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Diversity and Symbiotic Effectiveness of Indigenous Rhizobia-Nodulating *Adesmia bicolor* in Soils of Central Argentina

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Abstract Native perennial legume *Adesmia bicolor* reveals characteristics that are key to securing persistence under grazing. Literature on the diversity and symbiotic effectiveness of indigenous rhizobia-nodulating *A. bicolor* in central Argentina is limited. The purpose of this study was therefore to determine phenotypic and genotypic variability as well as biological N-fixation effectiveness in rhizobia isolated from *A. bicolor* nodules. To this end, repetitive genomic regions were analyzed using ERIC primers. In the greenhouse, plants were grown under a (i) N-fertilized treatment, (ii) N-free control treatment, and (iii) rhizobia inoculation treatment. Dry weight and N-content were analyzed. All isolates belonged to *Rhizobium* genus and showed high symbiotic effectiveness. The N-content/subterranean N-content ratio in aerial and subterranean parts of inoculated plants was higher than that observed in N-fertilized plants during the vegetative stage. Results from this study demonstrate that symbiosis between native rhizobial strains and *A. bicolor* is very effective.

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Introduction

Biological nitrogen fixation (BNF), which is the second most important biochemical process on Earth after photosynthesis [22], provides substantial amounts of N to plants and soils and therefore reduces the need for industrial fertilizer application [10]. In most cases, this process depends on the relationship between plants and bacteria. Legume-nodulating bacteria, often called rhizobia, usually live as saprophytes in the soil and in a facultative symbiosis with plants [7]. They induce the formation of nodules in the majority of legumes within which they fix N_2 and provide it to the plant in exchange for carbon compounds. Nodule structure and infection site are believed to be largely plant determined [20]. Nodule morphology and infection processes, within any given phylogenetic lineage, are generally the same regardless of which species or genus of rhizobia is involved.

Rhizobia are usually abundant in soils where legumes are either cultivated or form part of native flora [7]. Indigenous rhizobia are those found naturally in the soil of a given locality and widely distributed in the soils of diverse geographical areas around the world [22]. Soils containing indigenous rhizobia are, on the one hand, problematic as they create a barrier to effective inoculants in nodules of target host plants [46] while, on the other, they are not only highly adapted to their local soil environments but also have the ability to develop more effective symbioses with respect to commercial inoculants isolated from a distant soil environment.

In the last years, indigenous rhizobia have received much attention and, therefore, the number of identified species having the ability to associate with legumes has increased [22]. During the last years, research has provided interesting findings related to rhizobial taxonomy, such as the identification of β-Proteobacteria and new genera of

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α-Proteobacteria, both of which had not been previously reported as N_2-symbiotically fixing organisms [21, 25, 26, 35, 48, 52, 53]. The genera that have usually been considered as N_2-fixing organisms are *Rhizobium*, *Bradyrhizobium*, *Sinorhizobium*, *Mesorhizobium*, *Allorhizobium*, and *Azorhizobium* [23], and recently *Methylobacterium* [19], *Devosia* [42], *Blastobacter* [41], *Burkholderia* [10], *Cupriavidus* [30], *Ochrobactrum* [57], *Phyllobacterium* [24], and *Microvirga* [3] have also been reported as N_2-fixing organisms.

Rhizobia diversity is related to the high number of legume species with which rhizobia associate and to their geographical distribution. Interestingly, some rhizobia are particularly specific whereas others associate with different hosts [14, 22, 48, 51]. Although cross-inoculation with a legume [1] is possible, the wide diversity of rhizobia requires more precise matching in symbioses. This specificity guarantees maximal N_2-fixation [22]. Effective symbiosis allows legumes to colonize N-deficient soils, thus increasing their fertility.

Arid and semiarid areas occupy a surface portion on earth that is increasing with time. Although the range of legume flora in these areas is very diverse, little is known about their nodulating and N-fixing ability in their native habitats [36]. These species have to endure severe environmental conditions. Thus, rhizobia that are associated with legume flora growing in arid areas have the ability to establish symbiosis under stress environmental conditions [28, 29, 51, 55]. This is the reason why the selection of indigenous strains that have a high N_2-fixing ability and are adapted to a wide range of environmental conditions at a specific site which is key to maximizing legume production [15].

In central Argentina, new forage species adapted to arid and semiarid environmental conditions are needed. Studies have been conducted on *A. bicolor*, a species with valuable morphological and physiological characteristics which could help secure its persistence under intensive grazing [4, 5, 13, 50]. Further studies have also been carried out in order to determine both the nodulation pattern and N-fixation effectiveness under greenhouse conditions [44]. Nonetheless, the literature on the diversity of indigenous rhizobia-nodulating *A. bicolor* in central Argentina and their symbiotic effectiveness is still limited. In view of this, the purpose of this study was to determine: (i) the phenotypic and genotypic diversity in rhizobia from arid and semiarid locations from central Argentina and (ii) their nodular morphology and BNF effectiveness in association with *A. bicolor*.

Materials and Methods

Plants and seeds of *A. bicolor* were collected from Villa Rumipal, province of Córdoba, Argentina, at 32°11′S and 64°28′O, 550 masl. *Adesmia bicolor* grows in very fine sandy loam soils with pH 6.6 and 3.25 % organic matter.

Phenotypic and Genotypic Characterization of Strains

Nodules were obtained from plants collected in the field. They were surface-sterilized with ethanol for 1 min, 6 % sodium hypochlorite for 3 min and exhaustively washed in five changes of autoclaved distilled water. Intact surface-sterilized nodules were rolled and placed on yeast extract–mannitol (YEM) plates as controls to detect any possible contamination. Each nodule was crushed on a sterile plate, and bacteria were isolated on YEM agar plates [45]. Isolates were grown at 28 °C, and the purity of the colonies was checked by repeated streaking of single colonies on YEM plates and by microscopy examination. Pure cultures were preserved in 20 % glycerol at −80 °C. The phenotypic characteristics of these strains were determined estimating their growth velocity in YEM medium by the method of Vincent [39, 40].

In order to identify the strains that evidenced highest effectiveness, *A. bicolor* seeds were scarified and sterilized with ethanol for 1 min, 6 % sodium hypochlorite for 3 min and exhaustively washed in five changes of autoclaved distilled water. They were then kept at 30 °C until germination. After seed germination, they were put on sterile plates containing culture medium with the nutrients needed for plant growth [$CaCl_2{\cdot}2H_2O$, K_2HPO_4, KH_2PO_4, Fe-Citrate, $MgSO_4{\cdot}7H_2O$, K_2SO_4, $MnSO_4{\cdot}H_2O$, $ZnSO_4{\cdot}7H_2O$, H_3BO_3, $CuSO_4{\cdot}5H_2O$, $CoSO_4{\cdot}7H_2O$ ($CoCl_2{\cdot}6H_2O$), $Na_2MoO_4{\cdot}2H_2O$]. Plantlets were inoculated with the strains isolated from the nodules. Positive and negative controls were also carried out (culture media with N and without N, respectively). These plantlets were kept in growth chambers at 28 °C and under 12 h light/12 h darkness conditions until nodule emergence. Aerial biomass, subterranean biomass, and nodule number were determined in three individuals corresponding to each treatment. ANOVA and Scott & Knott multiple comparison test were carried out with INFOSTAT 2010/P.1 (Universidad Nacional de Córdoba, Argentina).

To determine the genotypic characteristics of these strains, bacteria were grown on YEM plates and colonies were collected, suspended in 300 µl of 1 M NaCl (picked up by using a plastic disposable loop), mixed thoroughly, and centrifuged at 14,000 rpm for 4 min. The supernatant was discarded and the pellet was suspended in 300 µl double-distilled sterile water. Once the pellet was mixed with distilled water and centrifuged as described before, the supernatant was removed and the pellet was suspended in 150 µl of 6 % (aqueous suspension) resin Chelex 100 (Bio Rad). This suspension was incubated at 56 °C for 20 min, followed by mixing and further incubation at 99 °C for 8 min [47].

The DNA sequences of enterobacterial repetitive intergenic consensus (ERIC) primers used in our study were those previously reported by Versalovic et al. [43]. Polymerase chain reaction (PCR) was performed in 12-µl reaction mixture containing 1× PCR buffer, 1.5 mM $MgCl_2$, 200 µM each nucleotide (Promega), 0.3 µM each primer, 1 U of *Taq* DNA polymerase (Invitrogen), and 18 ng of template DNA solution. The temperature profile was as follows: initial denaturation at 95 °C for 1 min, 35 cycles of denaturation at 94 °C for 1 min, annealing at 52 °C for 1 min, extension at 65 °C for 8 min, and a final extension step at 68 °C for 16 min. PCR amplifications were performed in a thermal cycler (Master cycle, Eppendorf, Germany). The ERIC amplification products were separated by horizontal electrophoresis on 1.5 % agarose gels and stained with ethidium bromide.

The band patterns of ERIC-PCR fingerprinting were converted into a binary matrix through a binary scoring system (one for the presence of a band and zero for the absence of bands). Computer-assisted analysis of the fingerprints was carried out using Cross-Checker system software 2.91 [9]. With the assistance of fingerprint analysis with missing data (FAMD) software package [33], a dendrogram was constructed from the distance matrix by means of the unweighted pair group method with arithmetic mean (UPGMA) algorithm.

The nucleotide sequence of purified PCR products was obtained from Macrogen Laboratories (Corea). Phylogenetic analysis was carried out using BLASTN algorithm [2]. Multiple alignments and phylogenetic trees were constructed using software packages BioEdit [17] and MEGA version 4 [38]. To select the most appropriate substitution model, the Model generator program was used (available at http://bioint.may.ie/software/modelgenerator). The reference sequences used were obtained from the GenBank database (www.ncbi.nlm.nih.gov/Genbank).

16S Amplified rDNA

Nearly full-length 16S rDNA gene was PCR amplified using primers pA and pH [16]. PCR was performed in 20-µl reaction mixture containing 2 µl PCR buffer, 1.5 $mmol^{-1}$ $MgCl_2$, 200 l mol^{-1} each nucleotide (Promega), 1 μmol^{-1} each primer, 1 U of *Taq* polymerase, and 5 µl of template DNA solution (5 ng DNA μl^{-1}). The temperature profile was as follows: initial denaturation at 95 °C for 3 min, 35 cycles of denaturation at 95 °C for 1 min, annealing at 55 °C for 1 min, extension at 72 °C for 2 min, and a final extension step consisting of 72 °C for 10 min. PCR products were purified using a QIAquick PCR Purification Kit (Qiagen), according to the manufacturer's instructions, and they were sequenced at the Macrogen Laboratories (Corea).

Nucleotide Sequence Accession Numbers

The nucleotide sequence of 16S rRNA gene from isolates S10, S14, S28, S33, S37, and S59 was submitted to the GenBank data bank under accession N° JX046045, N° JX046046, N° JX046047, N° JX046048, N° JX046049, and N° JX046050, respectively.

Data Analysis and Evaluation of Diversity

Adesmia bicolor isolates were grouped taking into account the degree of similarity of their ERIC-PCR banding patterns. A 90 % cut-off value was considered. Shannon–Weaver index was applied to determine the diversity index (H) [34] and was estimated by the following equation:

$$H = -\sum_{I}^{k} = p_i \cdot \ln p_i,$$

where k is the number of operational taxonomic units (OTU defined by similar ERIC-PCR profiles) and p_i is the relative abundance of isolates of each OTU [12].

BNF Effectiveness

Of the total seeds collected, five were randomly chosen, mechanically scarified, and subsequently seeded in each of the Leonard jars used [49]. After emergence, only one plant was used. Each pot constituted one experimental unit (EU), the total number of EU being 45. These plants were kept under optimal environmental conditions (constant temperature of 25 °C and 12 h photoperiod) in order to determine potential BNF effectiveness. The following treatments were applied:

(1) *N-free control treatment* The pots containing sterile vermiculite as substrate and non-inoculated seeds were irrigated with modified Munns nutrient solution without N.
(2) *N-fertilized treatment* The pots containing sterile vermiculite as substrate and non-inoculated seeds were irrigated with modified Munns nutrient solution with N.
(3) *Plant inoculation treatment* The pots containing sterile vermiculite as substrate and seeds inoculated with 1 ml of soil suspension were irrigated with modified Munns nutrient solution without N.

The modified Munns nutrient solution without N contained 34 g l^{-1} KH_2PO_4, 123 g l^{-1} $MgSO_4 \cdot 7H_2O$, 65 g l^{-1} K_2SO_4, 0.1 g l^{-1} $CaSO_4 \cdot 2H_2O$, 1.4 g l^{-1} $FeCl_3 \cdot 6H_20$, 1.7 g l^{-1} Na_2 H_2EDTA, 0.75 g l^{-1} KCl, 0.124 g l^{-1} H_3BO_3, 0.067 g l^{-1} $MnSO_4$ H_2O, 0.046 g l^{-1} $ZnSO_4 \cdot 7H_2O$, 0.01 g l^{-1} $CuSO_4 \cdot 5H_2O$, and 0.002 g l^{-1} H_2MoO_4, whereas the nutrient solution with N had the same chemical composition as that of the

solution without N plus 101 g l^{-1} KNO_3 and 133 g l^{-1} $(NH_4)_2SO_4$ [49].

At the vegetative, flowering, and fruiting stages, the nodules that grew on the main, lateral, and adventitious roots of inoculated plants were counted to determine the nodulation pattern. In addition, the aerial (leaves, shoots, flowers, and/or fruits) and underground parts (roots and nodules) belonging to five plants exposed to each treatment were destructively sampled. The samples were subsequently dried at 55 °C during 7 days and weighed in an analytical scale to determine aerial and underground dry matter. N-content was also determined in the dry matter samples after Kjeldahl digestion following the potentiometer method (HANNA-pH 211 with 0.1 mV sensitivity and NH_4 electrode) [8]. ANOVA and Scott & Knott multiple comparison test were performed with INFOSTAT 2010/P.1 (Universidad Nacional de Córdoba, Argentina).

Results

Phenotypic and Genotypic Characterization of Rhizobia Population

Ninety-nine isolates were obtained. Their colonies were light colored and semitranslucent. Some of them were aqueous while others were dry. All strains showed fast growth. Approximately, 93 % of the strains isolated from nodules proceeding from the field were observed to have the ability to nodulate *A. bicolor* plants (first inoculation), whereas only 74 % of the strains isolated from the nodules developed on these plants showed this ability (second inoculation).

The plants inoculated with these indigenous strains showed no significant differences in aerial biomass production ($P = 0.2241$) (Fig. 1a), while they did show significant differences in subterranean biomass production ($P = 0.0022$) (Fig. 1b). The strains that produced the highest subterranean biomass were: # 49, 1, 50, 67, and 35. Furthermore, although nodule number showed no significant differences among strains ($P = 0.6058$) (Fig. 2), strains # 3, 20, 28, 35, 37, 41, 49, 81, 84, and 90 were found to have the ability to nodulate 100 % of the inoculated plants. In line with this, strains # 35 and 49 were the most effective ones as they evidenced high infection ability and induced the production of the highest subterranean biomass in *A. bicolor* plants.

The dendrogram based on ERIC fingerprint patterns showed that the genetic diversity of isolates occupying *A. bicolor* nodules was low ($H = 0.169$). Thus, taking into account a similarity coefficient of 90 %, ERIC patterns could be grouped into 18 different operational taxonomic units (OTUs). From 53 strains analyzed, 50 % were observed to show an identical profile, which indicates the existence of a dominant bacterial genotype (Fig. 3a).

Furthermore, six isolates belonging to different fingerprint patterns (# 10, 14, 28, 33, 37, and 59) were selected for the phylogenetic analysis. They were all found to be genetically related and grouped into a single cluster corresponding to the genus *Rhizobium*. Such a low diversity could be either due to soil properties (pH, available phosphorus or iron, organic matter content, etc.) or the plant genetics, which may support the growth of only the best-suited genotypes.

The isolates selected were similar to the genera *Rhizobium*, *Sinorhizobium*, *Phyllobacterium*, *Mesorhizobium*, *Bosea*, *Afipia*, *Bradyrhizobium*, and *Blastobacter* (Fig. 3b). Furthermore, the strains that were found to evidence more similarities with *A. bicolor* isolates were *Rhizobium etli* CFN 42 T (U28916) isolated from *Phaseolus vulgaris*, *Rhizobium* sp. ORS 1466 (AY500264) isolated from the wild legume *Anthyllis henoniana* that grows in southern Tunisia arid area, and *Rhizobium* isolated from *Hedysarum coronarium sullae* (Fig. 3b). Other isolates which also evidenced similarities with *A. bicolor* strains were *Rhizobium* sp. SEMIA 6438 (FJ025120.1) isolated from *Adesmia latifolia* and *Rhizobium* sp. SEMIA 6436 (FJ025119.1) isolated from *Parapiptadenia pterosperma*.

Morphological Characteristics of *A. bicolor* Nodules

Mature *A. bicolor* nodules were observed to be small and oblate and they showed determinate growth (Fig. 4a). They were often associated with fine rootlets (Fig. 4b). Infected tissue was found to be located in the central part of these nodules with few uninfected cells. Nodules with these characteristics are classified as of aeschynomenoid type. Furthermore, nodules developed on the main and lateral roots and, later, on adventitious roots and they were found to be usually distributed in pairs.

It was also observed that the number of nodules increased as the plant grew (Table 1). In line with this, during the vegetative stage, the average nodule number was 53 (40 on the main root and 13 on lateral roots). During the flowering stage, the average nodule number was 103, of which 34 developed on the main root and 69 on the lateral roots. This is indicative of a 66 % increase over the vegetative stage. During the fruiting stage, the plant developed 84 nodules: 6 nodules were found on the main root, 3 on the lateral roots, and 75 on the adventitious roots. From the first growth stage until the last growth stage, the increase in the number of nodules reached 35 %. The number of nodules also showed statistically significant differences during the different growth stages ($P = 0.0002$).

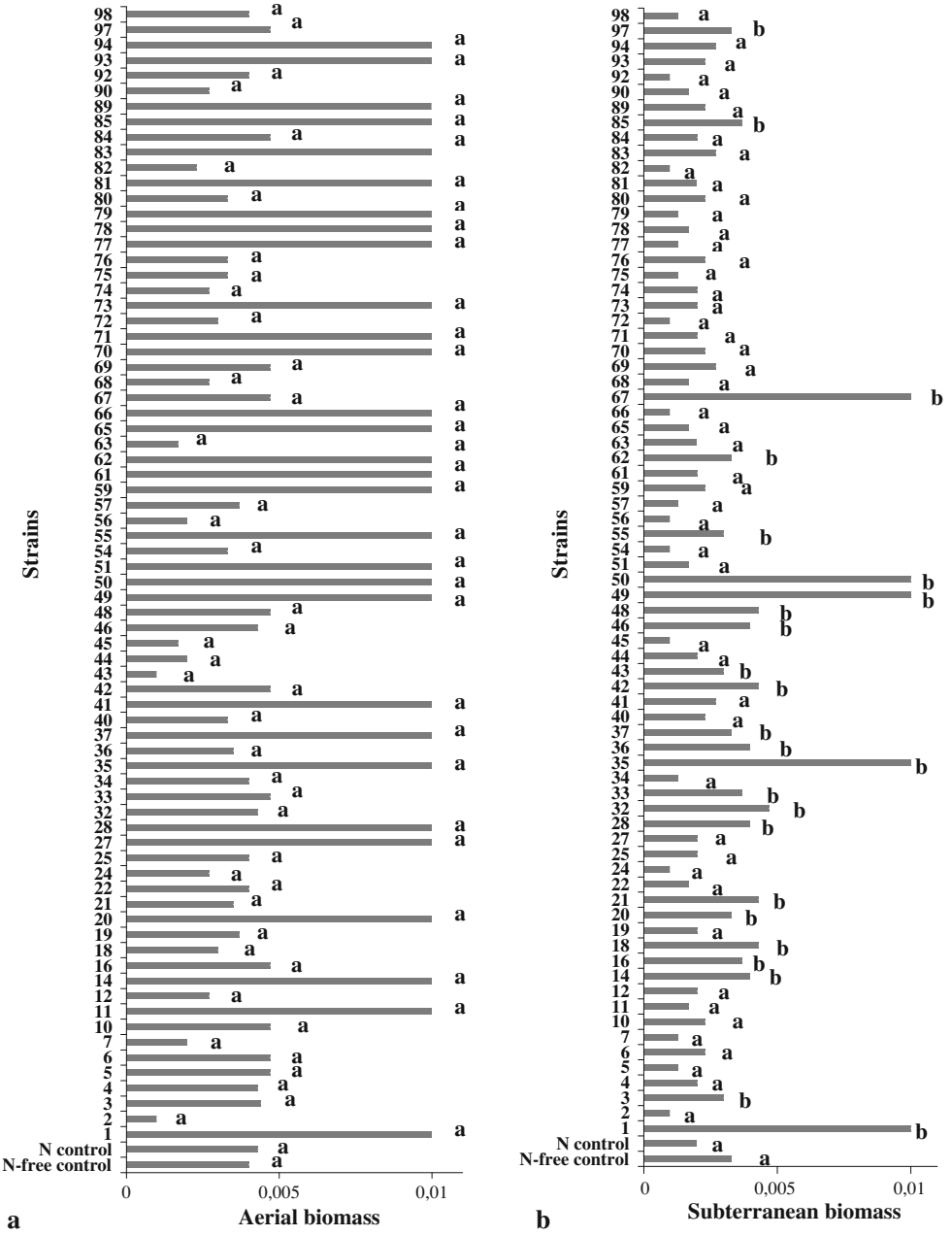

Fig. 1 **a** Average aerial biomass produced by *A. bicolor* plants ($n = 5$) inoculated with strains obtained from nodules from Villa Rumipal population, **b** Average underground biomass produced by *A. bicolor* plants ($n = 5$) inoculated with strains obtained from nodules from Villa Rumipal population. *Different letters* indicate significant differences among strains according to Scott & Knott multiple comparison test ($\alpha = 0.05$)

BNF Effectiveness

The aerial dry matter obtained during the different growth stages of *A. bicolor* showed significant differences among the treatments assayed (Table 2). At the vegetative and flowering growth stages, the aerial dry matter of inoculated plants and N-fertilized controls revealed no significant differences except for the differences with respect to the N-free controls ($P = 0.03$ and 0.005, respectively). In addition, even though no differences in dry matter production were observed between these two growth stages, N-content in the dry matter produced by the N control plants during the vegetative stage was higher (Table 3). During the flowering stage, no differences were found in the N-contents of both treatments. In contrast, at the fruiting stage, significant differences were found in aerial dry matter ($P = 0.008$) and N-contents from inoculated plants and N control plants.

At the fruiting stage, inoculated plant underground dry matter was significantly higher than N control underground dry matter ($P = 0.0001$). The aerial/underground N ratio in inoculated plants was higher than in N control plants during the vegetative stage, and the subsequent flowering and fruiting ratios of both treatments were found to be similar (Table 3).

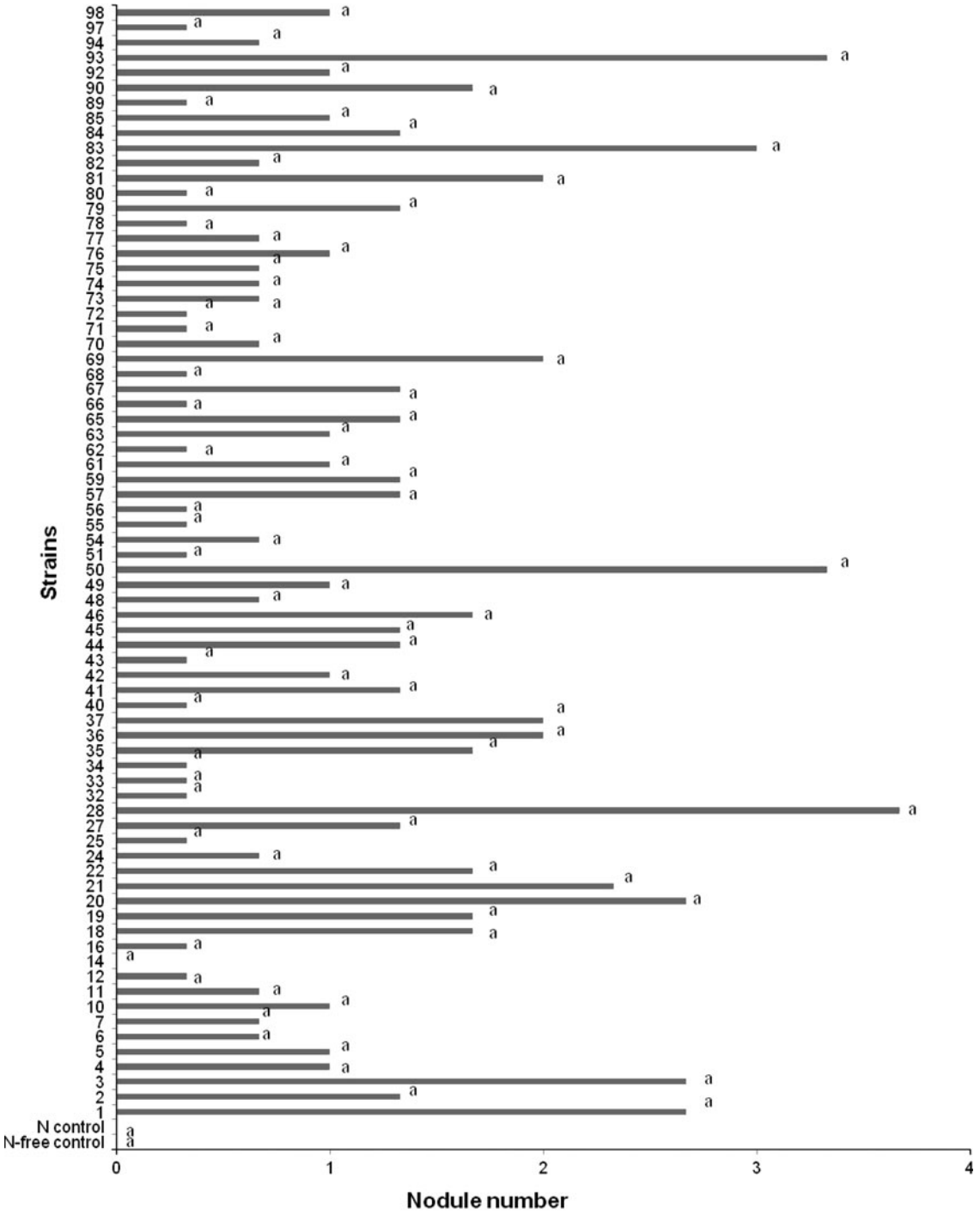

Fig. 2 Average number of nodules produced by *A. bicolor* plants ($n = 5$) inoculated with strains from nodules from Villa Rumipal population. *Different letters* indicate significant differences among strains according to Scott & Knott multiple comparison test ($\alpha = 0.05$)

As to the underground dry matter produced by N control plants, it was observed that it was significantly higher than that produced by inoculated plants and by N-free control plants during the vegetative and flowering growth stages ($P = 0.0028$ and 0.0004, respectively), whereas their N-contents were similar (Tables 2, 3).

Discussion

The strategy traditionally used to isolate rhizobial strains involves the isolation and cultivation of internal tissues of surface-sterilized nodules [45]. It is known that nodules can be colonized internally by several bacterial endophytes.

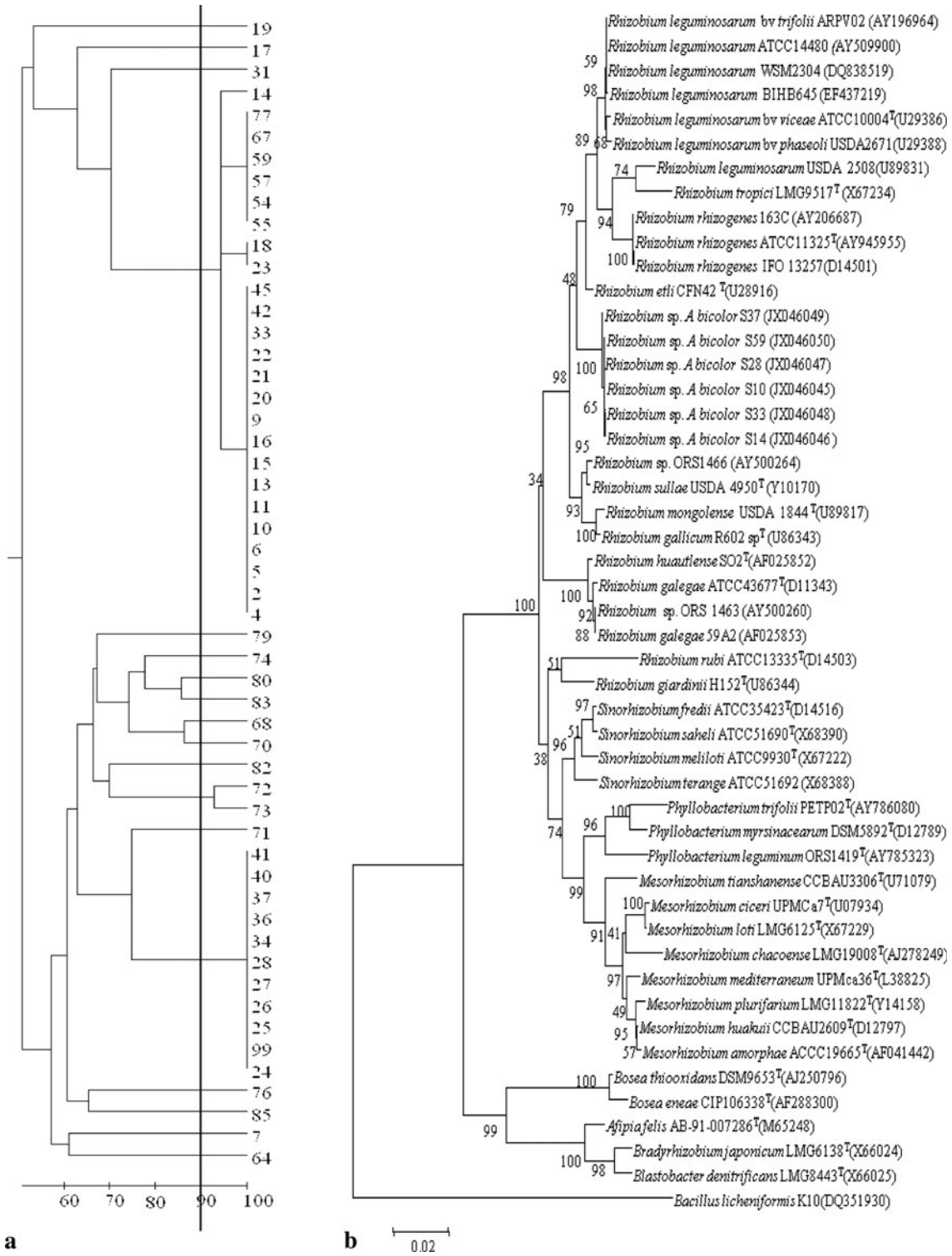

Fig. 3 **a** Dendrogram of similarity of 53 isolates of *A. bicolor* Villa Rumipal population, **b** Phylogenetic tree based on 1,500 bp alignment for the 16S gene sequences and inferred from the Tamura Nei substitution model + G. Bootstrap values (higher than 50 %) are shown for 1000 pseudoreplicates

In species, such as *Trifolium pratense*, Sturz et al. [37] reported the presence of not only rhizobial strains inside nodules but also of non-rhizobial endophytes. Furthermore, Philipson and Blair [27] observed the presence of diverse species in nodules of red clover, such as Gram-positive bacteria. In our study, all the strains isolated with the

Fig. 4 *Adesmia bicolor* nodules. **a** oblate nodules developed on the main root, *scale bar* 3 mm, **b** Nodules associated to rootlets, *scale bar* 3 mm

Table 1 Average number of nodules ($n = 5$) ± standard error developed on different types of roots during the growth cycle

Roots	Growth stages		
	Vegetative	Flowering	Fruiting
Main root	39.8 ± 5.0 b	33.8 ± 8.0 a	6 ± 6.0 b
Lateral roots	13.2 ± 6.1 a	68.8 ± 20.4 b	3 ± 3.0 b
Adventitious roots	0 ± 0.0 a	0 ± 0.0 a	74.8 ± 21.5 a

Different letters in the same column indicate significant differences among treatments according to Scott & Knott multiple comparison test ($\alpha = 0.05$)

This treatment does not have data at fruiting stage because plants without N die due to its absence

ability to nodulate *A. bicolor* belong to *Rhizobium* genus. Fifty percent of the strains analyzed were found to have an identical profile, thus indicating that they are phylogenetically related. In addition, as the novel groups of *Rhizobium* isolated in this study were different from the species described in previous studies, it would be important to find other markers that could help elucidate phylogenetic relationships [6, 56].

As stated above, it is known that nodule structure and infection site are largely plant determined [20] and that nodule morphology and infection processes, within any given phylogenetic lineage, are generally the same regardless of which species or genus of rhizobia is involved. Therefore, as aeschynomenoid nodules have been reported in two species of *Adesmia*, *A. lanata* and *A. volkmannii* [20], it could be hypothesized that *A. bicolor* develops aeschynomenoid nodules and that, in line with this, these nodules show the typical characteristics corresponding to this category. Results from our present study confirm this hypothesis.

In addition, the growth stage that yielded the highest number of nodules in *A. bicolor* coincides with other species of the genus, such as *Adesmia araujoi* and *A. latifolia* as well as other legume species, such as *Lupinus aureonitens*, *Rhynchosia hauthalli*, *Stylosanthes*, and *Trifolium leiocarpa* [31, 54]. Also, based on the high dry matter production yielded when *A. bicolor* grows in poor sandy soils, Coll and Zarza [11] claimed that this species has a high N-fixing ability. Inoculated plant N-content in our study confirmed these previous results on account of the fact that it was relatively high in comparison to that in the N control plants.

Furthermore, forage legumes may have up to 60 % of their fixed N in the root system [10]. Based on results from our study, it was possible to determine that *A. bicolor* allocated 55 % of its fixed N to subterranean parts during the vegetative and flowering stages. This could be explained by the fact that perennial forage legumes usually store N in their root systems to be able to initiate growth during the following growing season [10]. Unlike *A. bicolor*, other species of the

Table 2 Average aerial and underground dry matter accumulation of *A. bicolor* ($n = 5$) ± standard error at different growth stages

Growth stages	Treatments	Dry matter (g)	
		Aerial	Underground
Vegetative	Inoculated plants	0.228 ± 0.05 ab	0.021 ± 0.0043 a
	N control	0.766 ± 0.032 b	0.206 ± 0.06 b
	N-free control	0.004 ± 0.00071 a	0.0004 ± 0.00021 a
Flowering	Inoculated plants	1.202 ± 0.21 b	0.089 ± 0.02 a
	N control	1.613 ± 0.30 b	0.345 ± 0.07 b
	N-free control[a]	0.005 ± 0.001 a	0.001 ± 0.00024 a
Fruiting	Inoculated plants	2.626 ± 0.85 b	0.236 ± 0.01 b
	N control	0.775 ± 0.18 a	0.183 ± 0.02 a

Different letters in the same column and the same sampling date indicate significant differences among treatments according to Scott & Knott multiple comparison test ($\alpha = 0.05$)

[a] This treatment does not have data at fruiting stage because plants without N die due to its absence

Table 3 Aerial and underground nitrogen content of *A. bicolor* ($n = 5$) at different growth stages

Growth stages	Treatments	Nitrogen content (gN·100 g sample^{-1})	
		Aerial	Underground
Vegetative	Inoculated plants	2.63	3.19
	N control	4.15	3.11
Flowering	Inoculated plants	2.34	2.88
	N control	2.13	2.44
Fruiting	Inoculated plants	2.63	2.07
	N control	1.88	1.84

genus, such as *A. latifolia* [32, 54], showed higher N-content in the aerial parts than in the subterranean parts during the vegetative stage.

Finally, although *A. bicolor* inoculated plants were observed to show a higher N-content in subterranean parts than in aerial parts, N control plants showed only 43 % of its N in the stubterranean parts. Similar results were obtained by Jørgensen and Ledgard [18] in *Trifolium repens* fertilized with mineral N. They observed that these plants reduce the root/shoot N ratio and that during the fruiting stage N-content is highest in the aerial parts thanks to the N stored in fruits and seeds. Taken together, our findings lead us to confirm that native strains that are associated with *A. bicolor* show low diversity and high N-fixation effectiveness, both being highly desirable in a species with forage potential in arid and semiarid areas.

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