuous in nodulation and species of *Rhizobium*, *Mesorhizobium*, *Sinorhizobium*, *Azorhizobium* and *Agrobacterium* have been found as microsymbionts (Andrews and Andrews, 2017). Interestingly, the opposite is not true, and *Azorhizobium* has been only found associated with *Sesbania* spp. In Uruguay, two native *Sesbania* spp. have been described, *S. virgata* and *S. punicea*, that are perennial shrubs (Frioni et al. 2001). While *Azorhizobium* was the only genus recovered from nodules of *Sesbania* plants surveyed on the National Park of Esteros de Farrapos (Pereira-Gómez et al. 2020), Rodríguez-Blanco et al., 2008, reported the presence of rhizobial strains whose 16S rRNA sequence was closely related to *Rhizobium etli* and to *Agrobacterium* spp. in addition to *Azorhizobium doebereineriae* stains in agreement with the low specificity observed for this genus of legumes.

## Diversity of rhizobia that nodulate legumes of the Mimosoid clade

Classical legume classification divided the family into three subfamilies: Papilionoideae or Fabaceae, Caesalpinioideae, and Mimosoideae. However, current phylogenies divide the Leguminosae into six subfamilies, placing the mimosoid clade within the Caesalpinioideae subfamily (Azani et al. 2017). The so-called mimosoid clade comprises around 75% of the *Caesalpinioideae* species, including some of the largest genera in the legume family: *Acacia* (> 1,000 spp.), *Mimosa* (> 500 spp.), *Inga* (c.300 spp.), *Vachellia* (c. 160 spp.) and *Senegalia* (c. 220 spp.). Legumes of this clade are extremely diverse in height and growth habits, including lianas trees, shrubs, and herbaceous plants (LPWG 2022).

In Uruguay, 86 legume species belonging to the mimosoid clade have been described, genus *Mimosa* being the most diverse with around 50 species (Izaguirre and Beyhaut 2003). The presence of nodules was described in species of the genus *Mimosa*, *Parapiptadenia*, *Prosopis*, *Desmanthus*, *Neptunia*, *Albizia*, *Inga*, and *Calliandra* (Frioni et al. 1998; Pereira-Gomez et al. 2020; Platero et al. 2016; Taulé et al. 2012; Zabaleta 2015).

In 1998, Frioni et al. published a survey on the presence of root nodules of 17 Uruguayan woody legumes of the mimosoid clade, including *Vachellia caven*, *Inga vera*, *Calliandra tweedii*, *Parapiptadenia rigida*, *Senegalia praecox*, *Prosopis nigra* and *Prosopis affinis*. Nodules were collected from young plants (2–3 ys) growing in different native forests or from nurseries, and their ability to fix nitrogen was assessed through the acetylene-reduction assay. The ability to develop nitrogen fixing nodules was confirmed for rhizobia strains isolated from *V. caven*, *C. tweedii*, *P. rigida*, and *Prosopis* spp.. Seven isolates were further characterized according to their specific growth rate, carbon source utilization, and acid production, leading to its assignment to the fast-growing group of rhizobia (Milnitsky et al. 1997). The seven isolates could be distinguished according to their whole protein profiles in SDS-PAGE and plasmids presence, indicating that they represent unique strains. Additionally, promiscuity and effectivity were assayed for *V. caven* symbionts (Frioni et al. 1998). The authors concluded that *V. caven* was promiscuous for nodulation, being nodulated with both fast- and slow-growing strains but specific in effectivity since the amount of N<sub>2</sub> fixed markedly depended on the strain group.

Taulé et al. (2012) identified by 16S rRNA analysis, rhizobia found in nodules of the mimosoid legume tree *P. rigida*. The survey included samples obtained from trees present in native forests, from *P. rigida* plantations as well as plant-trap experiments. From the collection, 47 isolates were selected for further studies. According to ERIC-PCR

patterns and RFLP analysis of both, *nifH* and 16S rRNA genes, the isolates were grouped into seven genotypes, that included the genera *Paraburkholderia*, *Cupriavidus*, and *Rhizobium*, amongst which the *Paraburkholderia* genotypes were predominant. Representative strains of each genotype were selected for phylogenetic analyses of 16S rRNA, *nifH* and *nodA* genes. Results obtained indicated that symbiotic genes followed the evolutionary history of the housekeeping gene and suggested the presence of new beta-rhizobia species among both, *Paraburkholderia* and *Cupriavidus* genera. Overall, results indicated that beta-rhizobia were the main *P. rigida*-nodulating rhizobia in sites where this leguminous tree was naturally present, *Paraburkholderia* being the most represented genus. Nevertheless, *P. rigida* can also be nodulated by alpha-rhizobia belonging to *Rhizobium* and *Sinorhizobium* genera. To go deeper into the knowledge of the symbiotic traits encoded by these bacteria, two beta-rhizobia strains were selected for whole genome sequencing: strain UYPR4.13 belonging to the *Paraburkholderia* genus and strain UYPR2.512 belonging to the *Cupriavidus* genus (De Meyer et al. 2015a; De Meyer et al. 2015b).

The finding that beta-rhizobia were present as natural symbionts of *P. rigida* prompted the search for other legume hosts harboring this group of rhizobia. Beta-rhizobia had already been described as a common symbiont in many legumes, particularly in *Mimosa* spp. from the Cerrado and Caatinga biomes in Brazil (Bontemps et al. 2010; Do Reis Jr. et al. 2010). Uruguay is considered the southern limit for *Mimosa* spp. distribution. Sierra del Abra de Zabaleta in southern Uruguay contains a natural population of 5 *Mimosa* spp.: *M. magentea*, *M. ramulosa*, *M. schleidenii*, *M. reptans* and *M. amphigena*. Phylogenetic analyses based on sequences of the 16S rRNA, *recA*, and *gyrB* core genome and the *nifH* and *nodA* symbiosis-essential loci confirmed that all the rhizobia isolated from these legumes belonged to the *Cupriavidus* genus. However, none belonged to the well-described symbiotic species *Cupriavidus taiwanensis*. Instead, they were closely related to other species, such as *C. necator*, and to species not previously known to be symbiotic (or diazotrophic), such as *C. basilensis* and *C. pinatubonensis* (Platero et al. 2016). These findings support the idea that native legumes, particularly *Mimosa* spp. are natural reservoirs for novel rhizobia.

Further efforts to study the diversity of rhizobia symbionts of native legumes were centered in a protected National Park known as Esteros de Farrapos e Islas del Río Uruguay. Thirty native legume genera comprising 38 species were identified in this area including three *Mimosa* spp. (*M. uragiensis*, *M. adpressa* and *M. pilulifera*) as well as *V. caven*, *Albizia inundata*, *Inga vera*, *Calliandra parvifolia* and *Senegalia bonaerensis* from the mimosoid clade. These eight legume species were found nodulated in nature, however, no isolates could be obtained from *A.*



*inundata*, and *S. bonaerensis*. *C. parvifolia* was found nodulated by bacteria from the *Rhizobium* and *Paraburkholderia* genera. *Paraburkholderia* spp. strains were also found in all the inspected nodules obtained from *M. pilulifera* plants. Conversely *M. uragiensis* and *M. adpressa* were always found nodulated by *Cupriavidus* spp. Specificity in plant-rhizobia interaction was also observed for *I. vera*, which was always found associated with *Bradyrhizobium* spp. and for *V. caven*, which was found always associated with *Mesorhizobium* spp.. This selective association was also observed for other legumes outside the mimosoid clade, as for the Papilionoideae *Aeschynomene* and *Arachis* that were found associated only with *Bradyrhizobium* (Pereira-Gómez et al. 2020).

## Conclusions

The symbiotic nitrogen fixation supplies N to the plant and the soil in an environmentally friendly way. Thus, the rhizobia that establish symbiosis with legumes of agricultural interest such as lotus, clover, alfalfa, and soybean have been the most studied in Uruguay. The results showed that there is a diversity of autochthonous rhizobia strains that establish symbiosis with specific legumes, with equal or greater efficiency than the corresponding commercial inoculant. These promising rhizobia strains, belonging to the genera *Bradyrhizobium*, *Mesorhizobium*, *Rhizobium* and *Ensifer*, make up a collection whose field evaluation, which is at different stages of progress, should continue. It is interesting that, in some soils, good yields are obtained without the use of inoculants. But inoculation, a common practice in Uruguay for more than 50 years, is still recommended due to the risk involved in not doing it, particularly in crops such as alfalfa and soybeans.

Meanwhile, studies of alpha and beta rhizobia, also autochthonous, associated with various species of native and introduced legumes have been carried out due to their ecological importance and the interest to conserve native germplasm.

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## Declarations

**Conflict of interest** On behalf of all authors, the corresponding author declares that there is no conflict of interest. The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported.

**Research involving human participants and/or animals** This research has not involved Human Participants and/or Animals. The submitted work is original and was not submitted been published elsewhere in any form or language.

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