PHYLOGENETIC DIVERSITY OF RHIZOBIAL SYMBIONTS NODULATING *Vachellia karroo* (HAYNE) BANFI & GALASSO IN ALGERIAN ECOSYSTEMS: IMPLICATIONS FOR AFFORESTATION AND LAND RESTORATION

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Abstract. Despite their ecological and economic importance, the diversity of rhizobia associated with the introduced woody legumes in Algeria remains largely unexplored. The main objective of this research was to identify the rhizobial species associated with *Vachellia karroo* cultivated in ten seedling nurseries across different eco-climatic regions in Algeria, focusing on their diversity and distribution in relation to differing soil and climate conditions. Rhizobial strains were isolated from the root nodules of *V. karroo* seedlings grown in nurseries located in Saharan, sub-Saharan, and humid eco-climatic conditions. Molecular identification of 29 isolated isolates was undertaken using 16S rRNA gene sequencing to identify them and determine their phylogenetic position. Regardless of the soil and climate, the study found that *V. karroo* seedlings exhibited a strong ability to develop nitrogen (N)-fixing nodules. Three rhizobial genera, i.e., *Sinorhizobium*, *Mesorhizobium*, and *Bradyrhizobium*, were identified by molecular characterization. Of these, *Sinorhizobium* was the most commonly occurring genus (48%) and displayed greater diversity. Furthermore, the *Sinorhizobium* spp. were abundantly found in seedlings collected exclusively from the nurseries in Saharan and sub-Saharan regions, indicating a strong climatic adaptation to arid environments. These results highlight *V. karroo*'s broad ecological adaptation to diverse Algerian eco-climatic conditions and its potential for association with a wide range of rhizobial species. The *Sinorhizobium* spp. dominance in arid regions suggests its potential importance in biological N fixation under harsh environmental conditions. This research provides valuable information about the rhizobial diversity associated with *V. karroo*, which may help Algeria implement climate-resilient afforestation and sustainable land management strategies.

Keywords: *Acacia* tree; biodiversity; phylogeny; nodulation effectiveness; plant-microbe symbosis; woody legume flora.

INTRODUCTION

The erstwhile genus *Acacia* Miller (*s.l.*), within the *Fabaceae* family, subfamily *caesalpiniaceae,* has been subjected to significant reclassification. In the light of molecular phylogenetic studies, it has been spliced into five distinct genera: *Acacia*, *Senegalia*, *Acaciella*, *Mariosousa*, and *Vachellia* [24, 29, 31]. Genus *Acacia*, which mostly includes species native to Australia, remains the largest of these groups, while the primarily African genus *Vachellia*, with 161 species, is the smallest one. In Algerian flora, the *Acacia s.l.* (erstwhile genus) has 26 species with only six species (*A. ehrenbergina*, *A. nilotica*, *A. seyal*, *A. laeta*, and *A. tortilis*) which are endemic to the Algerian desert areas [41]. The rest of the species has been introduced from Australia and one species has originated from Africa; they are all well distributed in the northern part of the country [1].

The species *Vachellia karroo* (Hayne) Banfi & Galasso (*Fabaceae*), formerly known as *Acacia karroo* Hayne is a native from the desert of Karroo in South Africa; since 1865, it has been introduced into Algeria for ornamental purpose. For a long time, it has been locally confused with *A. horrida*. This Mimosoid shrub named *V. karroo*, also known as Sweet Thorn, is botanically recognized by its reddish underbark, long white prickles or stipular spines, yellow pompom-like globose inflorescences, and numerous free stamens [33, 36, 40, 48].

This plant is well adapted to a range of environments from South Africa to the Mediterranean basin mainly in dry semi-desert scrub habitats. It exhibits a high resilience to salinity, drought, and frost [14]. In South Africa, it is valued for its ethnomedicinal uses, including treatments for sexually transmitted infections and venereal diseases. Its bark, gum, and leaves serve as emollients and astringents for various ailments; its gum is edible because it is devoid of hydrogen cyanides sometimes present in other neighboring African species [2, 12, 14, 32]. In addition to their pastoral values due to their dense foliage, *V. karroo* bears melliferous flowers that attract insects and insectivorous birds. Ecologically, V. *karroo* is an adaptable pioneer species that can regenerate and spreading in overgrazed areas.Also, It contributes to soil stabilization and fertility by its strong tap root system and the success to perform nitrogen (N)-fixing symbiosis with rhizobial bacteria [16].

As most other *Fabaceae*, *Acacia s.l* legumes form N-fixing root nodules especially with soil rhizobia. A wide diversity of the Alpha- and Betaproteobacteria genera were already involved in these mutualistic symbioses [12, 42]. Species of the beta-rhizobial *Paraburkholderia* genus are the the predominantnodulating symbionts of certain Neotropical Mimosa species native to Brazil [4, 7, 26]. In contrast, in arid ecosystems such as the Indian Thar Desert and African deserts, *Acacia* species typically associate with genetically diverse and stress-adapted *Sinorhizobium* lineages [20]. Previous studies have revealed the intriguing symbiotic relationships between *V. karroo* and its rhizobial partners across various native and non-native ranges. Research focusing on *V.*

karroo in its South African primary area highlighted the plant's ability to form nodules with a diverse array of symbionts which are classified into two indicative categories as "fast" and "slow-growers", which express the dynamic of their symbiotic capabilities [38]. In facts, the distinction between fast and slow-growing rhizobia, corresponding to the alpha-rhizobial genera *Rhizobium* and *Bradyrhizobium*, respectively, emphasizes the complexity of these symbiotic interactions [26, 37]. This diversity was reflected in results from Libyan and Kenyan material, where *Rhizobium* and *Bradyrhizobium* strains were also isolated from *V. karroo* nodules, highlighting a consistent pattern of rhizobial association across different biogeographical regions [34, 47].

A pivotal study involving Algerian *V. karroo* samples, based on the 16S ribosomal RNA gene, has identified symbionts belonging to the genera *Rhizobium* and *Sinorhizobium*, expanding the known spectrum of the rhizobial partners of *V. karroo* [5]. More recently, Bontemps *et al.* [4] further broadened this spectrum by reporting the presence of several alpha-proteobacterial genera (*Bradyrhizobium*, *Sinorhizobium*, *Mesorhizobium*, and *Rhizobium*) and introduced species from the beta-proteobacteria class (*Paraburkholderia*) as new symbionts of *V. karroo*. The native and introduced Algerian floras, especially legumes as well as the accompanying rhizospheric bacteria, remain understudied despite the significant systematic, ecological and biogeographic diversity described since earlier works [1, 13, 15].

The identity and diversity of the rhizobia symbionts of cultivated *V. karroo* in Algeria are not yet well investigated, even though mutualistic interaction could be an important success factor in its successful introduction into a new environment [25]. In fact, the species *V. karroo* is a suitable candidate for afforestation programs, especially in an arid region that represents nearly 85% of Algerian land. Recent studies have shown that in mimosoid legumes, new rhizobial strains can evolve through horizontal gene transfer, enhancing their ability to adapt to local soil and climatic conditions [11].

Therefore, the present study attempts to explore plant-microbial interactions through multidisciplinary approaches, addressing the challenge posed by the diversity of Algerian samples in Mediterranean biotopes. To achieve successful afforestation, local inoculants that are capable of surviving the edaphic conditions of the arid regions are needed. However, there have been very little investigations on this subject.

To address this knowledge gap, present study, therefore, was aimed at: (1) isolating and characterizing rhizobial strains associated with *V. karroo* collectedfrom contrasting eco-climatic and edaphic regions across Algeria , and (2) identitying their phylogentic diversity using 16S rRNA gene sequencing to understand the ecological and evolutionary adaptation of these symbionts to different climat conditions.

MATERIALS AND METHODS

Symbiotic status

The evaluation of rhizobia strain diversity within nodules of *V. karroo* plants was carried out on 12 weeks old seedlings, sampled from 10 selected nurseries distributed across the northern and southern Algeria (Table 1). The seedlings were raised from seeds collected from a single tree of *V. karroo* located in the Baïnem forest (Algiers) (36° 48' 14.30" N, 2° 57' 28.03" E) during October 2021.

The seeds were first surface-sterilized by immersion in 5% calcium hypochlorite solution for 10 min, rinsed with sterile distilled water, and then were grown in perforated polyethylene bags (15 × 25 cm) filled with a mixture of nursery soil and river sand (2:1, v:v). The used sand was previously sterilized by autoclaving at 121°C (1 h at 121°C, three times spaced 24 h apart over a period of three days) as per Djouadi *et al.* [15] and Tak *et al.* [52]. The irrigation was performed every three days with a volume of water equivalent to 100% of the soil's water holding capacity.

From each of the 10 nurseries investigated, 15 randomly selected plants were harvested and examined in the laboratory. Root systems of the seedlings were carefully removed from the polyethylene bags, washed with distilled water to remove soil and then visually for the presence of nodules. The presence of nodules, their location, morphology, and efficiency [43] were noted for each plant.

Plant nodulation test and isolation of symbiotic bacterial strains

The collected root nodules were washed by immersion in 95% (*v*:*v*) ethanol for 30 sec, rinsed with distilled water, then immersed for 3 min in 5.25% $CaCl_2O_2$ and then rinsed in successive baths of sterile distilled water. After crushing in sterile physiological saline solution (NaCl 0.9%), the nodule homogenates were spread onto yeast extract-mannitol agar (YMA) plates [54] containing Congo red (0.00125%, *w/v*). One isolated colony per nodule homogenate was obtained from successive streaking on YMA plates, and purity of colonies was checked by phase-contrast microscopy. Colony shape and color and GRAM coloration were determined. Pure cultures were then cryogenically preserved (-80 °C) in yeast extract-mannitol (YEM) broth medium adjusted to 40% (v:v) glycerol.

The nodulation ability of the bacterial isolates obtained was checked by inoculating seedlings of *V. karroo*. collected, sterilized seeds were grown on a mixture of sterilized sandy soil and peat (2:1, v:v) in 250 mL plastic pots. One week after germination, each pot with five plants were inoculated with 3 mL of bacterial suspension 10^8 cells×mL^{-1} of each isolate grown on TY buffer . Uninoculated controls were also included. Each treatment, including the control, was performed in triplicate.

Plants were transferred to greenhouse set to 16 h light and 8 h dark photoperiod with an additional illumination of 8000 lux. Water loss due to evaporation and transpiration was determined every 48 h by weighing the pots and compensated by adding an equivalent weight of sterile distilled water to maintain a stable humidity equivalent to 80% of the soil's water holding capacity. At the period of 11 weeks after inoculation, plants were harvested to estimate the infectivity and effectivity of bacterial isolates based on the occurrence and color of nodules. The efficiency of the symbiotic couples was appreciated by the appearance of red or pink colour in nodule cross sections resulted from the accumulation of leghemoglobin, an hemoprotein found only in nitrogen-fixing nodules [17, 40].

Figure 1. Overview of the experimental setup under laboratory conditions (a) and development of root nodules on inoculated plants of *V. karroo*.

Bacterial DNA extraction, PCR, and Sequencing

Pure culture of each isolate was grown in 1.5 mL of Yeast extract Mannitol broth at 28 °C with shaking (150 rpm) for 4 days. Suspensions were centrifuged for 5 min at 6000 g and then washed twice, pellets were re-suspended. DNA was extracted using the same protocol adapted in [52].

The 16S rRNA gene primers *fD1* 5'-AGAGTTTGATCCTGGCTCAG-3' and rDl 5'-AAGGAGGTGATCCAGCC-3' [52] were used. PCR was performed for each sampled strain in a 50 µL of reaction mixture consisting of 10× Taq buffer, 5 µL of 2 mM of each dNTP, 0.5 µL of each primer (forward and reverse), 2.5 µL of Green Go Taq DNA polymerase (Promega), and 5 µL of extracted DNA as the template.

The PCR program was started by an initial cycle of denaturation at 94 °C for 3 min, followed by 35 cycles of denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min, extension at 72 °C for 1 min, and a final extension at 72 °C for 3 min. The obtained PCR products were purified using EZ-10 Spin Column PCR kits (BioBasic Inc.), and then checked by electrophoresis 0.8% Agarose gel and spectrophotometer (Nano Drop 2000, USA). The purified DNA fragments were sequenced by the ABI PRISM Big Dye Terminator v3.0 Cycle Sequencing Kit on an ABI 3100 automated Capillary sequencer (Applied Biosystems, CA, United States).

The 16S rRNA gene sequences of 30 new isolated *V. karoo* strains were deposited in the GenBank database under accession numbers according to Table 2. One stain (Ser2) was removed from the final alignment matrix (poor sequencing quality). The 16S rRNA gene sequences were analyzed using CHROMASPRO 4 (Technelysium Pty Ltd., Tewantin, Australia) than aligned using MEGA6 [50].

Phylogenetic analyses

The 16S rRNA gene sequences were submitted to a Basic Local Alignment Search Tool (BLAST) for the preliminary identification of the strains using the EzTaxon database [22]. All sequences of the type strains of the various genera used in this study were retrieved from the EzTaxon, except made for *Mesorhizobium erdmanii* and *M. jarvisii*, which were obtained from NCBI-GenBank (Table 2).

Phylogenetic analyses were inferred using the Maximum Likelihood (ML) method, performed following the procedure described in Harrison and Langdale (2006) [21]. For the ML analysis, the appropriate nucleotide substitution model of sequence evolution was determined using MEGA 6, based on the lowest value of the Bayes Information Criterion (BIC) [49]. Finally, the bootstrap analyses based on 1000 replications were performed. The strain of *R. tropiki* was used as out group.

Statistical analysis

The data for this study were subjected to analysis of variance one-way ANOVA and Fisher's Least Significant Difference (LSD) to evaluate significant differences among all treatments at the 5% significance level using R_Studio v.2.13 (R Development Core Team 2016).

RESULTS

Symbiotic status

The natural nodulation of *V. karroo* cultivated in nurseries was investigated here. To assess this, we examined the prevalence of nodulation in 12 weeks old seedlings from 10 selected nurseries situated in various regions of Algeria. The root systems of 150 seedlings were inspected by a binocular loupe. The results

revealed that 131 seedlings showed nodulation, with nodules on 115 seedlings being efficient. Significant differences were observed between seedlings from nurseries in the northern and southern regions. Seedlings from nurseries in the south exhibited lower nodulation rates (80%) and efficiency (75%) compared to those from the northern nurseries (95.3% and 98.7%, respectively). Both the nodulation and efficiency were significantly lower (p<0.05) with seedlings collected from the south. The nodules observed on the root systems of all seedlings were primarily located on the secondary roots and displayed an elongated lobe morphology that characterizes indeterminate nodules.

Strains collection and nodulation test

The trapping experiment carried out in the 10 selected nurseries resulted in the collection of 29 purified strains, all exhibiting colony characteristics typical of rhizobia. These 29 isolates underwent testing to assess their capacity for nodulation under laboratory conditions. Six weeks after inoculation, the plants were harvested, and the roots systems were observed. The results indicate that all the 29 isolates were able to induce efficient nodules in their host plant. The efficiency of all nodules was confirmed through qualitative assessments, primarily focusing on the pink color of the nodules (Table 1).

Phylogenetic analysis of bacterial strains

To assess bacterial diversity under *V. karroo*, phylogenetic analysis of the 16S rRNA gene sequences was inferred using Maximum Likelihood (ML). The final aligned data matrix of 71 sequences included sequences of the 29 isolates from this study and those from GenBank representing major previously described bacterial taxa of soil communities. The final alignment was 1446 bp long with 560 variable sites. For the ML analysis, the nucleotide substitution model selected by the lowest value of BIC (Bayesian Information Criterion) was General Time Reversible with discrete Gamma (GTR+G). For the MP analysis, the consistency index was CI=0.1655; the retention index was RI=0.921 and 337 sites were parsimony informative.

Phylogenetic trees based on the ML is presented here (Fig. 2). The two major clades and their subclades show moderately to well supported relationships indicated by percentage of bootstrap support (BS) in the Fig. 2. The clade I (100% BS) include the stains referred to the genus *Bradyrhizobium* (*Nitrobacteraceae*). The seven *Bradyrhizobium* isolates were separated into three species. The isolates Ben1, Ben2, Ben3, Med2, and Oul y2 were affiliated with *B. betae* supported by a bootstrap value of 88% for the main cluster and 66% for Oul y2 . Another isolate (Oul y3) within Subclade IA shows a genetic relationship with *B. guangzhouense*, with a bootstrap value of 81%. Additionally, there is one isolate (Rem1) identified as *B. japonicum* with a bootstrap value of 61%. (Fig. 2).

The clade II, show two main subclades (IIA, IIB) well supported (100% BS). The subclade IIA include all stain referred to the genus *Mesorhizobium* (Phyllobacteriaceae), while the subclade IIB clustered the stain from the genus *Sinorhizobium* (Rhizobiaceae). The subclade IIA includes eight isolates in the *Mesorhizobium* group with four strains (Ser1, BenA3, Rem2, and Rem3) close to *M. accaciae* with a robust bootstrap value of 100% and two strains (Med_1 and Oul1) closely related to *M. erdmanii* supported by a bootstrap value of 79%. The two last strains were affiliated to *M. albiziae* (Ser3) and *M. silamurunense* (Med3) with a robust bootstrap value of 99% and 79% respectively. All the strains belonging to *Mesorhizobium* and *Bradyrhizobium* were collected from geographical regions with a humid to sub-humid climate.

Table 1. Symbiotic status of *V. karroo* plants from different nurseries in Algeria

Region	Nurseries	Bioclimatic zone	Mean daily temperature (°C)	Annual precipitation (mm)	Soil texture	pH soil	Number of plants with nodules	Number of plants with efficient nodules	Nodule type	Position on the roots
North	Alger-Ben Aknoun	S.H	19.38	600-650	Clay-loam	6.8	**15**	**15**	Ind	RII
	Annaba-Serraidi	S.H	19.08	600-650	Clay	7.3	**15**	**15**	Ind	RII
	Blida-Oued Yaich	H	20.14	600-650	Clay	7.1	**13**	**12**	Ind	RII
	Médéa-Médéa	H	21.42	300-400	Marly-clay	7.2	**13**	**13**	Ind	RII
	Tlemcen-Remchi	S.H	19.20	200-450	Marly-clay	7.1	**15**	**15**	Ind	RII
							71 (95.3%a)	**70 (98.74%a)**		
South	Béchar-Beni Abbès	S	**28.56**	<50	Sandy	8.4	**11**	**8**	Ind	RII
	Biskara- Ouled Djellal	S	**25.32**	130-155	Sandy	8.3	**11**	**8**	Ind	RII
	Djelfa- Ain Oussara	S.A	**26.78**	320-350	Sandy-loam	8.3	**14**	**10**	Ind	RII
	El Menia-Belbachir	S	**27.66**	<50	Sandy-loam	8.2	**12**	**10**	Ind	RII
	Tamanrasset	S	**26.22**	40-60	Sandy	8.2	**12**	**9**	Ind	RII
							60 (80%b)	**45 (75%b)**		
Total							**131a**	**115a**		

Data were subjected to one-way analyses of variance (ANOVA) and Fisher's Least Significant Difference (LSD) tests at $p < 0.05$ threshold for all variables. Different letters (a,b,…) are significantly different. S.H: Sub-humid, H: Humid, S: Saharian, S.A: Semi-arid, Ind, Indeterminate, and RII: Secondary roots.

The *Sinorhizobium* cluster stands out as the most important group in our study, encompassing 14 strains. These strains represent 48% of the total isolated from *V. karroo* nodules. The 14 strains (Tam2, Tam3, Bel1, BenA2, BenA1, Tam1, Bir2, Bir3, Bir1, Bel2, El-K2, Bel3, El-K1, and El-K3) were all isolated from diverse geographic regions, yet they share the saharian to sub-saharian climate. Within the *Sinorhizobium* clade, two distinct subclades were identified, reflecting further genetic differentiation among the isolated strains. the subclade IIA supports three isolates (Bel1, Tam2, and Tam3) which they are associated with *Sinorhizobium fredii*, which is supported by a high bootstrap value of 98%, confirming their close genetic relationship. The subclade IIB includes two groups, the first one comprises one isolate (BenA2) which is identified as *Sinorhizobium garamanticum*, with a very high bootstrap support of 100%, underscoring the precision of its placement within the phylogenetic tree. The second one is a group of 6 strains (Bir1, Bel2, El-K2, Bel3, El-K1, and El-K3) that show a close genetic relationship with *Sinorhizobium numidicum*, supported by a bootstrap value of 77%. And four isolates (BenA1, Tam1, Bir2, and Bir3) are closely related to *Sinorhizobium meliloti*, with a robust bootstrap value of 89%, highlighting their genetic similarity.

To complement the general phylogeny presented in Fig. 2, separate 16S rRNA phylogenetic trees were also constructed for each genus: *Sinorhizobium* (Fig. 3), *Mesorhizobium* (Fig. 4), and *Bradyrhizobium* (Fig. 5) using extended reference datasets that include type strains of all described species within these genera. These genus-level analyses confirmed the placement of our isolates within their respective clades and provided higher-resolution support for species-level affiliations.

DISCUSSION

In this study, nodulation was observed in most plants of *V. karroo* that were collected from the nurseries. However, a significant decrease in the prevalence and efficiency of this symbiosis was noted in plants from nurseries located in the southern regions of Algeria. A nodule was considered as effecient when leghemoglobin was detected in their central zones, which was indicated by a characteristic reddish coloration. The sites vary in climatic features, such as average annual precipitation, temperatures and also in soil texture, and these factors most likely explain why in the sampling sites in the arid regions, where soil was sandy and poor in texture, that the % occurrence of nodulated plants and nodulation per plant was significantly reduced as compared to those from the other sampling sites, which are all humid and sub-humid with fertile soils. Many studies have been reported for *Vachellia* species growing in arid and semi-arid ecosystems, where nodulation efficiency is strongly influenced by soil moisture and physicochemical constraints [9, 10, 19, 47]. Consequently, mimosoid legume shrubs in seasonally dry environments will cease nodulation and/or shed nodules during dry periods [4].

Table 2. Identity of rhizobia strains isolated from nodules of *V. karroo* plants produced in nurseries

Nursery	Code isolate	Nodulation	Efficiency	Assignment based on 16S rDNA sequences	NCBI accession number
Alger-Ben Aknoun	Ben 1	+	+	*B. betae*	OQ119549
	Ben 2	+	+	*B. betae*	OQ119550
	Ben 3	+	+	*B. betae*	OQ119551
Annaba-Serraidi	Ser1	+	+	*M. acaciae*	OQ119570
	Ser3	+	+	*M. albiziae*	OQ119572
Blida-Oued Yaich	Oul y1	+	+	*M. erdmanii*	OQ119564
	Oul y2	+	+	*B. betae*	OQ119565
	Oul y3	+	+	*B. guangzhouense*	OQ119566
Médéa-Médéa	Med1	+	+	*M. erdmanii*	OQ119561
	Med2	+	+	*B. betae*	OQ119562
	Med3	+	+	*M. silamurunense*	OQ119563
Tlemcen-Remchi	Rem1	+	+	*B. japonicum*	OQ119567
	Rem2	+	+	*M. acaciae*	OQ119568
	Rem3	+	+	*M. acaciae*	OQ119569
Béchar-Beni Abbès	BenA1	+	+	*S. meliloti*	OQ119552
	BenA2	+	+	*S. garamanticum*	OQ119553
	BenA3	+	+	*M. acaciae*	OQ119554
Biskara- Ouled Djellal	El-K1	+	+	*S. numidicum*	OQ119558
	El-K2	+	+	*S. numidicum*	OQ119559
	El-K3	+	+	*S. numidicum*	OQ119560
Djelfa- Ain Oussara	Bir1	+	+	*S. numidicum*	OQ119555
	Bir2	+	+	*S. meliloti*	OQ119556
	Bir3	+	+	*S. meliloti*	OQ119557
El Menia-Belbachir	Bel1	+	+	*S. fredii*	OQ119546
	Bel2	+	+	*S. numidicum*	OQ119547
	Bel3	+	+	*S. numidicum*	OQ119548
Tamanrasset-Tamanrasset	Tam1	+	+	*S. meliloti*	OQ119573
	Tam2	+	+	*S. fredii*	OQ119574
	Tam3	+	+	*S. fredii*	OQ119575

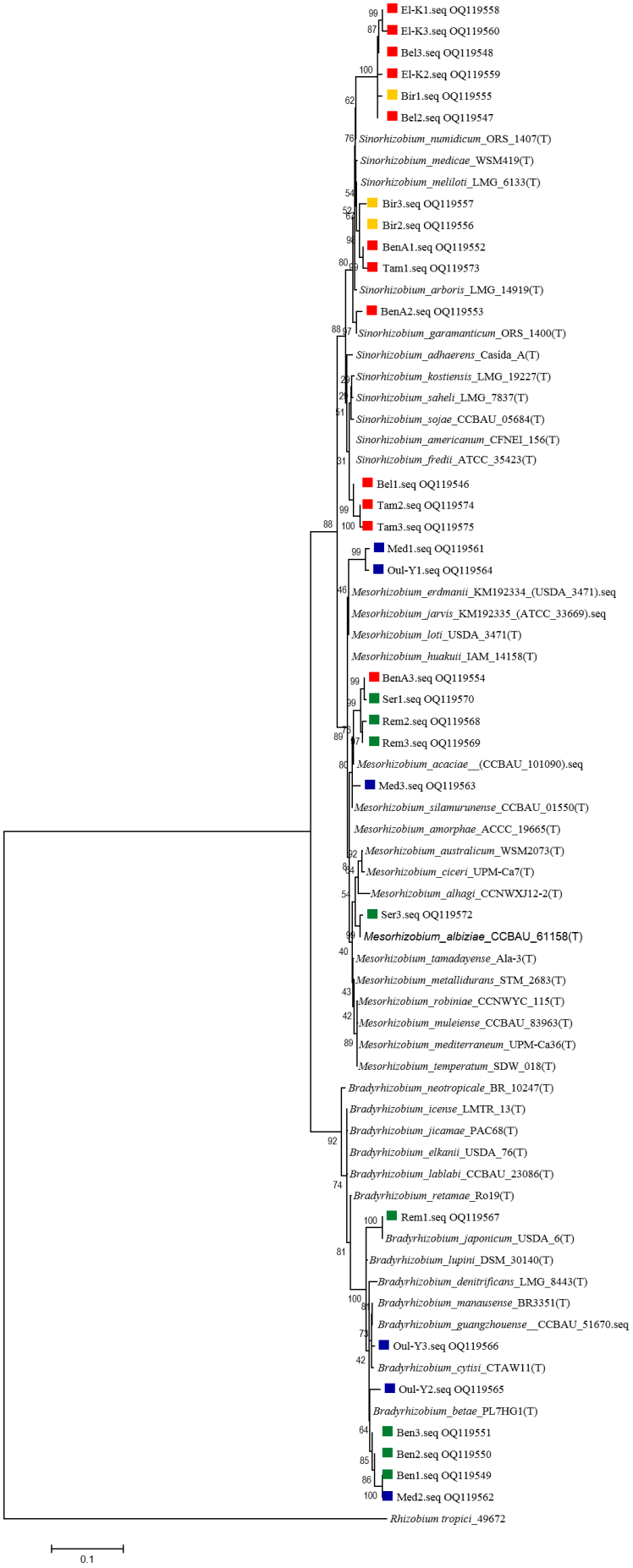

Figure 2. Phylogenetic tree based on the 16S rRNA gene sequences inferred by the Maximum Likelihood method for the bacterial Rhizobia. The bootstrap consensus tree was inferred from 1000 replicates. Isolates from this study are listed with colored labels according to color code of bioclimatic zones, while type strains of species appear with black labels. Branches with less than 50% bootstrap replicates are collapsed.
Arid Semi arid Subhumid
Humid Seq.: Sequences

Our results revealed that all the 29 isolates collected from the nodules of *V. karroo* demonstrated the capability to renodulate their original host plants in the laboratory under controlled bacteriological conditions. Consequently, these strains can be identified as rhizobia. Furthermore, the nodules formed on the roots of *V. karroo* in the laboratory displayed the presence of leghemoglobin in their central zones, indicating that the rhizobial strains obtained are efficient.

The analysis of the 16S rRNA gene sequences from the 29 strains belonged to three genera in the Alphaproteobacteria: *Sinorhizobium*, *Mesorhizobium* and *Bradyrhizobium*. Most of the isolates obtained from *V. karroo* nodules were members of the genera *Sinorhizobium* (14 isolates). The genus *Sinorhizobium* include a number of species that have been described from *Acacia sensu lato* species [3, 5, 8]. Our results indicate a considerable diversity within this genus compared to the other two genera represented in our collection. Four species within the genus *Sinorhizobium* where identified: *S. numidicum*, *S. meliloti*, *S. fredii*, and *S. garamanticum* with *S. numidicum* being the most prevalent, represented by six strains (El-K1, El-K2, El-K3, Bir1, Bel2, and Bel3). This species was first isolated from *Lotus arabicus* L. nodules in Senegal, a finding highlighted by Merabet *et al.* [35]. Following *S. numidicum* in dominance were *S. meliloti* and *S. fredii*, with each being represented by four and three strains, respectively. These strains are recognized as microsymbionts for a wide array of legumes, both has been reported in Algeria, Morocco, and Tunisia by several authors [1, 6, 18, 25, 39]. The 14 strains of *Sinorhizobium* species obtained from *V. karroo* in the current study are not common in all the biomes examined. They were found in nodules sampled only from arid regions. This exclusivity arises from the synergistic effects of rapid growth rates and the exceptional adaptability of *Sinorhizobium* bacteria to the harsh conditions prevalent in arid and semi-arid regions [53].

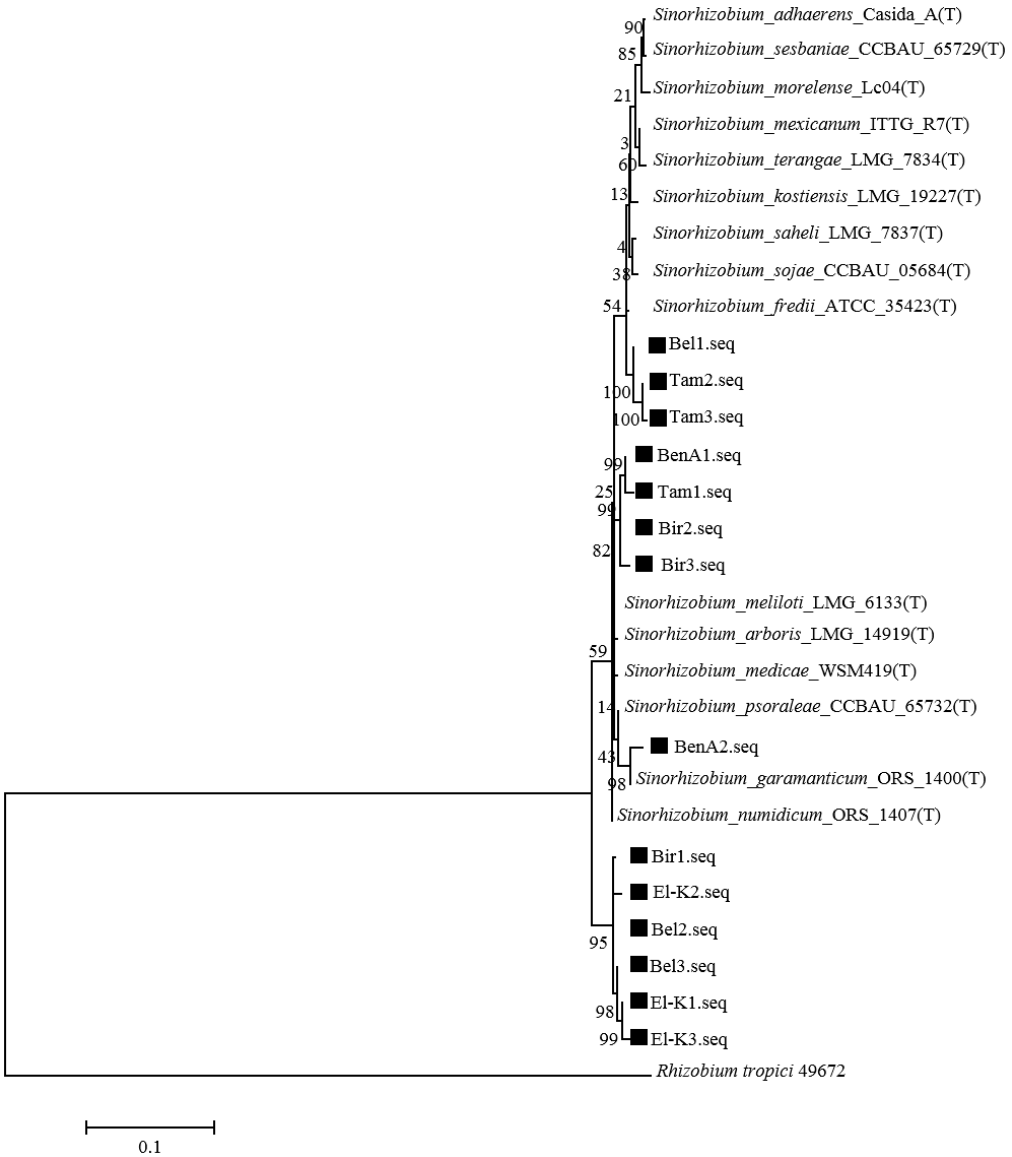

Figure 3. Phylogenetic tree of the locus 16S rRNA gene inferred by the maximum likelihood method for the *Sinorhizobium* genera. The bootstrap consensus tree was inferred from 1000 replicates. Isolates from this study are highlighted in bold. Seq.: Sequences.

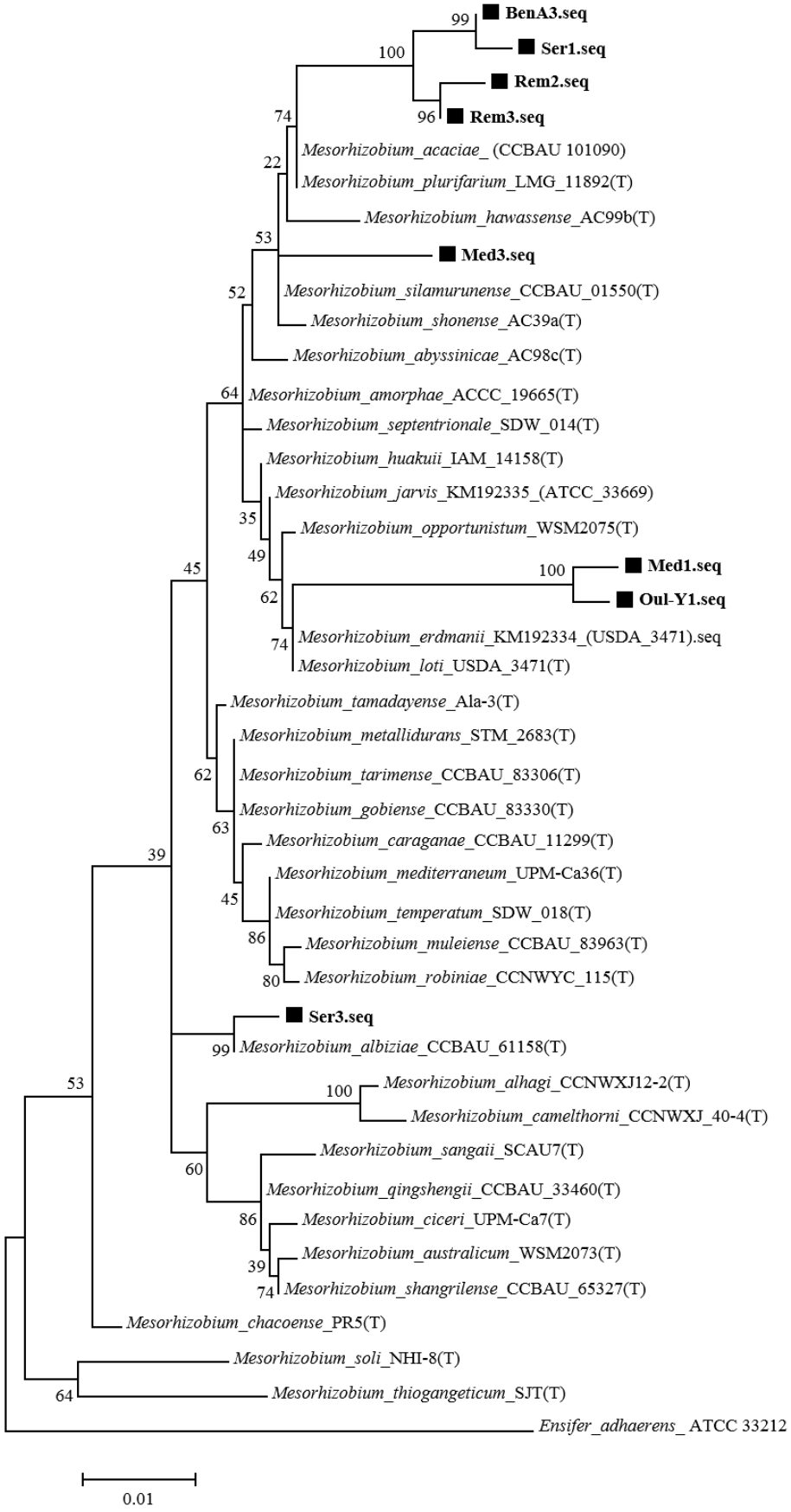

Figure 4. Phylogenetic tree of the locus 16S rRNA gene inferred by the maximum likelihood method for the *Mesorhizobium* genera. The bootstrap consensus tree was inferred from 1000 replicates. Isolates from this study are highlighted in bold. Seq.: Sequences.

Eight strains among the 29 strains collected from *V. karroo* (Fig. 2) are related to the genus *Mesorhizobium*. Although *V. karroo* in Algeria and Kenya have been reported to associate with *Rhizobium* and *Sinorhizobium* [6, 34], our study showed that in addition to *Sinorhizobium*, *Mesorhizobium*, and *Bradyrhizobium* also capable of nodulating *V. karroo*. This finding concord with Beukes *et al.* [3] who showed for the first time that *V. karroo* is nodulated by *Bradyrhizobium*, *Mesorhizobium*, *Paraburkholderia*,

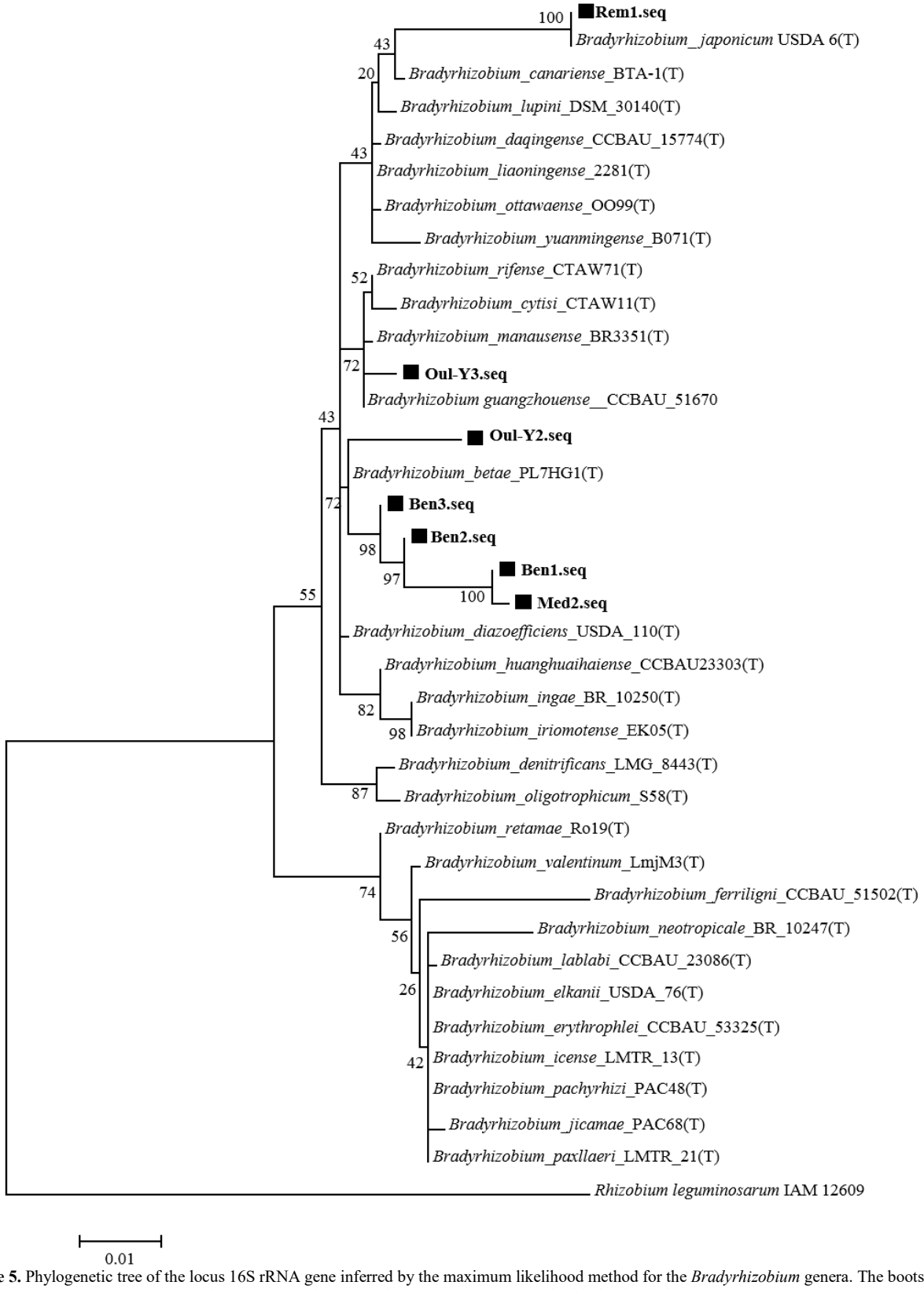

Figure 5. Phylogenetic tree of the locus 16S rRNA gene inferred by the maximum likelihood method for the *Bradyrhizobium* genera. The bootstrap consensus tree was inferred from 1000 replicates. Isolates from this study are highlighted in bold.

Sinorhizobium, and *Rhizobium*. Despite having fewer strains compared to *Sinorhizobium*, *Mesorhizobium* exhibits similar diversity with four species identified (*M. acaciae*, *M. erdmanii*, *M. silamuruense*, and *M. albiziae*). However, *M. acaciae* is the dominant species within the *Mesorhizobium* branch, represented by four strains (Ser1, BenA3, Rem2, and Rem3). This species was isolated from nodules of *Acacia melanoxylon* in Guangdong Province, China [58]. The strains Med1 and Oul-y1 are related to *M. erdmanii*, a species identified following genotypic analysis of various specimens of the type strain of the genus *Mesorhizobium* (*M. loti*), in which genotypic divergences were found [33].

The genus *Bradyrhizobium* is represented by seven strains (Rem1, Oul-Y3, Oul-Y2, Ben3, Ben2, Ben1, and Med2) isolated only from the northern regions of Algeria. The strains Ben3, Ben2, Ben1, and Med2 are related to *B. betae*, a species first isolated from tumor-like deformations on the roots of sugar beet (*Beta vulgaris* L.). One strain Rem1 is related to *B. japonicum*, which is considered the type species of the *Bradyrhizobium* genus. It is known to be the microsymbiont of herbaceous legumes, such as representatives of the genus *Lotus* [30, 49]. It has also been found as the microsymbiont of many woody legumes, including acacias [1, 44].

The *V. karroo* plants originating from the ten nurseries across three different eco-climatic regions are nodulated by various rhizobia genera. Notably, plants nodules from nurseries located in the northern regions, characterized by a humid to sub-humid climate, show the presence of slow growing and intermediate growing rhizobia, associated respectively with the genera *Bradyrhizobium* and *Mesorhizobium*. This can be attributed to environmental factors such as mild temperatures and high rainfall in the northern region, contrasting with limited adaptation to the edaphic and climatic conditions of the southern regions [57]. Numerous studies have highlighted the influence of environmental conditions, including temperature, rainfall, pH, and altitude of sampling sites, on rhizobia diversity [23, 28, 45].

In contrast, plants from nurseries in semiarid and Saharan bioclimatic regions in the south of the country exhibit nodulation by fast growing rhizobia associated with species of the genus *Sinorhizobium*, likely due to their adaptation to arid conditions, including high temperatures and drought [6, 8, 18, 27]. Moreover, several authors [51, 56] have suggested that the genotype of the host plant plays a crucial role in regulating genetic variation in rhizobia. Vuong *et al.* [55] demonstrated that the host species significantly influence rhizobia community structure in acacia nodules. Additionally, the identity of rhizobia species capable of forming a symbiotic relationship with the host plant is influenced by both the composition and structure of the indigenous microbial community [46] and the nature, structure, physical and chemical characteristics of the soil. In Algeria, research conducted by Djouadi *et al.* [15] revealed a predominance of the *Sinorhizobium* genus in nodules of *Hippocrepis* L. species from southern Algeria, while species belonging to the *Bradyrhizomium* genus colonize nodules of *Hippocrepis* species distributed in northern Algeria. Additionally, Amrani *et al.* [1] demonstrated that plants of *Acacia saligna* cultivated in Algerian nurseries are rarely nodulated by species of the *Bradyrhizobium*.

This study concluded that although the distribution of *V. karroo* is concentrated in the northern part of the country, under humid and sub-humid eco-climates, this plant exhibits an ability to thrive in a range of arid habitats with harsher climatic and edaphic conditions. Such adaptability reflects not only climatic tolerance but also the influence of soil factors, including pH and calcium content, which shape the diversity and efficiency of the rhizobial populations associated with *V. karroo*. In this context, the presence of both *Sinorhizobium* and *Bradyrhizobium* species, known to differ in their performance across soil types, may contribute to the observed ecological plasticity of this legume.This allows us to consider it a potential resource for soil rehabilitation and restoration. Furthermore, the genomic identification of associated rhizobia revealed their ability to interact with various symbionts, which certainly have a major role in their adaptive capacity. These insights have implications for enhancing the sustainability and productivity of agroecosystems through targeted inoculation strategies and the conservation of indigenous rhizobia populations. Moving forward, continued research efforts are warranted to elucidate the mechanisms underlying rhizobial symbiosis in woody legumes and to harness their potential for sustainable agricultural practices in Algeria.

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