PHENOTYPIC DIVERSITY AND PGPR TRAITS OF RHIZOBIA NODULATING PEANUT (*Arachis hypogaea* L.) GROWN IN ALGERIAN SANDY SOILS

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Abstract.- *In order to assess phenotypic variability of native rhizobia nodulating peanut (*Arachis hypogaea *L.), a collection of fourteen isolates obtained from effective root nodules of peanut, cultivated in two potential regions of Algeria (Sebseb and EL Mansoura), was subjected to phenotypic characterization using morphological, biochemical and physiological tests. Some Plant Gowth Promoting Rhizobacteria (PGPR) properties had also been investigated in this study for each strain. Furthermore, a representative strain (M044713), forming a separate cluster in the UPGMA dendrogram of API 20NE tests was chosen for phylogenetic analysis using 16S rRNA gene. The results showed that most of bacterial isolates are Gram-negative bacilli. They can be divided into slow-growing and fast-growing rhizobia. Their responses to the various tests as well as their PGPR characteristics were interesting, but variable. The phylogenetic distribution of the isolate M044713, based on 16S rRNA sequence analysis, revealed low similarity percentages with all strains previously isolated from peanut and the most important percentage of similarity was 94.5%, noted with* Pseudoxanthomonas koreensis *species.*

Key words: *Native rhizobia, peanut, root nodules, phylogenetic analysis*.

DIVERSITÉ PHÉNOTYPIQUE ET PROPRIÉTÉS PGPR DES RHIZOBIA NODULANT L'ARACHIDE (*Arachis hypogaea* L.) CULTIVÉE DANS LES SOLS SABLEUX ALGÉRIENS

Résumé.- *Afin d'évaluer la variabilité phénotypique des rhizobia natifs nodulant l'arachide (*Arachis hypogaea *L.), une collection de quatorze isolats obtenus à partir de nodules racinaires d'arachide, cultivés dans deux régions potentielles d'Algérie (Sebseb et EL Mansoura), a été soumise à une caractérisation phénotypique, en utilisant des tests morphologiques, biochimiques et physiologiques. Certaines propriétés PGPR (Plant Gowth Promoting Rhizobacteria) ont été également examinées dans cette étude pour chaque souche. De plus, une souche représentative (M044713), formant un groupe distinct dans le dendrogramme UPGMA des tests API 20NE, a été choisie pour l'analyse phylogénétique basée sur le gène ARNr 16S. Les résultats ont montré que la majorité des isolats bactériens sont des bacilles à Gram négatif. Ils peuvent être divisés en rhizobia à croissance lente et à croissance rapide. Leurs réponses aux différents tests ainsi que leurs caractéristiques PGPR étaient intéressantes, mais variables. Néanmoins, la distribution phylogénétique de l'isolat M044713, basée sur l'analyse de séquence d'ARNr 16S, a révélé de faibles pourcentages de similitude avec toutes les souches précédemment isolées de l'arachide et le pourcentage de similitude le plus important était de 94,5%, noté avec les espèces de* Pseudoxanthomonas koreensis.

Mots-clés: *Rhizobia natifs, arachide, nodules racinaires, analyse phylogénétique*

Introduction

Peanut (*Arachis hypogaea* L.) is an important food crop which is cultivated in tropical, subtropical and temperate zone [1]. It is one of the few crops that adapts well to the conditions of drought and variable soil fertility [2]. Originally from South America, peanut has been cultivated in Algeria for about a century, especially in sandy soils, of which several genotypes have been identified. Although the areas devoted are around 2000 ha, its yield hardly exceeded 20 quintals per hectare in 2018 (with a production of 3409.7 tons). The high instability of these yields is linked to the low fertility on limestone and alkaline soils. Indeed, on these soils nitrogen and phosphorus are the two factors limiting the productivity of crops [3]. Reasoned fertilization remains the most effective way to obtain optimal productivity. However, the effectiveness of fertilizers is closely dependent on the pH and the amount of limestone in these soils [4, 5]. In addition, the excessive use of inorganic fertilizers is currently responsible for serious threats to environment and human health [6].

Even in arid environments, peanut have a high symbiotic N_2-fixing capacity and its contribution of biological N_2-fixing (BNF) was 40.9 kg N ha^{-1} [7, 8]. The supply of nitrogen can occur by means of biological nitrogen fixing through symbiosis with efficient rhizobia dispensing or reducing the application of nitrogen fertilizers and enhancing ability of legumes, such as peanut, to withstand stress [9].

Several types of native rhizobia are widely distributed in various geographical and ecological areas of the world [10-13]. Peanut has been reported to form effective root nodules with slow-growing rhizobia [14-17]. These strains are classified into the genus of *Bradyrhizobium* [18]. Until now, new species of *Bradyrhizobium* have been isolated from *Arachis hypogaea*, including *Bradyrhizobium lablabi* [19], *B. arachidis* [20], *B. subterraneum* [21], *B. guangxiense*, *B. guangdongense* [22], *B. vignae* [23] and *B. yuanmingense* [24]. Though, Santos et al [25] reported the predominance of fast-growing bacteria that acidify the medium forming symbiosis with peanut grown in the soils of Northeastern Brazil. Previously, Taurian et al [26] also found that peanut forms symbiosis with fast-growing bacteria closely related to *Rhizobium giardini* and *R. tropici.*

Nodulation of peanut by indigenous bacteria is usually assumed to be adequate, and inoculation is seldom practiced. While, typical environmental stresses faced by the legume nodules and effective functioning of rhizobia populations may include high soil temperatures, salt and osmotic stress, soil acidity and alkalinity, pesticide and fungicide applications and nutrient deficiency stress [27, 28].

In order to assess the diversity of peanut rhizobial strains in the peanut producing area of the province of Ghardaia (Algeria), fourteen isolates from effective root nodules were collected from two geographical regions in southern Algeria. Isolates were subjected to phenotypic characterization, plant growth promoting rhizobacteria features and genotypic variability using PCR-amplified 16S rRNA gene.

2.- Materials and methods

2.1.- Bacterial isolates and nodulation test

Isolates collected from two geographical regions representing the potential peanut culture zone of Algeria (Sebseb and EL Mansoura) (fig. 1). In which, soils are sandy with

an alluvial supply, characterized by a sandy-silty texture (tab. I). Fourteen isolates obtained from effective root nodules were kept on desiccated $CaCl_2$ using standard method [29]. Strains were isolated from sterilized nodules and maintained on yeast mannitol agar (YMA) medium. Bacterial cultures were incubated at 28°C for 7days. Rhizobial colonies were selected and streaked on YMA medium, several times to obtain pure cultures. To test isolates nodulation, 140 seeds of a local peanut genotype (Sebseb) were sterilized in sodium hypochlorite (13%) and germinated on sterile sand at 28°C. After germination, seedlings were inoculated with a bacterial culture of each isolate and then transferred to Gibson tubes containing the nutrient solution (60 ml) [30]. Plants were placed in a culture chamber at 28 °C, under 400 W m^{-1} for 16 hours in the light and 50% humidity. After 35 days of inoculation, plants were harvested for root nodule observation.

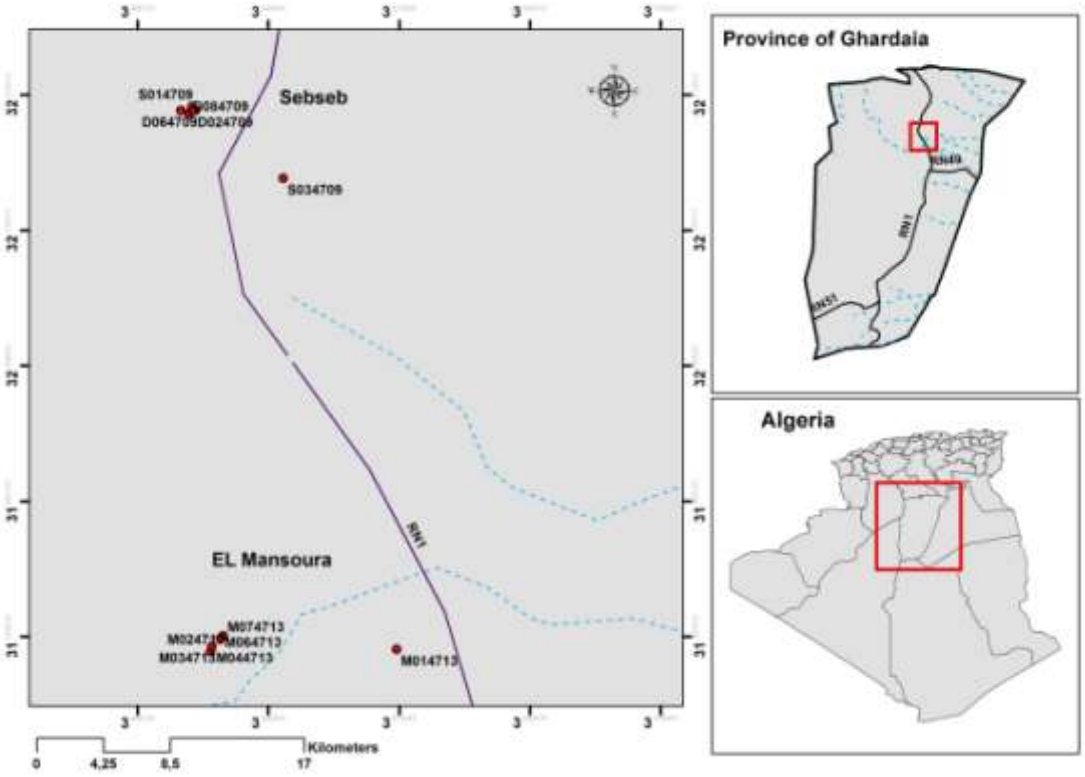

Figure 1. - Geographical location of the fourteen isolates collected from two agricultural areas in the province of Ghardaia, based on WGS 84 projection system
(Original figure designed on ArcGIS software)

Table I.- Granulometric and chemical soils properties [2]

Geographic region	Coarse sand (g kg^{-1})	Fine sand (g kg^{-1})	Silt+Clay (g kg^{-1})	pH	CE (ds cm^{-1})
Sebseb	451±1.1	483±1.0	66±0.3	8.64±0.22	0.138±0.03
EL Mansoura	527±1.4	392±0.8	81±0.5	7.86±0.13	0.146±0.04
Geographic region	Active- $CaCO_3$ (g kg^{-1})	OM (g kg^{-1})	Total-N (g kg^{-1})	P_2O_5 (mg kg^{-1})	K_2O (mg kg^{-1})
Sebseb	36.9±0.01	6.8±0.54	5.9±0.01	6.43±1.35	67±1.84
EL Mansoura	34.8±0.02	9.3±0.46	3.7±0.04	9.22±0.87	79±2.56

Data are means and SD of 3 replicates for soils from two geographic regions

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2.2.- Phenotypic tests

Phenotypic features of isolates were determined according to the procedure described by Wdowiak and Malek [31]. The tested features included: (i) utilization as sole carbon sources of D-galactose (0.1%), glucose (0.1%), lactose (0.1%), fructose (0.1%) and mannitol (0.1%); (ii) utilization as sole nitrogen sources of L-glutathione (0.1%), casein hydrolysate (0.1%), L-tryptophan (0.1%), L-cysteine (0.1%) and L-leucine (0.1%); (iii) tolerance to sodium chloride (0.01, 1, 5%); (iv): capacity to grow at different pHs (4.5, 7, 9, 11) and (v): intrinsic antibiotic resistance to ampicillin (10 µg ml^{-1}), amoxicillin (25 µg ml^{-1}), gentamicin (10 µg ml^{-1}) and nalidixic acid (30 µg ml^{-1}). Biochemical tests, including activities of catalase, oxidase, amylase, gelatinase, reduction of litmus milk and Gram reaction, were also performed according to SMIBERT and KRIEG (1994) [32]. Similarly, assimilation of substrates was determined using API 20E and API 20NE kit for all isolates.

2.3.- Phosphate-potassium solubilizing ability

The ability of the isolates to solubilize inorganic phosphate was checked on two media: Pikovskaya (PVK) medium [33] and NBRIP medium [34], both containing 10 g l^{-1} of tricalcium phosphate ($Ca_3(PO_4)_2$). To test potassium solubilization, bacteria were regrown using Aleksandrov solid medium [35]. An aliquot of 10 µl of fresh bacterial culture was spotted onto these plates and incubated at 28°C for 7days. Formation of halo around the colonies indicated solubilizing ability [36,37].

2.4.- Siderophores production

Siderophores production has been assessed using the method described by Schwyn and Neilands [38], based on chrome azurol S (CAS) and hexadecyltrimethylammonium bromide (HDTMA) as indicators. The CAS/HDTMA complexes tightly with ferric iron to produce a blue color. When a strong iron chelator such as a siderophore removes iron from the dye complex, the color changes from blue to orange. Chrome azurol S agar plates were inoculated with bacterial cultures and incubated at 28°C for 2-7 days. Development of yellow-orange halo around the colonies indicated siderophores production [39].

2.5.- Indole acetic acid production

Indole acetic acid (IAA) production was detected by the method of Bric et al [40]. Isolates were inoculated on Luria Bertani (LB) medium supplemented with glucose (5 g l^{-1}) and tryptophan (0.01 g l^{-1}) at 28°C for 72 h with shaking (180 rpm). Cultures were centrifuged at 1000 rpm for 15 min and 1 ml of the supernatant was recovered and added to 2 ml of the Salkowsky reagent. The mixture was then incubated in the dark for 20 min and AIA production has been evaluated by appearance of pink color.

2.6.- Hydrogen cyanide acid production

Hydrogen cyanide acid (HCN) production was determined according to the procedure described by Lorck [41]. Bacterial isolates were streaked on nutrient agar supplemented with glycine (4.4 g l^{-1}). A Whatman paper (N°42), saturated with alkaline picrate was placed in the lids of plates, sealed with para-film and inversely incubated at 30°C for 96 h. HCN production was indicated by changing of paper color to yellow or orange.

2.7.- DNA extraction and PCR amplification

Total genomic DNA for isolate M044713 was extracted in macrogen laboratory using InstaGene^{TM} Matrix protocol according to BIO-RAD catalog (732-6030). The supernatant containing total DNA was recovered and placed at -20 °C until required. DNA was isolated using MG Tissue SV kit (Doctor protein INC, Korea, Cat. no. DR00302). 16S rRNA gene has been amplified by DNA Engine Tetrad 2 Peltier Thermal Cycler using Dr. MAX DNA Polymerase (Doctor protein INC, South Korea, Cat. no. DR00302). The PCR conditions were as follows: initial denaturation (5 min at 95°C), 35 cycles each consisting of denaturation (30 s at 95°C), annealing (30 s at 55°C), extension (1 min 30 s at 72°C) and final extension for 7 min at 72 °C. PCR product was purified by multiscreen filter plate and sequenced by ABI PRISM 3730XL Analyzer (96 capillary type) using BigDye (R) Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). Sequences were finally analyzed with Variant Reporter Software Version 1.1 (Applied Biosystems).

2.8.- Phylogenetic and data analysis

16S rRNA gene sequence was aligned using MEGA 5.05. Comparisons of the obtained sequence and reference sequences in the public databases were performed using EzBioCloud databases (https://www.ezbiocloud.net/identify). Phylogenetic trees were carried by the neighbor-joining and maximum likelihood models using MEGA 5.05 software. Bootstrap confidence levels were based on 1000 permutations of the data sets.

Data obtained for the various tests were subjected to heatmap analysis using heatmap2 function under R.3.5.2. An analysis of similarity (ANOSIM), according to the Bray-Curtis method was carried using Past 3.

3.- Results

3.1.- Phenotypic diversity

Although, all colonies were white and had a smooth, shiny appearance. Macroscopic analysis of bacterial isolates revealed variation in shape, opacity, consistency and texture. However, microscopic observation showed that isolates were mainly Gram negative, except M064713 and M074713 which were Gram positive. Peanut isolates could utilize most of the carbon sources including lactose, glucose, fructose, D-galactose and mannitol. Casein and L-tryptophan are the two most assimilated sources of nitrogen, whereas isolates D044709 and S034709 did not assimilate any source used. Most of them could grow on the YMA medium at pH 9 and up to 5% (W/v) of NaCl. Their spectrum of intrinsic antibiotics resistance was relatively narrow ampicillin (10 µg ml^{-1}) for all isolated strains and amoxicillin (25 µg ml^{-1}) for those isolated from EL Mansoura. Bacterial isolates could be divided into strict aerobic and positive catalase reaction for most isolates, except for M064713 and M074713 which were aero-anaerobic and possessing a catalase negative reaction. Reduction of litmus milk gave, however, a negative reaction for all isolates tested (tab. II).

Similarity analysis based on API 20E, using the Bray-Curtis method according to the UPGMA algorithm, grouped bacterial isolates into two main clusters. The first cluster formed by D054709 showing only positive reactions with ONPG, TDA and IND tests, having the lowest similarity index (0.25 to 0.70). The second grouped other isolates,

having variable responses and with a similarity index varying between 0.64 and 1 (Fig. 2). Identity analysis using Apiweb 20E V5.0 (https://www.apiweb.biomerieux.com), showed up to 96.4% of identity with *Ochrobactrum anthropi* for this group. For API 20NE, isolates could be divided into S014709, S034709, D044709 and D054709 that revealed positive reactions for NO_3, ESC and Ox tests. More of these tests, M044713 strain reacted positively with ARA, MNE NAG, GNT and MLT tests. Both had low similarity indices (0.19-0.55). Other isolates showed variable responses for all tests (Fig. 3). However, the identity profile from Apiweb 20NE V8.0 showed 98.9% of identity with *Rhizobium radiobacter*.

3.2.- Plant growth promotion properties

The study of PGPR properties based on heatmap analysis of five characters divided bacterial isolates into: M024713 could further solubilize phosphorus, potassium and produce considerably AIA and HCN, but not for sidérophores; M034713, D024709, D064709 and M064713 group which did not solubilize potassium, having a low AIA and sidérophores production; D044709 had a high of siderophores and AIA production but with a low capacity of the phosphate-potassium solubilizing. Other isolates revealed, however, variable results for all properties examined (fig. 4).

3.3.- Phylogenetic analysis

A representative strain, M044713, forming a separate cluster in the UPGMA dendrogram of API 20NE tests was chosen for phylogenetic trees. Analysis of 16S rRNA sequence using the BLAST function in EzBioCloud databases, according to the neighbor-joining and maximum likelihood models for nearly complete 16S rRNA gene sequences of several species of *Bradyrhizobium*, *Rhizobium*, *Sinorhizobium* and *Mesorhizobium*, that have been isolated from peanut and other legumes, had shown low similarity distances. However, the most important percentage of similarity was 94.5%, noted with *Pseudoxanthomonas koreensis* (fig. 5, 6).

4.- Discussion

In this study, physiological and biochemical characterization of fourteen isolates from effective peanut nodules were carried. The results showed both morphologically and physiologically variations. The majority of bacterial isolates were Gram-negative bacilli. They could be divided into slow-growing and fast-growing rhizobia. Several reports describe that peanut has been found to form effective nodule with both fast and slow-growing rhizobia [24]. Most rhizobial isolates belong to *Bradyrhizobium* [22,23,42]. Although some other fast-growing effective rhizobia have been also reported, classified as *Rhizobium* [43,44]. Many isolates studied could utilize the various carbon sources, but a few of them that could grow on nitrogen sources. They could also grow up to 5% of NaCl and from 4.5 to 11 of pH. Variability of native rhizobia in terms of their tolerance to salt, pH or other factor is conditioned by the specific environmental conditions of their natural habitats, which suggests that soil and climatic properties affect the diversity and distribution of indigenous rhizobia [45, 46].

Table II.- Phenotypic features for peanut isolates collected from effective root nodules (+: positive; ±: weakly positive; -: negative)

Characteristics	M014713	M024713	M034713	M044713	M064713	M074713	D024709	D034709	D044709	D054709	D064709	D084709	S014709	S034709
Sole carbon sources:														
D-galactose	±	±	+	±	±	+	±	±	±	±	+	+	±	±
Glucose	+	+	+	+	+	+	+	±	±	+	+	+	+	±
Lactose	+	+	+	+	±	±	±	+	+	+	+	+	+	+
Fructose	-	+	+	±	-	+	+	+	-	±	±	±	+	±
Mannitol	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Sole nitrogen sources:														
L-Glutathione	-	-	-	-	-	-	-	+	-	-	±	±	+	-
Casein	-	±	-	-	-	-	-	-	-	+	+	+	-	-
L-Tryptophan	-	-	±	-	±	-	+	-	-	-	-	-	+	-
L-Cysteine	-	+	-	-	±	-	-	-	-	-	-	+	-	-
L-Leucine	±	-	-	+	±	+	-	-	-	-	±	-	-	-
Grown at/in:														
pH 11	±	±	-	±	-	±	-	+	-	±	±	±	±	-
pH 9	+	+	+	+	+	+	+	+	±	+	+	+	±	±
pH 7	+	+	+	+	+	+	+	+	+	+	+	+	+	+
pH 4,5	+	+	+	+	+	+	+	+	±	+	+	+	+	+
0,01% (W/v) NaCl	+	±	+	±	+	+	+	+	±	-	+	±	+	-
1% (W/v) NaCl	±	+	±	+	±	±	+	+	-	±	±	+	+	+
5% (W/v) NaCl	-	±	+	+	+	±	-	-	-	+	+	+	±	-
Resistance to (µg ml^{-1})														
Ampicillin (10)	+	+	+	+	+	+	-	+	+	+	-	+	+	+
Amoxicillin (25)	+	+	+	+	+	+	-	-	+	-	-	-	-	-
Gentamicin (10)	+	+	-	-	-	-	-	-	-	-	-	-	-	-
Nalidixic Acid (30)	-	-	-	-	-	-	-	-	-	-	-	+	-	-
Oxidase	-	-	-	-	-	-	-	-	-	+	-	-	-	+
Catalase	+	+	+	+	-	-	+	+	+	+	+	+	+	+
Amylase	+	+	-	+	+	-	+	-	-	+	-	+	-	-
Gelatinase	+	+	-	-	-	+	-	-	-	-	+	+	-	-
Nodulation	±	+	±	±	±	+	±	+	+	+	±	+	±	±
PVK-solubilization	+	+	+	+	+	+	+	+	+	+	+	+	+	+
NBRIP-solubilization	+	+	+	+	+	+	+	+	+	+	+	+	+	+

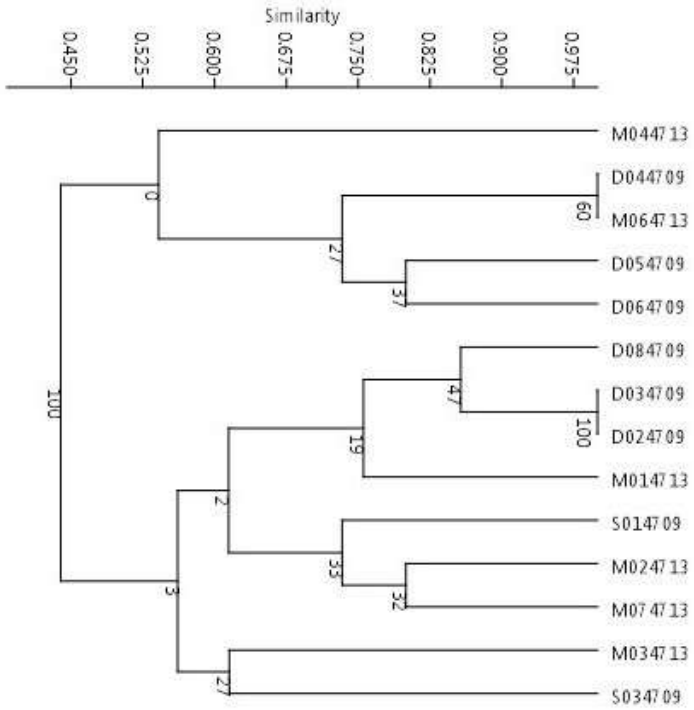

Figure 2.- Similarity analysis of isolates based on API 20E using the Bray-Curtis method according to the UPGMA algorithm, applied on Past 3.

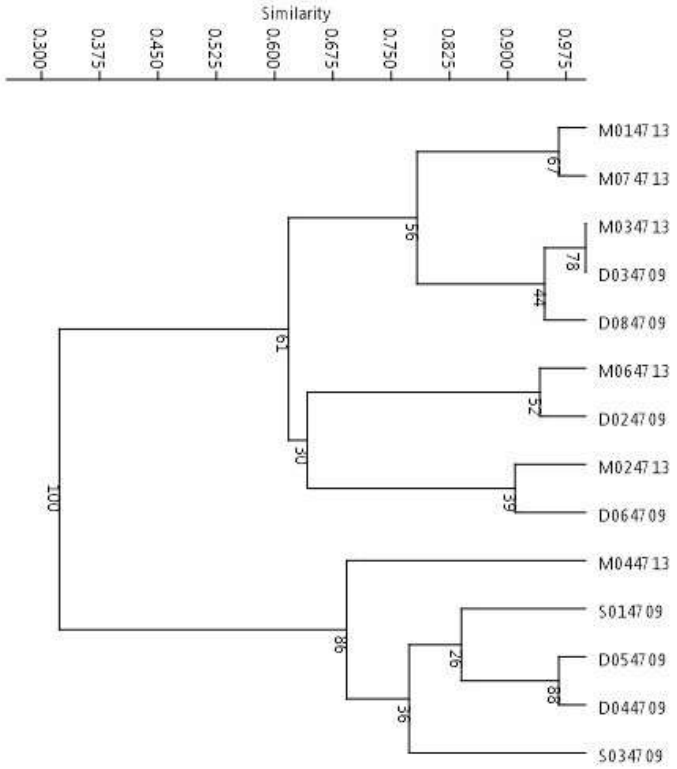

Figure 3.- Similarity analysis of isolates based on API 20NE using the Bray-Curtis method according to the UPGMA algorithm, applied on Past 3.

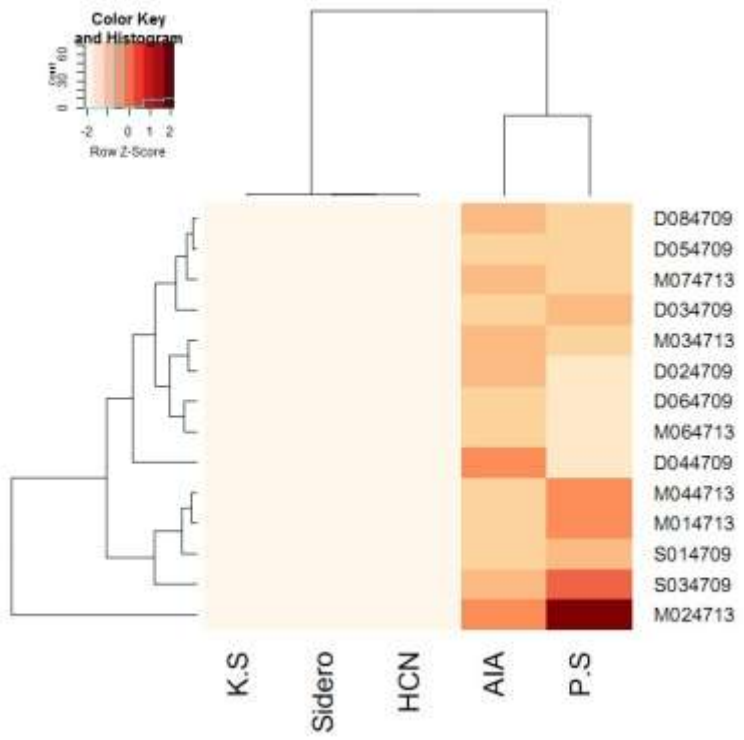

Figure 4.- Heatmap grouping the fourteen isolates based on their plant growth promotion properties, using the heatmap2 function on R 5.3.2.

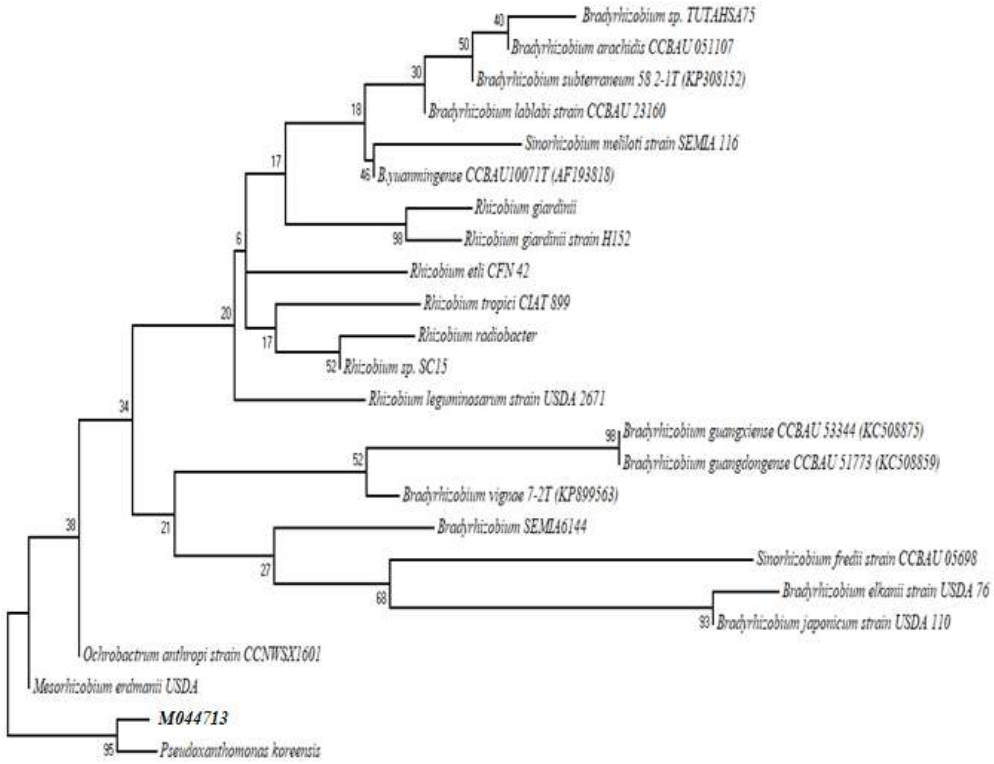

Figure 5.- Neighbor-joining phylogenetic tree based on 16S rRNA gene sequence of M04471 and most representative strains isolated from root nodules of *A. hypogaea*. Bootstrap confidence levels were derived from 1000 replications and those greater than 60% are indicated at the internodes. The bar represents two estimated substitutions per 100 nucleotide positions.

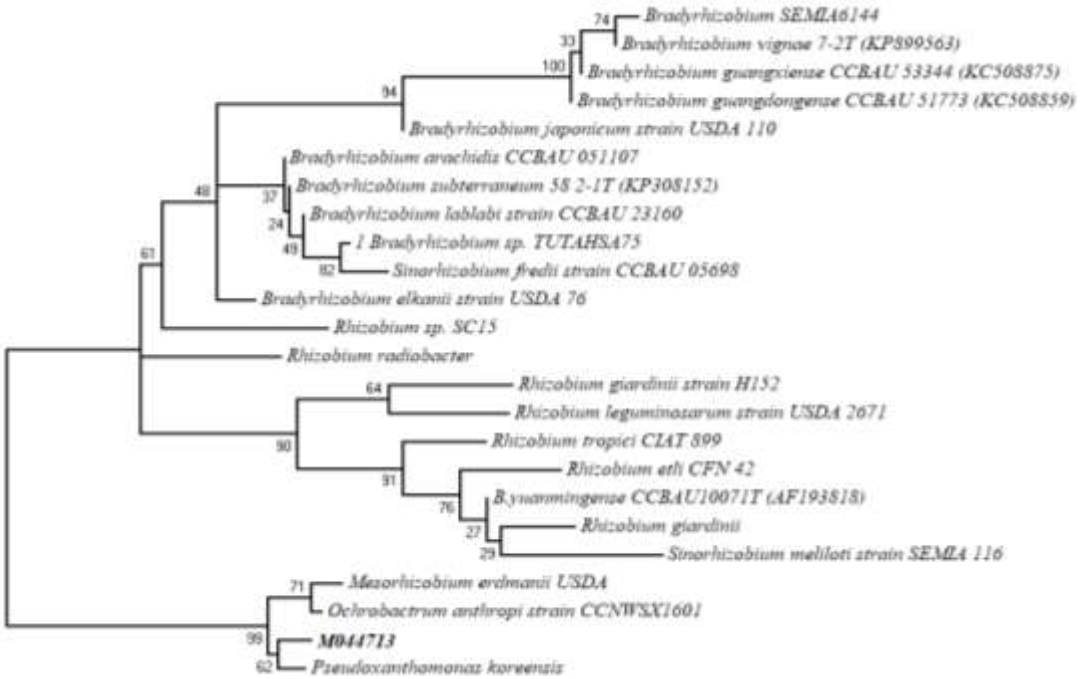

Figure 6.- Maximum Likelihood phylogenetic tree based on 16S rRNA gene sequence of M04471 and most representative strains isolated from root nodules of *A. hypogaea*. Bootstrap confidence levels were derived from 1000 replications and those greater than 60% are indicated at the internodes. The bar represents two estimated substitutions per 100 nucleotide positions

The PGPR properties tested in this work revealed that almost all rhizobial isolates could dissolve tricalcium phosphate, produced siderophores and AIA molecules. The results were, on the other hand, variable for hydrogen cyanide acid production and potassium solubilization. The use of beneficial microorganisms to increase crop yields has been reported as an ecological alternative. Plant growth-promoting bacteria (PGPR) may facilitate plant growth either indirectly or directly by several ways [47]. Phosphate Solubilizing Bacteria (PSB) are capable to convert insoluble phosphate in to soluble forms through the production of organic acids, chelates formation, exchange reaction and protons H^+ release [48,49]. Potassium Solubilizing Bacteria (KSB) can dissolve K-minerals such as mica, illite and orthoclase in the soil through the excretion of organic acids and production of capsular polysaccharide [50,51]. This minerals solubilizing making them more readily available for plant growth [52]. The PSB and KSB can be potentially useful, as an alternative solution for the problem of P and K availability in various soils and can be developed as biological fertilizers [37]. Siderophores which can sequester iron from the soil and provide it to plant cells as a siderophore–iron complex, have been also implicated in the ability of certain strains to trigger induced resistance in plants [53,54]. While phytohormones synthesis, such as AIA, can enhance or regulate various stages of plant growth [55]. Studies carried out by MARTÍNEZ-VIVEROS *et al*. (2006) had shown that AIA-producing bacteria stimulate seed germination, division, cell and tissue enlargement, leaf expansion and root elongation [56]. In this work, rhizobial bacteria showed the significant capacity of phosphate-potassium solubilizing, siderophores and phytohormone production, what makes them beneficial to promoting plant growth such as *Pseudomonas, Bacillus, Enterobacter, Azotobacter, Agrobacterium, Achromobacter, Rhizobium, Burkholderia, Flavobacterium, Micrococcu*s and other species defined as PGPR [57].

The Identification of isolates based on API 20E and API 20NE tests developed the significant percentage of similarity with *Ochrobactrum anthropi* (96.4%) and *Rhizobium*

radiobacter (98.9%), respectively. Although they are the pathogenic strains, the two genera contain several species considered as PGPR [58,59]. Nevertheless, the molecular level of the isolate M044713, assessed through 16S rRNA sequence analysis, showed low similarity distances with *Bradyrhizobium*, *Rhizobium*, *Sinorhizobium* and *Mesorhizobium* species. Indeed, the most important distance (94.5%) was that noted with *Pseudoxanthomonas koreensis*, which is long away for considering them as related species, even though several species of *Pseudoxanthomonas* had been reported as rhizobacteria [1, 60].

Conclusion

This work is the first to report the rhizobia diversity nodulating peanut grown in Algerian sandy soils, through phenotypic and genotypic approaches. The results obtained for the fourteen isolates showed morphological and biochemical variability. All isolates presented significant capacities of PGPR, which can be utilized as bio-inoculant for improving plant growth and nodulation of legumes. The phylogenetic distribution of the isolate M044713, based on 16S rRNA sequence analysis, revealed low similarity percentages for all strains previously isolated from peanut. Although, other molecular analysis, especially, symbiotic genes nodC, 16S-23S ITS rRNA region, atpD, glnII and recA fragments need to be sequenced in order to approve the genetic affiliation of this strains collection.

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