PREVALENCE OF EFFECTIVE FIXING NODULATION AND CHARACTERISATION OF ASSOCIATED RHIZOBIA IN *Leucaena leucocephala* (LAM.) De Wit SEEDLINGS FROM ALGERIAN NURSERIES

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Abstract. This study investigates the diversity and nodulation efficiency of rhizobial endophytes associated with *Leucaena leucocephala* nodules across 12 nurseries located in different climatic regions of Algeria. Rhizobial trapping revealed that 82.14% of strains were able to induce nodulation, with a noticeable decline in nodulation frequency and effectiveness in southern arid regions. Environmental factors such as soil texture,pH, precipitation, and temperature were key determinants influencing symbiotic performance. Strains displayed varied growth rates and host ranges; in the north, both fast- and slow-growing rhizobia nodulated *L. leucocephala*, whereas in the south, only fast-growing strains were effective. Fast growing strains exhibited broader host ranges and greater tolerance to salinity and drought compared to slow-growing. Phylogenetic analysis identified four genera involved in nodulation, including *Bradyrhizobium*, *Sinorhizobium*, *Mesorhizobium* and *Rhizobium*, with *Sinorhizobium* predominating in harsher southern soils. These findings highlight the impact of environmental stressors on rhizobial diversity and nodulation patterns and emphasize the importance of selecting stress-tolerant symbiotic partners for successful legume inoculation in arid and semi-arid regions.

Keywords: *Leucaena leucocephala*; nitrogen; nurseries; rhizobium; 16S rRNA; symbiosis.

INTRODUCTION

The flora of Algeria includes a wide variety of introduced leguminous trees that play a significant role in enhancing the country's tree biodiversity. Most of these species are concentrated in the northern regions of Algeria, where the humid climate provides favorable conditions for their establishment [1, 46]. Among them, the subfamily Caesalpinioideae is particularly well represented by genera such as *Acacia*, *Vachellia*, *Albizia*, *Prosopis*, and *Leucaena* [40].

One notable species is *Leucaena leucocephala* (Lam.) de Wit, native to Mexico and introduced in Algeria as early as 1850 [25, 26, 40]. Known for its high resilience, this species thrives in challenging environments, tolerating salinity, drought, and elevated temperatures. Its adaptability has contributed to its widespread and prevalence in Algeria and globally [4, 11, 13, 59].

Leucaena leucocephala is a branched shrub or small tree legume belonging to the genus *Leucaena*, family Fabaceae, subfamily Caesalpinioideae comprising around 32 species. *Leucaena leucocephala* is the most important among them [54]. Its leaves are bipinnate with 6-9 pairs of pinnae bearing 11-23 pairs of leaflets, each 8-16 mm long. Flowers are numerous, white to yellowish white, formed in axillary, cream colored to yellowish-white, globose, and form in axillary clusters [35]. These are followed by flat brown pods 13-18 mm long containing 15-30 seeds [35].

This species exhibits several key traits that contribute to its multiple ecological and agricultural roles [39]. As a fast-growing legume with a robust root system, *L. leucocephala* enhances soil structure and promotes nitrogen fixation, thereby enhancing soil fertility [4, 13]. These features make it highly effective for land reclamation, stabilization of sandy soils, and combating desertification. Additionally, its leaves and pods are protein-rich, providing valuable fodder for livestock and enhancing agricultural productivity. Its rapid biomass production makes the species an important source of firewood and charcoal for rural communities [16, 31]. The tree also offers additional agroforestry benefits, including functioning as a windbreak, providing shade, and serving as a visual screen [31].

Research on rhizobia associated with *L. leucocephala* has mainly been concentrated in its native Mexico [37, 38, 43], the species' primary distribution center [42] as well as in South America [43, 51, 64], Asia [15, 66] and Africa [17].

These studies reveal that *L. leucocephala* is a promiscuous legume, capable of being nodulated by various rhizobia species, including fast-growing genera like *Rhizobium* [6, 66], *Sinorhizobium* [7, 24, 63] and *Mesorhizobium* [6, 62], as well as slow-growing *Bradyrhizobium* [60].

Nevertheless, some studies indicate a preference for specific rhizobia, particularly fast-growing strains [5, 58, 66]. Ribeiro *et al.* (2012) [51] highlighted the high specificity of *L. leucocephala* in recruiting rhizobia species, with *Rhizobium leucaenae* sp. nov. representing 79% of the nodules in the Cerrados savannah [45]. This strain has also been found to nodulate other legumes such as *L. esculenta*, *Gliricidia sepium*, and *Phaseolus vulgaris*.

Interestingly, introduced legumes often bring their native symbionts with them into new environments, where they interact with local rhizobia [2, 65]. For instance, *Mesorhizobium* was the dominant rhizobial genus in *L. leucocephala* nodules from subtropical China [62], whereas in the Panxi region and in India, the main symbionts were from the genus *Sinorhizobium* [5, 15].

Despite the species ecological and agricultural importance and its wide distribution across Algeria, no

research has yet been conducted on the distribution of *L. leucocephala* in Algeria or the nature of its associated rhizobia. To address this knowledge gap, the present study aims to characterize and identify rhizobia associated with *L. leucocephala* by sequencing the 16S rRNA gene from root nodules collected in twelve nurseries across various Algerian regions, which were selected based on their climatic conditions and soil characteristics.

This research builds on previous work by Amrani *et al.* (2010) [2] and seeks to deepen our understanding of rhizobial biodiversity associated with introduced legume species in Algeria. It also aims to explore how plant and soil factors influence rhizobial community composition and whether *L. leucocephala* shows specificity in its rhizobial associations or is more influenced by the environmental availability of native strains. Ultimately, the identification and characterization of rhizobia associated with *L. leucocephala* could reveal promising bacterial strains for use as bio-inoculants. These could significantly enhance nitrogen fixation and improve legume productivity under harsh environmental conditions, particularly in arid regions such as the Algerian Sahara.

MATERIALS AND METHODS

Evaluation of rhizobial symbiosis and nitrogen fixation prevalence

The evaluation of rhizobial symbiosis and nitrogen fixation prevalence in *L. leucocephala* nodules was carried out on 16 weeks old seedlings grown in 12 nurseries located in both northern and southern regions of the country (Fig.1).

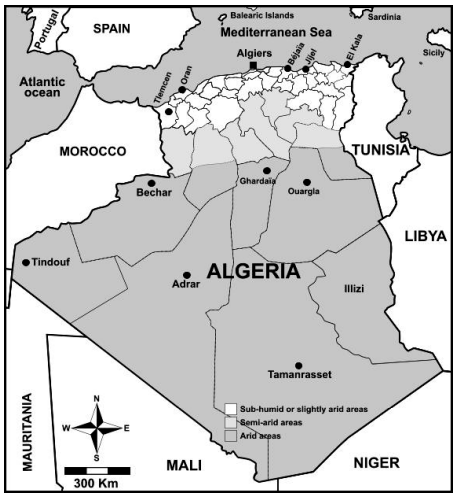

Figure 1. Geographic location of the 12 nurseries from which *Leucaena leucocephala* seedlings were produced and collected for use in the recovery of associated rhizobia.

The seedlings were produced from seeds collected from a single tree of *L. leucocephala* located in the Baïnem forest (Algiers) (36°48'14.30"N, 2°57'28.03"E) in June 2022.

The seeds were surface-sterilized by immersion in 5% calcium hypochlorite solution for 10 min, rinsed thoroughly sterile distilled water, and then were grown in perforated polyethylene bags (15x25 cm) filled with a mixture of nursery soil and river sand (v/v, 2/1). The sand was previously sterilized by autoclaving at 121°C for1 hour, repeated three times at 24-hour intervals. Irrigation was performed every three days using a volume of water corresponding to 80% of the soil water holding capacity.

From each of the 12 nurseries investigated, 10 randomly selected plants were harvested and examined in the laboratory. The root systems of the seedlings were carefully removed from the polyethylene bags, washed with distilled water to remove soil, and then visually inspected for the presence of nodules. The presence of nodules, their location, morphology, biomass and, apparent efficiency were recorded for each plant.

Isolation of rhizobia associated strains

The collected nodules were treated using the same protocols as described by Djouadi *et al.* (2017, 2021) [18, 19]. The resulting strains were purified by repeated streaking of a single colonies on YMA medium and were checked for purity by light microscopic of living cells and Gram staining [61]. They were then stored at 4°C on YMA slants and at -80°C as aliquots in Tryptone Yeast Extract (TY) liquid cultures supplemented with of 25% (v/v) glycerol [8].

Determination of the osmotolerance of the strains

Tolerance of the strains to osmotic stress (salinity or drought) was evaluated in liquid minimal medium MAS [27] supplemented with 20 mM glucose as carbon source. Salinity stress was imposed by adding NaCl to the MAS basal medium to obtain different concentrations (0, 100, 200, 400 and 500 mM). Drought stress was simulated by adding PEG 6000 to the medium at final concentration ranging from 0% (control) to 25%. Three replicates were prepared for each strain and each treatment. Each isolate was inoculated in 5 mL of MAS medium under corresponding condition and incubated for 48 h at 28°C with shaking at 180 rpm. Bacterial growth was quantified by measuring the optical density at 600 nm (OD600) using a UV/Visible spectrophotometer (Jenway™ 6305).

For simplicity and comparative purposes, strain tolerance was reported qualitatively, based on OD600 values and visual inspection. Three categories were used: (+): clear turbidity and OD600 > 0.2 (active growth), (±): weak turbidity and OD600 ≈ 0.1–0.2 (delayed or partial growth), (–): OD600 < 0.1 (no visible growth).

Nodulation and effectiveness test

Strains were tested for their nodulation and nitrogen fixation ability on young plantlets of *Leucaena leucocephala, Acacia saligna* and *Vachellia tortilis* subsp. *raddiana*. Seeds were provided from National Forest Research Institute, Baïnem-Algiers and scarified before use with a cutter blade to break the dormancy. They were then surface sterilized using the method described above, except that immersion into sodium

hypochlorite was maintained for 10 min. Surface sterilization of both nodules and seeds was confirmed by rolling them onto Nutrient Agar (NA) plates followed incubation at 28°C. Seeds were pre-germinated on Bacto water agar (0.75% w/v) for three days at 25°C and then transferred to plastic pots containing 300 g of sterilized mixture of river black sand and peat (2/1 v/v). Three seedlings were planted per pot.Plants were inoculated with 3 mL of early stationary phase rhizobial culture (10^8 to 10^9 cells mL^{-1}) previously grown in TY broth at 28°C. Five replications were carried out for both inoculated and non-inoculated plants (negative controls). Plantlets were harvested two months after inoculation and examined for root nodulation. The efficiency of the symbiotic couples was assessed 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 [21, 47].

16S rRNA gene sequencing and phylogenetic analysis

Total genomic DNA was extracted from 1.5 mL cultures grown in TY medium using the Genomic DNA Purification Kit (SpinClean™ Genomic DNA Purification Kit – Mbiotech, Korea). The 16S rRNA gene amplification was conducted using the universal forward and reverse primers: fD1: 5′-AGAGTTTGATCCTGGCTCAG-3' and rD1: 5′-AAGGAGGTGATCCAGCC-3′ [56] provided by MWG Biotech (Germany). Polymerase chain reaction (PCR) was carried out for each strain in a 50 µL reaction volume in presence of 1 µL of extracted DNA as the template, 5 µL of ten-fold concentrated PCR buffer (Invitrogen, USA), 5 µL (25 mM) of $MgCl_2$, 8 µL (1.25 mM) of dNTP mix, 2.5 µL (12 µM) of each primer and 0.5 µL (1.25 U) of *Taq* DNA polymerase. The PCR reaction temperature used was 94°C for 3 min followed by 35 cycles consisting of 95°C for 1 min, 50°C for 1 min, 72°C for 1 min and a final extension step at 72°C for 3 min. Reaction efficiency was estimated by horizontal electrophoresis on 1% (w/v) agarose-gel (1% w/v) containing 1 $mg \times mL^{-1}$ ethidium bromide.

Nearly the full 16S rRNA gene sequence of 22 selected strains was obtained using the primers 16F27 (5′-AGAGTTTGATCMTGGCTCAG-3'), 16R1488 (5′-CGGTTACCTTGTTACGACTTCACC-3'), 16R343 (5'-ACTGCTGCCTCCCGTA-3') and 16F530 (5′-TTCGTGCCAGCAGCCGCGG-3') (MWG Biotech, Germany)**.** Sequencing was performed on an automated DNA sequencer (model 3100, Applied Biosystems, USA) by the company Newbiotechnic (Seville, Spain). Amplified sequences of 16S rRNA gene were assembled using the Auto-Assembler tool of ChromasPro Program (Technelysium Pty. Ltd., Australia) and aligned using the Clustal-W method of the MEGA4 software [57]. The phylogenetic tree was constructed by using the neighbour-joining method of the MEGA program and the stability of the grouping was estimated by running 1000 pseudoreplicates. The 16S rRNA gene sequences of rhizobial type strains related to the isolates were retrieved from the EZTAXON database [12] and included in the phylogenetic analysis.

Statistical analysis

Data from 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% level using RStudio v.2.13 (R Development Core Team, 2016).

Due to the qualitative nature of osmotolerance data (growth scoring as "+", "±", or "−"), comparisons between strain groups (Group I vs Group II) were carried out using the non-parametric Mann–Whitney U test to assess differences in tolerance frequencies at increasing concentrations of NaCl and PEG 6000. Results were considered statistically significant when p-values < 0.05.

RESULTS

Prevalence of rhizobial symbiosis

The prevalence of nodulation was assessed in 144 *Leucaena leucocephala* seedlings of 16 weeks old harvested from the 12 explored nurseries. Results (Table 1) showed that 112 seedlings (77.77%) harboured in their root system the characteristic nodules of rhizobial-legumes symbiosis. From the 112 nodulated seedlings, 90 seedlings (62.5 %) were considered effective, as nodule cross-sections showed a pinkish or reddish coloration attesting the presence of leghemoglobin, which is produced only by nitrogen fixing nodules [20, 32]. However, we noticed that the prevalence of nodulation and nitrogen fixation as well as nodules biomass were significantly different ($P<0.05$) whether it were originated from nursery situated in the northern part or the southern part of the country. Indeed the average proportion of nodulated seedlings and within the later the proportion of efficient nodulation were much more higher for plants from the northern part of the country 93 % and 84 % respectively than for the plants produced by nurseries from the southern part of the country with only 62.5 % nodulated plants and 40.27 % of efficient nodulated plants.

Nodules on the root systems of all seedlings were primarily located on the secondary roots and displayed an elongated lobe morphology that characterizes indeterminate nodules. The only significant difference observed was in nodule biomass. Nodules biomass reached an average of 95.31±47.25 mg for northern nurseries while it was significantly lower ($P<0.05$) 35.48±21.23 mg of for southern nurseries.

Strains collection and characterization

From the root nodules of *Leucaena leucocephala* cultivated in the 12 nurseries around the country, 28 purified strains were collected. Only 22 strains showed the typical colonial characteristics of rhizobia, including transparent to creamy colonies on YMA

plates, pink colour of cells in Gram test and negative catalase reaction [23].

The 22 strains were classified into two groups based on their growth in YMB at 28±2°C. Group I included 17 strains from both northern and southern nurseries and showeded fast growth, the strains extracted, forming visible colonies within 1.9 to 4.7 h of incubation, Group II consisted of five strains, isolated only from northern nurseries, which exhibited slow growth, forming colonies after 8.7 to 9.4 hours (Table 2). These observations indicate that the collection included both fast- and slow-growing rhizobia [6].

The overall analysis of the phenotypic profiles obtained allowed us to distinguish between the strains of the two groups previously defined on the basis of their growth speed. All strains were able to grow in MAS without NaCl or PEG. Group I strains tolerated NaCl concentrations of 100, 200 and 400 mM, with 100% growth rate 100%, whereas, group II strains showed lower tolerance, with growth rates of 80%, 40% and 0% at the same concentrations. Strains, OUA-2, TIN-1, TIN-2 and TAM-2 showed the ability to tolerate the high concentration of 500 mM NaCl. In contrast, the isolates ALG-1, ALG-2, JIJ-1, BJA-1 and ELK-1 were unable to grow to 400 mM and 500 mM NaCl and therefore were consider as salt sensitive.

Table 1. Geographical origin, growth, symbiotic characteristic of rhizobial strains associated to *Leucaena leucocephala.*

	Nurseries	Bioclimatic zone	pH Soil	Mean daily temperature during the experimentation (T°C)	Number of nodulated seedlings	Main nodule localization	Number of nitrogen fixing seedlings	Nodule number	Plant dry weight (g/seedlings)	Nodule dry weight (mg/seedlings)
Northen nurseries	Algiers – Baînem	S.H	6.6	19.38	12	IIR	12	36.58^{ab}	36.58^{ab}	36.58^{ab}
	Béjaia (Souk El Tenine)	S.H	7.1	19.08	12	IIR	10	28.42^{bc}	28.42^{bc}	28.42^{bc}
	El Kala	H	7.6	19.56	12	IIR	12	46.25^{a}	46.25^{a}	46.25^{a}
	Jijel	S.H	7.3	19.54	12	IIR	12	32.25^{bc}	32.25^{bc}	32.25^{bc}
	Oran (Messerghine)	S.H	7.5	19.66	9	IIR	9	27.25^{bc}	27.25^{bc}	27.25^{bc}
	Tlemcen	S.H	7.3	19.20	10	IIR	6	11.50^{d}	11.50^{d}	11.50^{d}
Sothern nurseries	Adrar (desertic)	H	8.4	28.56	6	C	5	2.75^{e}	2.75^{e}	2.75^{e}
	Bechar (desertic)	H	7.9	25.32	7	IIR	4	3.42^{e}	3.42^{e}	3.42^{e}
	Ghardaia (desertic)	H	8.3	26.78	11	IIR	7	10.33^{d}	10.33^{d}	10.33^{d}
	Ouargla (desertic)	S	8.5	23.66	8	IIR	4	6.36^{de}	6.36^{de}	6.36^{de}
	Tamanrasset (desertic)	S	8.3	26.22	4	C	4	2.17^{e}	2.17^{e}	2.17^{e}
	Tindouf (arid)	S	7.8	27.22	9	IIR	5	4.92^{de}	4.92^{de}	4.92^{de}

S.H: Sub Humid, H: Humid, **S**: Saharan. **IIR**: Secondary root, **C**: Collar region.Values within each column followed by the same letter are Values within each column followed by the same letter are not significantly different according to Fisher's Least Significant Difference (LSD) test at $P < 0.05$. Different letters indicate significant differences among means.

Table 2. Effect of salinity (NaCl), and drought (PEG6000) stress on growth of *Leucaena leucocephala* associated Rhizobia in glucose MAS minimal medium.

			NaCL (mM)				PEG6000 (%)			
	Code	Generation time (h)	100	200	400	500	5	10	15	25
Groupe II	**ALG-1**	9.1	+	-	-	-	+	-	-	-
	ALG-2	9.3	-	-	-	-	+	-	-	-
	JII-1	9.4	+	+	-	-	+	+	-	-
	BEJ-1	8.9	+	-	-	-	+	+	+	-
	ELK-1	8.7	+	+	-	-	+	+	+	-
Groupe I	**ELK-3**	3.1	+	+	+	-	+	+	+	-
	JIJ-2	2.3	+	+	+	-	+	+	+	-
	OUA-2	2.7	+	+	+	+	+	+	+	+
	TIN-1	3.3	+	+	+	+	+	+	+	+
	TIN-2	3.5	+	+	+	+	+	+	+	+
	TAM-1	2.8	+	+	+	-	+	+	-	-
	TAM-2	3.2	+	+	+	+	+	+	+	+
	TAM-3	3.2	+	+	+	-	+	+	+	-
	ORA-2	3	+	+	+	-	+	+	-	-
	BEJ-3	4.2	+	+	+	-	+	±	-	-
	TLE-1	4.3	+	+	+	-	+	±	-	-
	TLE-2	4.1	+	+	±	-	+	+	-	-
	TLE-3	3.9	+	+	+	-	+	+	-	-
	BEC-2	4.4	+	+	±	-	+	+	+	-
	BEC-3	4.7	+	+	+	-	+	+	+	-
	ADR-1	3.7	+	+	+	-	+	+	-	-
	ADR-3	3.9	+	+	+	-	+	-	-	-

(+): clear turbidity and OD600 > 0.2 (active growth), **(±)**: weak turbidity and OD600 ≈ 0.1–0.2 (delayed or partial growth), **(–)**: OD600 < 0.1 (no visible growth).

Group I and II strains showed variations similar to those observed for salinity. Group I strains showed very high tolerance to PEG 6000 at 5%, 10%, and 15%, but low resistance at 25%. In contrast, Group II strains were highly sensitive to all PEG concentrations. Four highly osmotolerant strains (OUA-2, TIN-1, TIN-2, and TAM-2) were able to grow at all PEG concentrations tested. Strains JIJ-2, ELK-3, TAM-3, BEC-2, and BEC-3 displayed intermediate tolerance, whereas two Group II isolates (BEJ-1 and ELK-1) were able to tolerate only the lower PEG concentrations of 5%, 10%, and 15%.

To evaluate the osmotolerance of rhizobial strains, the tolerance profiles of Group I and Group II strains were statistically compared under salinity (NaCl) and drought (PEG 6000) stress using the MannWhitney U test. The results revealed significant differences in salt tolerance between the two groups. Group I strains exhibited significantly higher tolerance to 100 mM ($U = 135.0$; $p = 0.0013$), 200 mM ($U = 153.0$; $p = 7.5\times10^{-5}$), and 400 mM NaCl ($U = 180.0$; $p = 9.8\times10^{-7}$) compared to Group II strains. These results highlight a greater saline resilience in Group I strains.

In terms of drought stress, induced by PEG 6000, Group I strains alsoexhibited significantly higher tolerance at 5% PEG ($U = 126.0$; $p = 0.005$) and 10% PEG ($U = 140.0$; $p = 0.0059$) compared to Group II strains. However, no statistically significant differences were detected between the two groups at the highest PEG concentration tested (25%) or under NaCl stress at 500 mM. These findings suggest that Group I strains are better adapted to moderate osmotic stress conditions.

Symbiotic efficiency of the isolates

The 22 strains were tested under controlled laboratory conditions of temperature (25°C) and humidity (70%) for their infectivity and effectiveness to fix atmospheric nitrogen in symbiosis with young plantlets of *L. leucocephala*, *A saligna* and *V. tortilis* subsp. *raddiana* (Table 3). The authentication test on *Leucaena leucocephala* was performed alongside the two species that show the highest responsiveness in Algeria. *Acacia saligna* introduced species most responded in the northern regions [1, 2] and *Vachellia tortilis* subsp. *Raddiana,* the native species most responsive in the southern region [46].

Results showed that the 22 strains were authenticated as rhizobia, as they successfully induced nodulation in *Leucaena leucocephala*. The strains exhibited variable host ranges. Strains of Group I were able to induce effective nodules in *Leucaena leucocephala*, *Acacia saligna*, and *Vachellia tortilis* subsp. *raddiana*, demonstrating a broad host spectrum. In contrast, Group II strains displayed a narrower nodulation spectrum, as they were only able to nodulate the introduced species *Leucaena leucocephala* and *Acacia saligna*. These Group II strains were primarily isolated from nurseries located in the northern region of the country.

Phylogenetic analysis of bacterial strains

Phylogenetic trees based on the Neighbor-joining and Maximum Parsimony methods show sensibly the same topologies and all major relationships were supported by the two methods. Only Neighbor-joining cladogram is presented here (Fig. 2).

The two major clades and their subclades show moderate to strong support, as indicated by the bootstrap values (BS) in the Figure 2. Clade I (100% BS) include strains belonging to the genus *Bradyrhizobium* (Bradyrhizobiaceae). The five *Bradyrhizobium* strains were separated into two distinct species.

Table3. Identity of rhizobia strains isolated from nodules of *Leucaena leucocephala* plants produced in nurseries

Nursery	Code isolate	*L. leu*	*A. sal*	*V. tor*	Assignment based 16S rRNA sequences	NCBI accession number
Algies-Bainem	ALG-1	+	+	-	*B. nitroreduceus*	OQ324734.1
	ALG-2	+	+	-	*B. nitroreduceus*	OQ324733.1
Béjaia-Djebira	BEJ-1	+	+	-	*B. nitroreduceus*	OQ324722.1
	BEJ-3	+	+	+	*R. mesoamericanum*	OQ324726.1
Jijel – Tamila	JIJ-1	+	+	-	*B. guangxiense*	OQ324728.1
	JIJ-2	+	+	+	*S. medicae*	OQ324737.1
El Kala-Souk Reguibat	ELK-1	+	+	-	*B. guangxiense*	OQ324731.1
	ELK-3	+	+	+	*S. medicae*	OQ324732.1
Tlemcen-Tlemcen	TLE-1	+	+	+	*R. leucaenae*	OQ324721.1
	TLE-2	+	+	+	*R. azibense*	OQ324718.1
	TLE-3	+	+	+	*R. azibense*	OQ324714.1
Oran-Es senia	ORA-2	+	+	+	*S. medicae*	OQ324717.1
Bechar-Abadla	BEC-2	+	+	+	*R. tropici*	OQ324716.1
	BEC-3	+	+	+	*R. yanglingense*	OQ324716.1
Ouargla-Ouargla	OUA-2	+	+	+	*S. medicae*	OQ324711.1
Tindouf-Oum El Assel	TIN-1	+	+	+	*S. medicae*	OQ324735.1
	TIN-2	+	+	+	*S. medicae*	OQ324736.1
Adrar-Adrar	ADR-1	+	+	+	*M. plurifarium*	OQ324724.1
	ADR-3	+	+	+	*M. plurifarium*	OQ324727.1
Tamanrasset-Tamanrasset	TAM-1	+	+	+	*S. medicae*	OQ324713.1
	TAM-2	+	+	+	*S. medicae*	OQ324712.1
	TAM-3	+	+	+	*S medicae*	OQ324715.1

R, Rhizobium; *M, Mesorhizobium*; *B, Bradyrhizobium; S, Sinorhizobium; S, Sinorhizobium; L. leu, Leucaena leucocephala; A. sal; Acacia saligna; V. tor; Vachellia raddiana* subsp *tortilis.*

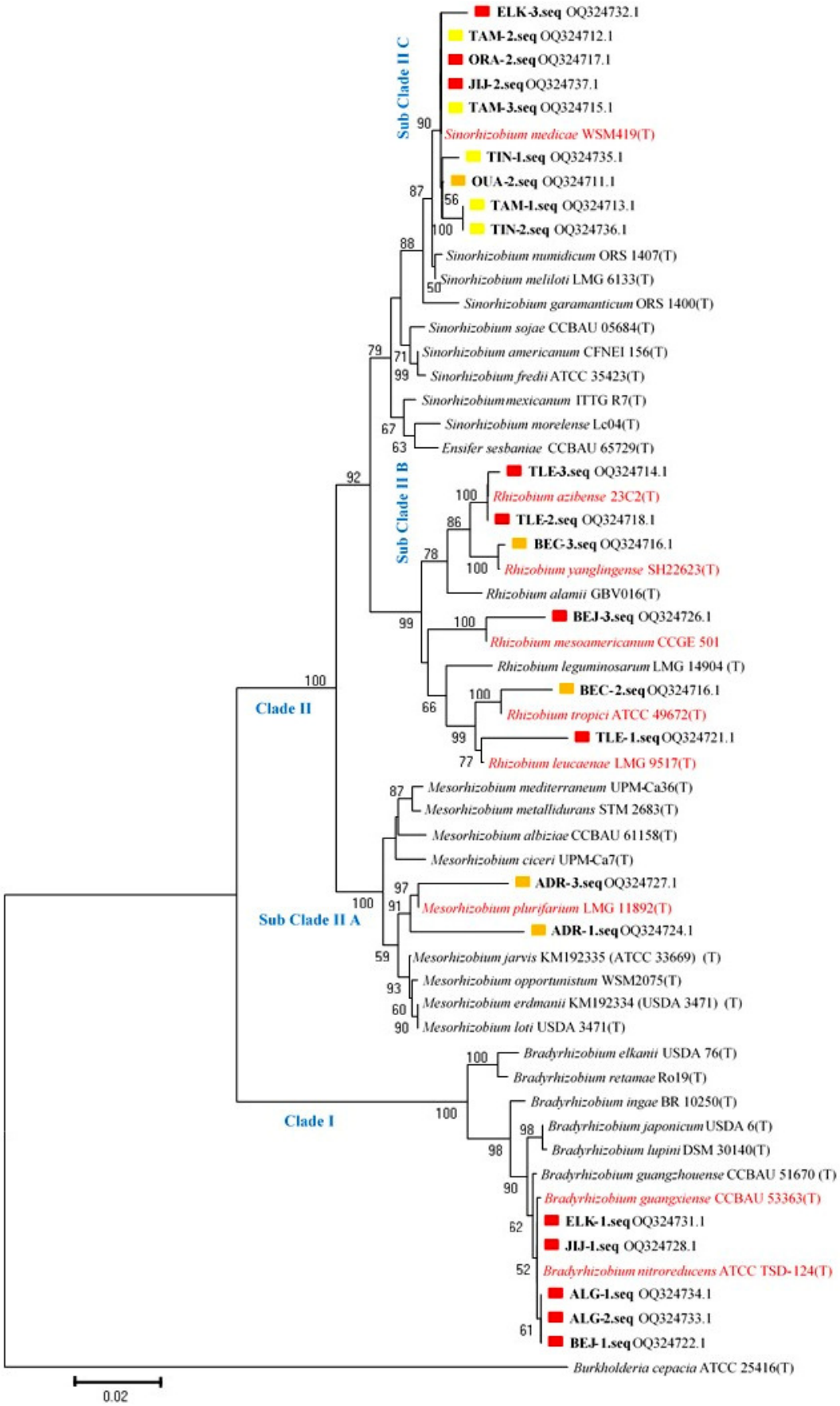

Figure 2. Phylogenetic tree based on the 16S rRNA gene sequences inferred by the Maximum Likelihood method for the bacterial Rhizobia. The bootstrap consensus tree was inferred from 1000 replicates. Isolates from this study are listed with colored labels according to color code of bioclimatic zones,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

The strains ELK-1 and JIJ-1 were affiliated with *Bradyrhizobium guangxiense*, originally isolated from peanut (*Arachis hypogaea*) nodules in Guangxi Province, southern China, with a bootstrap value of 62%. The strains ALG-1, ALG-2 and BEJ-1 were affiliated with *Bradyrhizobium nitroreducens*, supported by a bootstrap value of 61% (Fig.2).

The clade II, show three main subclades (IIA, IIB and IIC) with strong bootstrap support of 100%, 99% and 79% BS respectively. The subclade IIA include all strains referred to the genus *Mesorhizobium* (Phyllobacteriaceae), while the subclade IIB clustered the strains from the genus *Rhizobium* (Rhizobiaceae) and the subclade IIC clustered the strains from the genus *Sinorhizobium* (Rhizobiaceae).

The subclade IIA includes two strains in the *Mesorhizobium* group with two strains (ADR-1 and ADR-3) closely related to *Mesorhizobium plurifarium* with bootstrap value of 97% and 91% respectively. The subclade IIB includes six strains in the *Rhizobium* group, two strains (TLE-2 and TLE-3) were closely related to *Rhizobium azibense* with a bootstrap value of 100%.BEC-3 was related to *Rhizobium yanglingense* with a robust bootstrap value of 100s. The etrain BEJ-3 was close related to *Rhizobium mesoamericanum* with 100% bootstrap value. One strain (BEC-2) was related to *Rhizobium tropici*, TLE-1 was close related to *Rhizobium leucaenae* with bootstrap value of 77%.
Nine strains (ELK-3, TAM-2, ORA-2, JIJ-2, TAM-3, TIN-1, OUA-2, TAM-1 and TIN-2) were related to *Sinorhizobium medicae* with a bootstrap value of 90%.

DISCUSSION

The present study provides the first comprehensive assessment of the nodulation capacity and rhizobial diversity associated with *Leucaena leucocephala* seedlings cultivated in 12 nurseries across various bioclimatic zones of Algeria. Our results provide new insights into how environmental conditions and regional microbial communities influence symbiotic performance in this introduced legume. A clear biogeographic pattern in nodulation efficiency was observed. Seedlings from northern nurseries (humid and sub-humid zones) showed significantly higher nodulation and nodule effectiveness compared to those from southern arid and desert regions. In addition to climatic differences, soil characteristics such as texture and pH are likely contributing factors: northern soils were generally fertile, with a slightly acidic to neutral pH, whereas southern soils were sandy, nutrient-poor, and more alkaline. These edaphic differences may influence the distribution and selection of rhizobial species, consistent with global studies showing that legume-rhizobia associations are strongly affected by soil pH and other edaphic factors [50].These findings align with global studies indicating that nodulation is generally suppressed under drought and salinity stress [9, 10, 55]. However, the present work is among the few to document such variability in nodulation of *L. leucocephala* across a north-south aridity gradient within a single country.

In addition, we show that *L. leucocephala* in Algeria associates with both fast- and slow-growing rhizobia in the north, while only fast-growing strains are found in the south. This supports prior findings by Amri-Tiliouine (2008) [3, 52], and adds new evidence of the ecological filtering effect in rhizobial community composition due to abiotic constraints. Similar trends have been observed in tropical soils of Brazil and China, and desert regions,where fast-growing *Sinorhizobium* and *Rhizobium* species dominate under arid conditions [14, 34, 51, 53, 62, 66].The nodulation tests confirmed that Group I strains possess a broader host range, effectively nodulating *L. leucocephala*, *Acacia saligna*, and *Vachellia tortilis* subsp. *raddiana*. In contrast, Group II strains were restricted to the first two species [2, 22, 36]. These results reinforce the flexibility and promiscuity of *L. leucocephala*, previously observed in other regions [6, 42], but provide new comparative data from Algeria, where local rhizobial strains show selective symbiotic patterns depending on environmental origin.

Abiotic stress tolerance was another critical aspect of this study. Salinity and drought are major constraints in arid and semi-arid agricultural systems [48, 67]. A key contribution of our study lies in the characterization of rhizobial osmotolerance. Fast-growing strains (Group I) demonstrated significantly higher tolerance to both salinity and drought stress compared to slow-growers (Group II) [22, 33]. This phenotypic trait is essential for survival and symbiotic performance in degraded soils, and positions these strains as promising candidates for biofertilizer development. To our knowledge, this is the first report in Algeria linking physiological resilience of rhizobia to their biogeographic origin and host range within *L. leucocephala*.

Phylogenetic analysis revealed the presence of four main rhizobial genera: *Bradyrhizobium*, *Mesorhizobium*, *Sinorhizobium*, and *Rhizobium*. Interestingly, *Sinorhizobium medicae* was the most dominant species, especially in southern nurseries, confirming its ecological fitness under arid conditions. While *Bradyrhizobium* was mostly recovered from northern nurseries [2, 29, 41], its absence in southern sites may reflect competition or environmental limitations. Similar patterns have been described in China and India, where *Sinorhizobium* outcompetes *Bradyrhizobium* under drought and salinity stress [5, 34, 66].The prevalence of fast- versus slow-growing rhizobia under stressed conditions has also been documented in India, including in studies on *Leucaena leucocephala* and the mimosoid legume *Dichrostachys cinerea*, where environmental and climatic factors shaped rhizobial community composition and facilitated lateral gene transfer among *Ensifer* strains [14, 53].

Lastly, the observed specificity of *L. leucocephala* toward *S. medicae* suggests that *S. meliloti* is less

competitive for nodulation compared to *S. medicae* [30, 49], is consistent with previous competition experiments in North Africa [19, 28, 44], and highlights the importance of targeted strain selection in rhizobial inoculation programs.

In conclusion, this study highlights the influence of bioclimatic factors on nodulation performance and rhizobial diversity associated with *Leucaena leucocephala* in Algeria. The identification of fast-growing, stress-tolerant rhizobia with broad host ranges particularly those isolated from arid nurseries represents a significant step toward developing tailored bio-inoculants for sustainable legume cultivation in harsh environments. This is the first report documenting the diversity and symbiotic potential of *L. leucocephala*-associated rhizobia across Algeria, and contributes to the global understanding of rhizobia-legume interactions under abiotic stress. The strains described here offer promising applications in agroforestry and land rehabilitation programs in arid and semi-arid regions.

Conflict of interest. There is no actual or potential conflict of interest in relation to this article.

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