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Contributed Paper

Biochemical and Molecular Characterization of Native Rhizobia Nodulating *Leucaena leucocephala* with Potential Use as Bioinoculants in Yucatan, Mexico

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Abstract

The legume-rhizobium association is widely used around the world to improve crop production via nitrogen fixation (N_2-fixation). Inoculation with suitable strains is required in order to establish effective symbioses in soils. Plant growth-promoting rhizobacteria (PGPR) are of biotechnological interest since they can improve plant health and soil fertility. A collection of root-nodulating bacteria was isolated from *Leucaena leucocephala* in the Yucatan Peninsula in Mexico. The isolates were characterized based on their phenotypic features including growth rate, acidification in culture media, utilization of carbon sources and salinity stress tolerance. The antibiotic resistance level of selected strains was tested against five different antibiotics and showed that most of the strains were sensitive to kanamycin (30 μg) but resistant to ampicillin (10 μg) and trimethoprim/sulfamethoxazole (1.25/23.75 μg). Isolates 40a and 74 produced substantial amounts of indole-3-acetic acid (IAA), and other isolates produced siderophores. These isolates were used to verify their nodulation capacity on *L. leucocephala*. The 16S rRNA gene products were sequenced to determine the identity and phylogenetic diversity of the isolates, placing them in the genera *Rhizobium* and *Sinorhizobium* (syn. *Ensifer*). Based on this study, we propose that isolates *Sinorhizobium* (40a) and *Rhizobium* (74) are potential candidates to be used as inoculants to enhance symbioses and N_2-fixation using *Leucaena leucocephala* in karst soils.

Keywords: *Rhizobium*, plant growth promotion, nodulating bacteria, indole-3-acetic acid, 16S rRNA gene

1. INTRODUCTION

The Yucatan Peninsula is characterized by karst soil, low organic matter content and low fertility. However, the Peninsula is one of the richest areas in terms of plant diversity with approximately 260 legume species that have multiple uses[1]. *Leucaena leucocephala* is an important local legume species which has been recently used in the Yucatan Peninsula, not only as fodder, but also in an effort to restore degraded soil[2]. *L. leucocephala* offers multiple benefits for the producers but mainly for the environment, as it protects the soil from erosion, increases the amount of organic matter, improving soil physical and chemical properties and provides feed and shade for animals all year round, thus contributing towards the mitigation of greenhouse gas emissions using carbon sequestration, N_2-fixation and the reduction of methane emissions in ruminant production [3].

Rhizobia, in turn, are of great interest due to their symbiotic N_2-fixing capacity and are known to promote plant growth via direct and indirect mechanisms including the synthesis of plant hormones, solubilization of phosphates and the biosynthesis and release of siderophores, compounds that supply iron or phosphates to the plant when they are not available in the soil [4].

Rhizobia, also known as plant growth promoting rhizobacteria (PGPR), can act as biofertilizers, improving soil fertility and increasing the biomass yields of crops in addition to increasing the nutrient content by producing compounds that facilitate an improved uptake of nutrients[5,6]. However, the establishment of legumes depends to a large extent on the effective symbiosis with strains of specific rhizobia [7,8]. The isolation of native Rhizobium strains provides specific locally adapted inoculants that boost agri-productivity [9]. The objective of this study was to isolate and characterize native strains of Rhizobia that nodulate *L. leucocephala* in the Yucatan state, Mexico.

2. MATERIALS AND METHODS

2.1 Collection and Analysis of Soil and Nodules

Nodules were collected in three different locations of the municipality of Mérida: (1) Xmatkuil (20º 51' 59.9" N; 89º 37' 25.6" W), (2) Caucel (21º 01' 13.2" N; 89º 42' 29.7" W) and (3) Motul (21º 04' 07.2" N; 89º16' 45.7" W). The collected nodules were surface sterilized with 90% ethanol (v/v) for 10 sec and by immersion in 3% NaOCl (v/v) for 3 min., followed by rinsing with sterile distilled water. Nodules were crushed and inoculated in plates of yeast extract mannitol (YEM) medium containing 25 $\mu g \cdot mL^{-1}$ Congo red [10]. The bacterial isolates obtained from crushed nodules were purified and used for further analysis.

Concurrently, soil samples were taken from the same location where the nodules were collected. These samples were dried and sifted (< 2 mm) to determine the pH (1:2 KCl), electric conductivity (EC; 1:5 H_2O), soil texture and total nitrogen and phosphate content [11].

2.2 Production of 3-Ketolactose

To determine the presence or absence of *Agrobacterium*, isolates were grown on yeast lactose agar and incubated at 28 ºC for 3 to 10 days. After this period, 10 mL of Benedict's reagent were added to the plate and incubated for 10 min. A color change in the medium to a bright, yellow color was used to confirm the presence of *Agrobacterium* [12].

2.3 Morphology of the Colonies and Growth Rate

The morphological properties of the colonies (shape, size, color, edge, elevation and consistency) were determined by seeding each isolate into YEM plates at pH 6.8. Bacterial characterization was done 4 to 8 days after inoculation. The growth was determined in the same medium by measuring the incubation time for each isolate until the colonies appeared [12].

2.4 Biochemical Tests

To determine the media acidification, each isolate was cultivated in YEM plates (pH 6.8) with bromothymol blue (0.5% (v/v)) incubated at 28 ºC from 1 to 3 days depending on the growth of the specific strain. A change in the color of the media was determined post incubation. Tolerance to different pH was determined by inoculating ± 10^8 cells·mL^{-1} in 10 mL liquid culture medium (YEM) at pH values 5, 7 and 9. Cultures were incubated at 28 ºC for up to 10 days, and measured by turbidity; three replicates were used for each treatment. Salinity stress tolerance was performed on YEM media (pH 6.8), containing NaCl at concentrations of 1, 2 and 3% (w/v). A bacterial suspension was diluted in sterile distilled water at a concentration of ± 10^8 cells·mL^{-1} and spread onto the plates using sterile swabs. The plates were incubated at 28 ºC, and the bacterial growth was evaluated for 10 days. Growth on different carbon sources was carried out on YEM media at pH 6.8, exchanging mannitol for various individual carbon sources at concentrations of 10 g·L^{-1} (sucrose, glucose, rhamnose, lactose and galactose). A sterile water suspension of ±10^8 cells·mL^{-1} of each isolate was spread on the plates using sterile swabs [13].

2.5 The Ability to Ferment Sugars

The ability of the isolates to ferment sugars was determined by color change, using Kligler iron agar (KIA), a complex red differential medium that consists primarily of glucose 0.1% (w/v), lactose 1% (w/v), sodium thiosulphate, iron citrate and phenol red [14].

2.6 Sensitivity to Antibiotics Test

The sensitivity or resistance of the isolates to antibiotics was evaluated on YEM plates. A bacterial suspension containing ± 10^8 cells·mL^{-1} of each isolate was spread on the plates using sterile swabs. Sensi-Discs with different types and concentrations of antibiotics were placed on top, including nalidixic acid (30 µg), kanamycin (30 µg), trimethoprim/sulfamethoxazole (1.25/23.75 µg), gentamicin (10 µg), chloramphenicol (30 µg) and ampicillin (10 µg). Plates were incubated at 30 ºC. After 48 h, the diameter of the inhibition zone was measured and compared with the reference tables. The bacteria were reported as being susceptible (S), intermediate (I) or resistant (R) to each of the antibiotics used.

2.7 Cluster Analysis

The data of the phenotypic characterization and antibiotic resistance properties were used for cluster analysis. A similarity matrix was calculated using the Dice coefficient and employed later in a cluster analysis performed by the Unweighted Pair-Group Method with Arithmetic Averaging using PAST software [15]. Branch support of the tree was assessed by bootstrapping using 1,000 replications [16].

2.8 Production of Indole-3-Acetic Acid (IAA) and Siderophore Production

The amount of IAA produced by each strain was quantified in a 125-mL flask with 50 mL YEM medium supplemented with 0.1 mg·mL^{-1} L-tryptophan. Bacterial isolates were inoculated (±10^8 cells·mL^{-1}) and incubated at 30 ºC and 100 rpm. After this, an aliquot of the culture was centrifuged at 13,000 rpm for 3 min, and 2 mL Salkowski reagent was added to the supernatant. The IAA was quantified at 530 nm using a spectrophotometer (Thermo Scientific, Genesys 20). Sampling was performed every day and for each strain until a decrease in IAA production was observed. Subsequent experiments were conducted to evaluate the maximum production of IAA using the best strains on medium that was enriched with L-tryptophan at 2.5 mg·mL^{-1}. Experiments were conducted three times [17].

Additionally, bacterial growth was quantified during IAA production by spectrophotometry and plate seeding. Briefly, 1 mL of bacterial culture from each sample was taken to measure the optical density at 540 nm. A serial dilution was carried out from 10^{-1} to 10^{-8} and seeded onto plates with

YEM medium (0.1 mL of the suitable dilutions with regard to growth time) to determine the number of CFU. Quantification of the bacterial growth was performed in triplicate each day until a decrease in IAA production was observed.

Siderophore production was qualitatively assayed as described by Schwyn and Neilands [18], with some modifications according to Pérez-Miranda [19]. Briefly, cultures were inoculated onto a chrome azurol S (CAS) agar plate and incubated for 72 h at 30°C. Formation of an orange halo surrounding the colonies was indicative of siderophore production. For the preparation of one liter of medium, the following reagents were needed: Chrome azurol S (CAS) 60 mg, hexadecyltrimethyl ammonium bromide (HDTMA) 72.3 mg, piperazine-1,4-bis(2-ethanesulfonic acid) (PIPES) 30.24 g and 1 mM FeCl3.6H2O in 10 mL 10 mM HCl. Agarose (0.9%, w/v) was used as the gelling agent. Ten milliliters of CAS medium were placed in Petri dishes 80 mm in diameter. The isolates were selected by pricking the center of the box. Plates were incubated at 30 °C and observed at 96 h. Formation of an orange halo in the culture was considered to be a positive response to the test.

2.9 Nodulation Assay

Isolates 26, 40, 46, 74 and 34b were selected, based on their production of IAA or siderophores, for a nodulation assay. Seeds of *L. leucocephala*, were previously disinfected and scarified prior to their use. Stone powder was sterilized in an oven at 120°C for 24 hours and used as the substrate. The sterilized substrate was deposited in clean plastic bags, where three seeds of *L. leucocephala* per bag were planted. Afterwards, 1 ml of the inoculum of interest, diluted in saline solution at a concentration of 10^7-10^9 cells/ml/seed was added. One week after sowing, re-inoculation with 1 ml of the inoculant at the same concentration was done. The plants were grown under greenhouse conditions, and irrigation was performed with a nitrogen free nutrient solution. Sampling was performed at 45, 75 and 105 days after inoculation. Also was included a non-native strain belonging to the species *Rhizobium tropici* (RT) and an uninoculated control.

2.10 Molecular Identification of Isolates

2.10.1 DNA extraction

The strains were cultivated in YEM supplemented with agar at 30 ºC during 48 h to extract their DNA. Each colony was sampled using an inoculating loop, and DNA extraction was performed using a commercial kit (PureLink™ Genomic DNA Mini Kit, Invitrogen™).

2.10.2 Amplification and sequencing of the 16S rRNA gene

PCR was used to amplify the 16S rRNA gene from *Rhizobium*. The amplification reaction was carried out with the primer oligonucleotides fD1(5'-CCGAATTCGTCGACAACAGAGTTT GATCCTGGCTCAG-3') and rD1(5'-CCCGGG ATCCAAGCTTAAGGAGGTGATCCAGCC-3') as described by Weisburg et al [20]. The primers amplified fragments of 1500 pb. PCR was carried out in a volume of 50 µL containing 0.5-1 µL of DNA at a concentration of 50 ng·mL^{-1}, 1.5 µM $MgCl_2$, 50 mM KCl, 20 mM each deoxyribonucleotide triphosphate, 1.25 U Taq DNA polymerase and 0.5 µM each primer. PCR conditions were as follows: initial denaturalization for 4 mins at 94 °C followed by 30 cycles of 2 min each at 95 °C, 30 sec at 42 ºC, 4 min at 72 °C and a final extension of 7 min at 72 °C. Subsequently, a 5 µL aliquot of the PCR was used to visualize the amplification products on a 2% agarose gel in TBE 1X buffer. The PCR products were sequenced at Langebio-CINVESTAV Genomic Services Laboratory (Irapuato, Mexico). Alignment was performed using the BioEdit Sequence Alignment program [21].

2.10.3 16S rRNA gene sequence analysis

Sequences were then compared against reference sequences downloaded from the GenBank

Database. Data were aligned and analyzed using the Molecular Evolutionary Genetic Analysis (MEGA) software version 7.0 [22]. Distances were calculated using the Jukes-Cantor model (assumes equal probability of independent change at all sites), and a tree was produced using the Neighbor-Joining method. A bootstrap analysis with 1,000 re-samples was conducted. The sequences of *Bradyrhizobium* spp. were used as the outgroup.

3. Results

3.1 Morphology of Colonies and Growth Rate

A total of 23 rhizobia isolates (9 from Xmatkuil, 10 from Caucel and 4 from Motul) that did not absorb Congo red and tested negative for the production of 3-ketolactose, it allowed to discard *Agrobacterium* and another non-rhizobia strains. Eighty two percent of the isolates were cream colored with low amounts of exopolysaccharides. The remaining isolates were white or transparent. Ninety one percent of the isolates had circular colonies with convex elevation (Table 1) with a colony diameter ranging between 1 and 3 mm. All isolates were classified as growing rapidly (1-3 days).

Table 1. Morphological characterization of colonies grown in YEM media.

Isolate	Site	Color	Consistence	Diameter (mm)	Shape	Elevation	Edge
20	Caucel	transparent	watery	2	fusiform	raised	whole
26	Caucel	cream	watery	1-2	circular	convex	whole
34 a2	Caucel	cream	creamy	2-3	circular	convex	whole
34 b	Caucel	cream	watery	2-3	circular	convex	whole
37 b	Caucel	cream	watery	2-3	circular	convex	whole
40 a	Caucel	cream	watery	3-5	circular	convex	whole
41(1)	Caucel	cream	watery	1-2	circular	convex	whole
41(2)	Caucel	cream	watery	1-2	circular	convex	whole
45	Caucel	transparent	watery	3-7	circular	convex	whole
46	Caucel	cream	watery	2-3	circular	convex	whole
47A	Xmatkuil	cream	watery	3-4	circular	convex	whole
47B	Xmatkuil	cream	watery	1-2	circular	convex	whole
51A	Xmatkuil	cream	watery	1-2	circular	convex	whole
51B	Xmatkuil	cream	watery/elastic	1-2	circular	convex	whole
52B	Xmatkuil	cream	creamy	1-2	circular	raised	whole
54A	Xmatkuil	cream	watery	1-2	circular	convex	whole
54B	Xmatkuil	cream	creamy	2-3	circular	raised	whole
57B	Xmatkuil	cream	watery	2-3	circular	convex	whole
59A	Xmatkuil	cream	watery/elastic	1-2	irregular	convex	whole
72	Motul	transparent	watery	1-2	circular	convex	whole
74	Motul	transparent	watery	1-2	circular	convex	whole
75	Motul	cream	watery	1.5-2	circular	convex	whole
76	Motul	cream	watery	1.5-2	circular	convex	whole

3.2 Physical and Chemical Soil Properties

All three sampling sites were classified as soils with neutral to slightly alkaline pH (7.2-8.0). The soil textures were clay and clayey-sandy in Xmatkuil and Caucel and loamy-sandy in Motul. The EC was 3.95 $mS \cdot cm^{-1}$ for Xmatkuil, 2.82 $mS \cdot cm^{-1}$ in Caucel and 3.55 $mS \cdot cm^{-1}$ for Motul.

The nitrogen content varied between Caucel and Motul and was approximately 0.848-1.086% and 0.037% for Xmatkuil. The phosphate content at Motul and Xmatkuil was 4.74 ($mg \cdot kg^{-1}$) with no data available for Caucel.

3.3 Culture Medium Acidification

Isolates were classified into two groups according to the pH modification of the medium. Fifty six percent of the isolates were characterized as excreting acidic substances, lowering the pH value to below 6, thus causing a change in media color (yellow). The other 44% of the isolates excreted alkali with pH above 7.6 (Table 2).
[Table 2]

3.4 Growth Testing at Different pH Values

All of the isolates grew on YEM medium between pH 7-9. Seventeen of the isolates grew at pH 5, while five isolates (strains 26, 40a, 46, 75 and 76) excreting acidic substances did not grow in the acidic media (Table 2).

3.5 Saline Tolerance Stress Testing

Thirteen isolates were tolerant towards 3% NaCl, while three strains were intolerant to concentration of 1% or upper. All of the isolates from Xmatkuil were tolerant to 3% salinity. (Table 2)

3.6 Growth with Different Carbon Sources and Ability to Fermentation Capacity

In addition to mannitol, all of the isolates utilized rhamnose and sucrose as carbon sources. Lactose was utilized by all of the isolates except 34b. Maltose was also utilized by most of the isolates, except 20, 34b, 41(1) and 45. Xylose was the least assimilated carbohydrate, used only by isolate 52B from Xmatkuil. Ten isolates fermented either one or both carbohydrates, while none of the Motul isolates carried out fermentation (Table 2).

3.7 Sensitivity to Antibiotics

The ability to grow in the presence of antibiotics in the culture media was evaluated. A total of 43% of the isolates were sensitive to nalidixic acid, 70% to kanamycin, 43% to trimethoprim sulfamethoxazole, 22% to gentamicin, 48% to chloramphenicol and 43% to ampicillin. Isolates from Xmatkuil were more susceptible, while isolates from Motul exhibited greater levels of resistance (Table 2).

3.8 Phenotypic Analysis

Phenotypic variation among the isolates was used to perform a cluster analysis. According to this clustering, the strains in this study were divided into two major groups (Figure 1). The clustering showed an association according to the geographical origin of the isolates, since cluster A comprises eight of the nine isolates from Xtmakuil, and only two of the ten isolates from Caucel. In contrast, cluster B contains the remaining eight isolates from Caucel and all of the isolates form Motul (four, in total).

The isolates from Xmatkuil were divided into five subgroups, one of them including isolates 51A, 54A, 57B and 59A with a similarity coefficient of 1. A distinguishing characteristic of this subgroup was its capability to ferment only glucose. Isolates 51B and 54B were part of another subgroup, also with a similarity coefficient of 1. This subgroup could ferment both glucose and lactose. Isolates 47B and 52B were separated from the previous subgroups. Both isolates could assimilate glucose and lactose. Among the Xtmakuil isolates, strain 52B was the only one unable to grow at low pH. When examined for antibiotic resistance, 47B exhibited sensitivity to gentamicin, while the other seven isolates within this cluster had an intermediate resistant to gentamicin but were susceptible to the other five antibiotics tested.

Table 2. Main phenotypic traits of the Rhizobia isolates.

Isolate	Acid/ alkali production	Growth at different pH values	Glucose & lactose fermentation	NaCl Tolerance %	Assimilation of different carbon sources					Susceptibility to antibiotics					
					Xyl	Rha	Lac	Suc	Malt	NA	Kan	Trim/ Sulfa	Genta	Chlora	Amp
20	Alkaline	5, 7, 9	Glu & Lac	2	-	+	+	+	-	S	R	S	S	S	S
26	Acidic	7 & 9	-	-	-	+	+	+	+	R	S	R	I	R	R
34 a2	Acidic	5, 7, 9	-	3	-	+	+	+	+	S	S	S	S	S	S
34 b	Acidic	5, 7, 9	-	1	-	+	-	+	-	R	S	R	I	R	R
37 b	Acidic	5, 7, 9	-	1	-	+	+	+	+	R	I	R	R	R	R
40 a	Acidic	7 & 9	-	2	-	+	+	+	+	R	S	R	R	S	R
41(1)	Acidic	5, 7, 9	-	-	-	+	+	+	-	R	S	R	I	R	R
41(2)	Acidic	5, 7, 9	-	3	-	+	+	+	+	R	S	R	S	R	R
45	Acidic	5, 7, 9	-	1	-	+	+	+	-	R	S	R	S	R	R
46	Acidic	7 & 9	-	3	-	+	+	+	+	R	I	R	R	R	R
47A	alkaline	5, 7, 9	Glu	3	-	+	+	+	+	R	R	R	R	R	R
47B	alkaline	5, 7, 9	Glu & Lac	3	-	+	+	+	+	S	S	S	S	S	S
51A	alkaline	5, 7, 9	Glu	3	-	+	+	+	+	S	S	S	I	S	S
51B	alkaline	5, 7, 9	Glu & Lac	3	-	+	+	+	+	S	S	S	I	S	S
52B	alkaline	7 & 9	Glu & Lac	3	+	+	+	+	+	S	S	S	I	S	S
54A	alkaline	5, 7, 9	Glu	3	-	+	+	+	+	S	S	S	I	S	S
54B	alkaline	5, 7, 9	Glu & Lac	3	-	+	+	+	+	S	S	S	I	S	S
57B	alkaline	5, 7, 9	Glu	3	-	+	+	+	+	S	S	S	I	S	S
59A	alkaline	5, 7, 9	Glu	3	-	+	+	+	+	S	S	S	I	S	S
72	Acidic	5, 7, 9	-	2	-	+	+	+	+	R	R	R	R	R	R
74	Acidic	5, 7, 9	-	2	-	+	+	+	+	R	R	R	R	R	R
75	Acidic	7 & 9	-	3	-	+	+	+	+	R	I	R	R	R	R
76	Acidic	7 & 9	-	-	-	+	+	+	+	R	S	R	I	R	R

Note: Xyl = xylose; Rha = rhamnose; Lac = lactose; Suc = sucrose; Malt = maltose; NA = nalidixic acid 30 µg; Kan = kanamycin 30 µg; Trim/Sulfa = trimethoprim/ sulfamethoxazole 1.25/23.75 µg; Genta = gentamicin 10 µg; Chlora = chloramphenicol 30 µg; Amp = ampicillin 10 µg; S = susceptible; R = resistant; I = intermediate

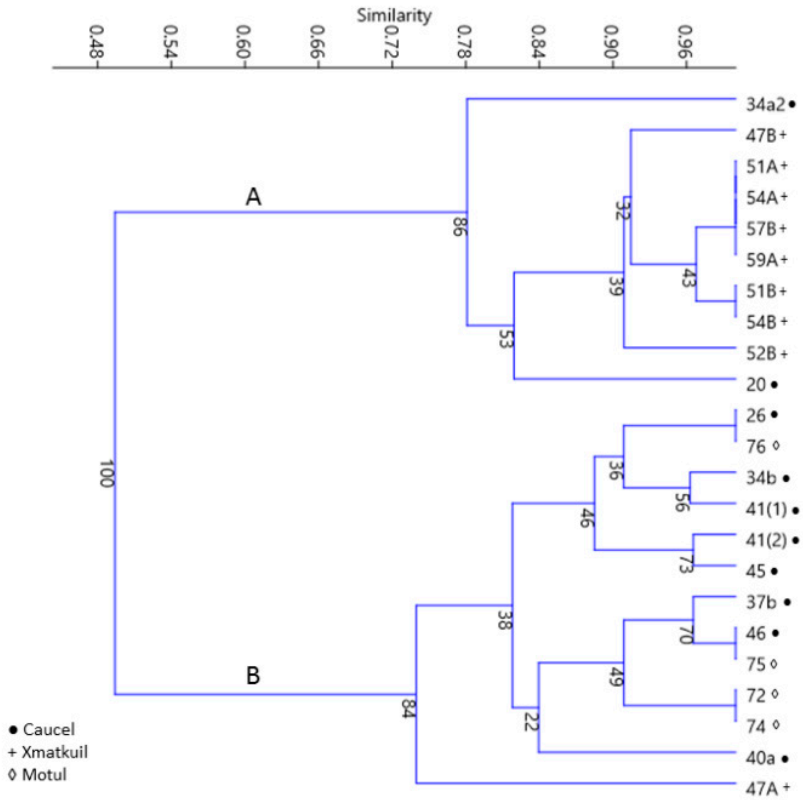

Figure 1. UPGMA dendrogram showing the phenotypic diversity of Rhizobia from *L. leucocephala* obtained from the Yucatan peninsula.

Isolate 47A clustered into a separate group (B) and was unique in being resistant to all of the antibiotics tested.

Isolates 34a2 and 20 from Caucel were located in cluster A along with the Xmatkuil isolates. However, the isolates were in separate subgroups. A distinctive trait of these strains compared with the other isolates from the same locality was their sensitivity to almost all of the antibiotics tested. Another feature of isolate 20 was its ability to ferment glucose and lactose, since all of the Caucel isolates were classified as non-fermenting bacteria.

Isolates comprised in cluster B were delineated by their antibiotic resistance. Another remarkable feature of the strains within this group was their ability to acidify the surrounding medium during their growth in addition to their inability to ferment glucose and/or lactose.

Isolates 26 from Caucel and 76 from Motul clustered together with a similarity coefficient of 1. Additionally, isolates 37b, 46 and 75 formed a well-supported cluster indicating that, according to the features evaluated, these isolates were almost identical despite their different geographical origins.

3.9 Production of Indole-3-Acetic Acid and Siderophores

When the medium was supplemented with 0.1 $mg \cdot mL^{-1}$ of L-tryptophan, isolates 26, 34b, 37b, 40a, 41(1) 45 and 46 from Caucel produced indole compounds in addition to isolates 72 and 74

from Motul. Bacterial growth and IAA production started immediately after inoculation. The isolates produced the highest amount of IAA on day 3 when they reached their stationary growth phase. After this period, the levels of IAA decreased in the media (Figure 2). The highest IAA levels at 24 h were produced by strains 40a (21.40 $\mu g \cdot mL^{-1}$) and 74 (15.91 $\mu g \cdot mL^{-1}$). However, on subsequent days, strain 74 was the only isolate that produced the greatest amount of IAA with a maximum of 40.39 $\mu g \cdot mL^{-1}$ at 72 hours. Significant differences ($P < 0.05$) were observed in IAA production between the different rhizobia strains (Table 3).

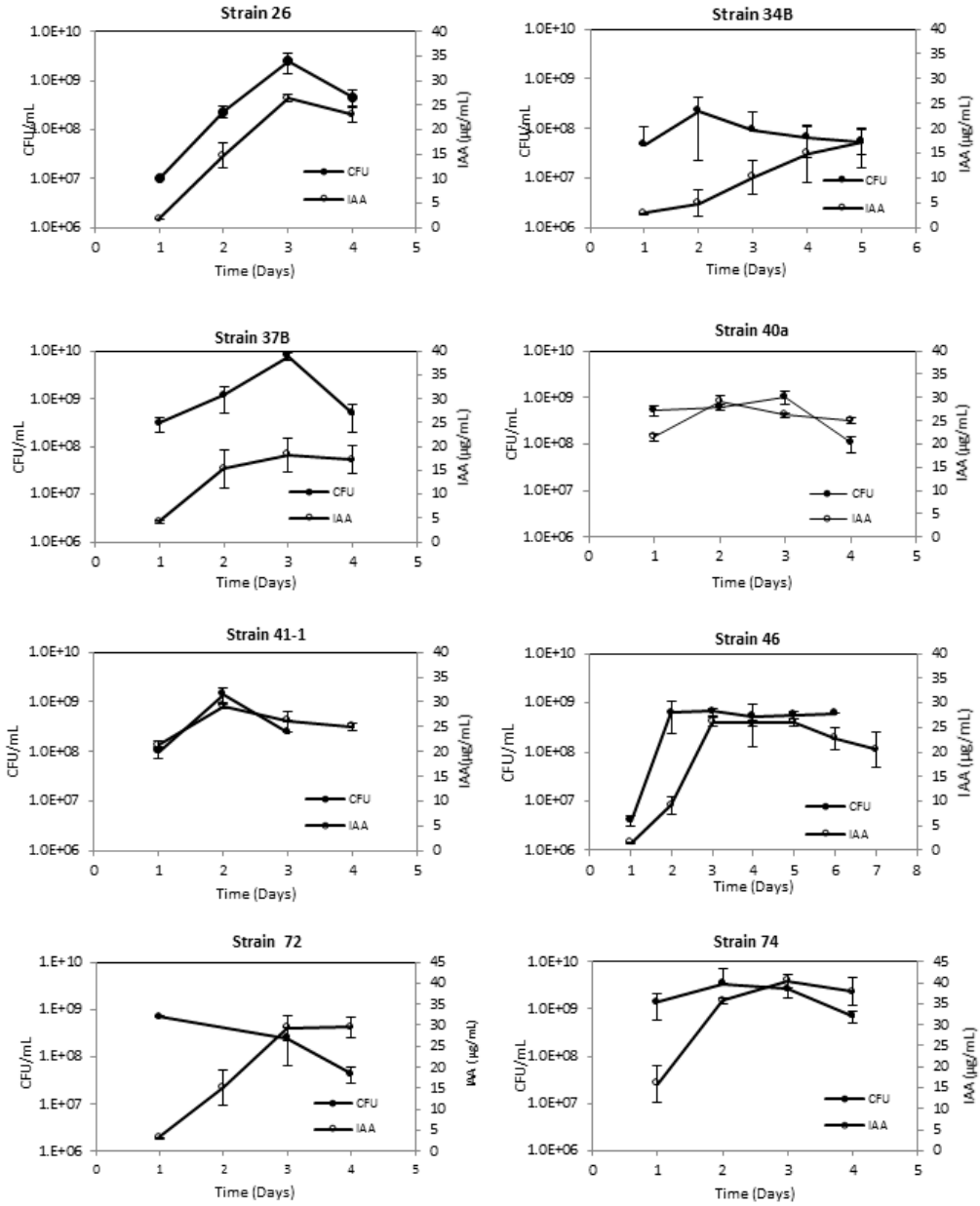

Figure 2. Production of IAA and cell growth by isolates, using 0.1 $mg.mL^{-1}$ L-tryptophan as precursor.
Note: The values shown are mean ± SD of three replicates of the experiments

Table 3. Production of IAA by isolates that used 0.1 mg.mL^{-1} of L-tryptophan as a precursor.

Strains	Production of IAA (µg•ml-1)			
	24 hrs	48 hrs	72 hrs	96 hrs
26	1.780 ±0.000e	14.607 ± 2.560c	26.423 ± 0.681b	23.117 ± 1.599d
34b	2.923± 0.070de	4.913± 2.553d	10.213 ± 3.422d	14.843 ± 5.658e
37b	4.207 ± 0.322de	15.257 ± 3.935c	18.210 ± 3.553c	17.213 ± 2.873e
40a	21.403 ± 0.875a	29.177 ± 1.203b	26.160 ± 0.428b	25.000 ± 0.644cd
41(1)	5.983 ± 0.671dc	26.143 ± 0.534b	27.837 ± 0.195b	24.883 ± 0.667cd
46	9.243 ± 4.168c	10.687 ± 1.113c	23.390 ± 6.929cb	29.680 ± 0.336cb

Note: Different letters indicate a significant difference. The values shown are mean ± SD of three replicates of the experiments. The data were subjected to analysis of variance (ANOVA), and differences between the means were determined using Tukey's test ($P \leq 0.05$) via SAS statistical analysis software (SAS Institute, Cary, NC, USA).

Strain 74 exhibited its maximal IAA production after 4 days of incubation with 2.5 mg·mL^{-1} L-tryptophan. Maximal IAA production was 187.0 µg·mL^{-1} which then started to decline. Strain 40a exhibited similar behavior with 173.0 µg·mL^{-1} of maximal IAA production.

Some strains were able to produce siderophores. The formation of an orange halo around the colonies indicates that the iron contained in the medium had been removed. Seven isolates were able to produce some type of siderophore. Strains 34b, 41(1), 41(2) from Caucel and strains 47b, 51a, 51b and 59a from Xmatkuil could produce siderophores. Isolates from Motul were unable to produce siderophores.

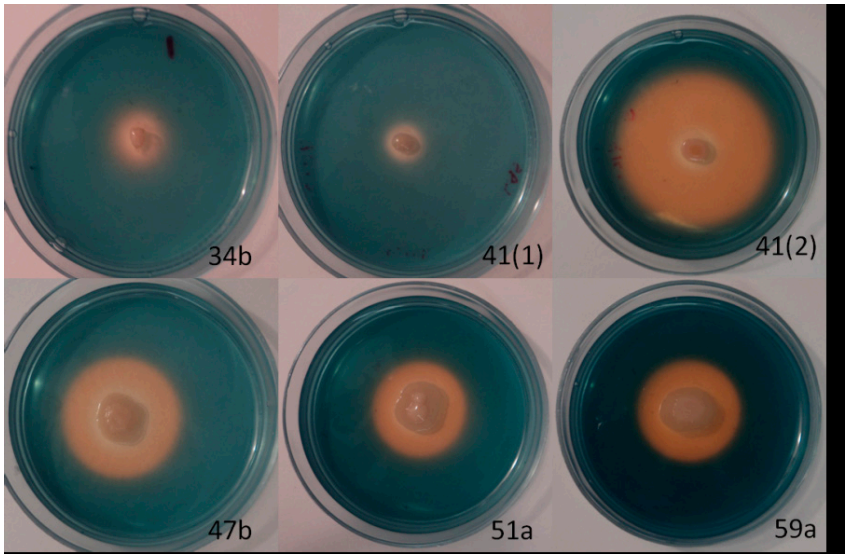

Figure 3. O-CAS assay performed on different Strains. Orange halo in the petri dish is considered positive. Three replicates were conducted for each assay.

3.10 Nodulation Assay

All the isolates tested were capable to nodulate *L. leucocephala* seedlings (Table 4). Nodules were brown; oval shaped, and had 2 to 3 mm in diameter. Although scarce, nodules were visible 45 days after inoculation with isolates 40, 46 and 74. Seedlings inoculated with strains 40, and 46 had the greatest number of nodules after 75 days, with an average of 8.33 ± 4.10, and 10 ± 3.33 nodules per plant, respectively. However, a decrease in the nodulation was observed at the 105 day measurement for these treatments. The number of nodules observed in the seedlings inoculated with isolates 26, 34b and 74 increased gradually over time 105 days after inoculation, reaching a maximum average of 9.8 ± 4.1 (Strain 26), 6 ± 2.4 (strain 34b). Strain 74 produced 11.2 ± 2.8 nodules, which was the highest number of nodule formation among all treatments (Table 4). The non native strain (RT) had the lowest number of nodules (1.17±0.83 and 2.8±1.33 after 75 days and 105 days respectively)

Table 4. Number of nodules of *L. leucocephala* inoculated with different rhizobia strains at 75 and 105 days after planting date.

Strains	Average of Number of Nodules			
	75 days	EE	105 days	EE
26	2.30	±1.96	9.8	±4.1
40	8.33	±4.10	3.2	±2.0
34b	2.33	±0.76	6.0	±2.4
46	10.0	±3.33	7.5	±2.3
74	3.80	±1.11	11.1	±2.8
RT	1.17	±0.83	2.8	±1.33
Control	0	0	0	0

3.11 Molecular Characterization

Isolates obtained from the nodules of *L. leucocephala* were identified via sequencing and analysis of their 16S rRNA. The selection of the isolates for the molecular characterization was based on the nodulating strains with biotechnological potential (IAA Production). The 16S rRNA gene of the isolates 26, 34b, 37b, 40a, 41(1), 46 and 74 was successfully amplified and sequenced (GenBank AN. KY940278 to KY940284).

Seventeen reference sequences of strains from the rhizobial isolates were downloaded from the NCBI database and used in this study: JN009838, JN009836, NR_113892, NR_026096, KU862337, KU862336, KU862334, KR051017 and KF836057 for *Sinorhizobium*, FJ430076, JQ659634, JQ659781, JQ659635 and JQ659519 for *Rhizobium* and NR_028768, FJ540938 and AB231916 for *Bradyrhizobium*.

The evolutionary history was inferred using the Neighbor-Joining method. The evolutionary distances were computed using the Jukes-Cantor model and are in the units of the number of base substitutions per site. The analysis involved 24 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 1345 positions in the final dataset. Phylogenetic analysis revealed well-supported clades as shown in Fig. 4. A phylogram identified strain 74 as a species of *Rhizobium*. Isolates 34b, 41(1), 40a, 37b and 46 were identified as species of *Sinorhizobium*, while strain 26 was shown to be *Sinorhizobium fredii* with a 100% bootstrap support. [Figure 4 near here]

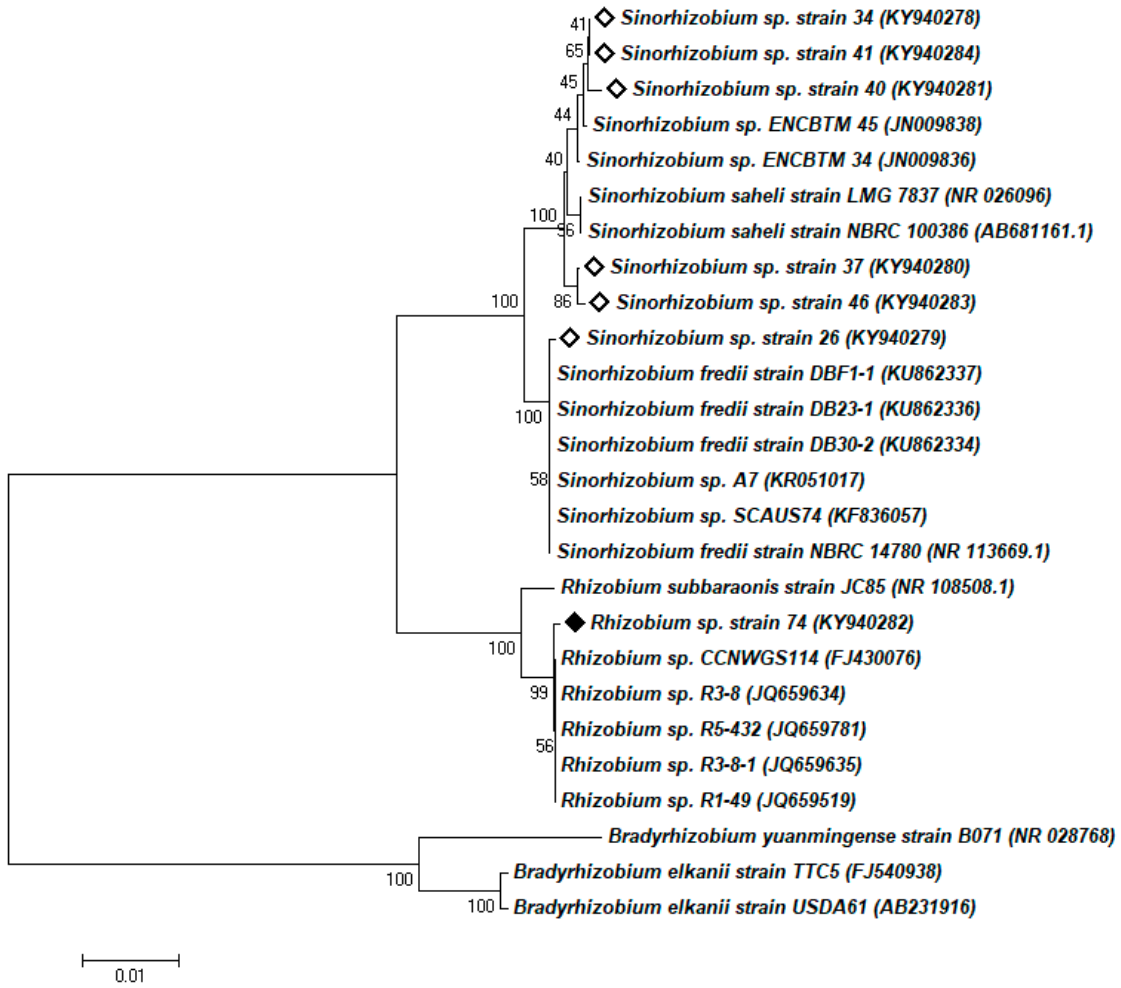

Figure 4. Phylogenetic tree using the Neighbor Joining (NJ) method based on the Jukes Cantor model for the 16S rRNA sequences. Values above the branches show bootstrap support. *Bradyrhizobium* spp. were used as the outgroup.

Isolates 34b and 41(1) were almost identical, and thus clustered together. In addition, isolates 37b and 46 clustered together as previously shown in the dendrogram. These results support the clusters obtained with the phenotypic characterization.

4. Discussion

The establishment of legumes in a given area depends to a large extent on the effective symbiosis with rhizobia strains to maximize N_2-fixation and increase usable biomass production. This requires that rhizobia compatible with the specific legume host be present in the soil [8]. The 23 isolates obtained in this study could grow at pH > 7, suggesting that the soil where the nodules were collected was alkaline. This is an important trait since inoculant strains not only have to be specific and selected for their high efficiency at N_2-fixation and as biofertilizer, they also need to be adapted to soil conditions such as drought, temperature extremes, salinity, acidity and alkalinity to persist in the soil and assure that sufficient

quantities of rhizobia are present. However, soil pH is a parameter that correlates positively with the amount and genetic diversity of the rhizobia populations present in the soil [23].

The properties of the isolates did not correlate with their sites of origin. In addition, none of the sampling sites contained a predominant strain. However, in previous studies, rhizobia strains obtained from *L. leucocephala* produced exclusively alkaline substances in the media, while only 44% of the total isolates in this study exhibited this property [24].

Isolates of *Rhizobium* spp. obtained from *Phaseolus mung* and *Cajanus cajan* were notable for their high IAA production (138 $\mu g \cdot mL^{-1}$ and 142 $\mu g \cdot mL^{-1}$, respectively) in media supplemented with L-tryptophan (2 $mg \cdot L^{-1}$) [25]. Rhizobia isolates in this study produced large amounts of indole compounds when the medium was supplemented with L-tryptophan (2.5 $mg \cdot mL^{-1}$) and produced 35% more than other rhizobia strains isolated from *Phaseolus mung* and *Cajanus cajan*. IAA production in this study started immediately after inoculation and then decreased during the cell decline phase of growth kinetics. This decrease in IAA production could be due to the presence of IAA degrading enzymes such as oxidases, peroxidases and polyphenol oxidases [26, 27]. Isolates producing a large quantity of IAA included *Rhizobium* sp. (isolate 74) and *Sinorhizobium* spp. (isolate 40a). Both strains have been reported as being involved in specific symbioses with *L. leucocephala*. *Sinorhizobium* spp. has been reported to predominate in alkaline soils and serve as a main symbiotic strain for *L. leucocephala* in Yucatan [28].

Some strains were able to produce other growth promoters in addition to IAA such as siderophores (41(1) and 34b) that contribute to a more effective establishment of the plants under field conditions where these bacteria can act as biocontrol agents using their siderophores to limit the proliferation of phytopathogens [29]. The application of native PGPR is an effective, economical practice and protects the environment by reducing the use of agrichemicals [5].

All native strains tested were able to nodulate, strains 40 and 46, belonging to the genus *Sinorhizobium* sp. had the highest nodules number. *Sinorhizobium* is a genus of bacteria, which has been reported as nodulant of *L. leucocephala*, however, not all strains belonging to this genus are able to nodulate *L. Leucocephala* [30]. In the case of *Rhizobium*, it has been widely reported as a nodulant of *L. leucocephala* [9]. On the other hand, the strains used belong the *Sinorhizobium* and *Rhizobium* are producers of growth promoting compounds such as IAA and siderophores and it has been shown that the effect of multiple hormones produced by rhizobia strains affects not only nodulation, also other processes such as germination and plant growth [31]. These native rhizobia are able to establish effective symbiosis, they contribute to increase the amount of fixable N_2.

Treatment with strain 74 (*Rhizobium*) reached its maximum nodulation at 105 days after seed inoculation; it seems that nodulation had a positive correlation with time. The results of the study by da Silva [32] showed no differences in the first two evaluations (30 and 60 days), but they found higher average number of 23 nodules/plant at 90 days after establishment. The treatment (strain 74) with the greatest average nodulation in our trial was 11 nodules/plant at 105 days. The non-native strain (*Rhizobium*), was below the average of the all native strain tested in our trial. These results are similar with those from da Silva [32] were the native *Rhizobium* strain developed more nodules number than the non-native strain.

5. Conclusions

Phenotypic characterization of rhizobia isolates from *L. leucocephala* nodules indicated that they are highly tolerant to abiotic conditions including varying pH values and salinity. Two isolates were identified as highly effective at producing indole compounds. Genotypic characterization showed that these isolates belong to different genera: *Sinorhizobium sp.* (isolate 40a) and *Rhizobium* sp.

(isolate 74). Both genera have been reported as effective at nodulating *L. leucocephala*. The isolates could be used to increase N_2-fixation in *L. leucocephala* when used as inoculants to enhance symbioses and nitrogen availability in soils with poor nutrition conditions.

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