ISSN 2348-313X (Print)
International Journal of Life Sciences Research ISSN 2348-3148 (online)
Vol. 3, Issue 1, pp: (140-148), Month: January - March 2015, Available at: www.researchpublish.com

Molecular Characterization of Bacteria Isolated From Groundnut (*Arachis Hypogaea* L.) Root Nodules

[1]Dr. Abdelmalik O. A. Idris, [2]Prof. Awad M. Abdel Rahim, [3]Prof. Abdelmoniem E. Suliman, [4]Prof. Michael Gottfert

[1] Department of Biology, Faculty of Education, University of Kordofan, El-Obeid, Sudan
[2]Biosciences and Biotechnology Center, Faculty of Engineering and Technology, University of Gezira, Wad-Medani, Sudan
[3] Department of food Technology, Faculty of Engineering and Technology, University of Gezira, Wad-Medani, Sudan
[4] Institute of Genetics, Technical University of Dresden, Dresden, Germany

Abstract: **This study was carried out to characterize three strains of bacteria isolated from groundnut grown in different soils in the Sudan using 16SrRNA gene sequences. The analysis of the sequences revealed that all the three isolates and the reference strains are *Klebsiella* spp. with similarity ranging from 98% to 99%. However when the sequences were aligned, it was found that there are differences between the different isolated and the reference strains in three positions namely 867, 955 and 964. The differences in the three positions resulted in differences in the translated amino acids of the sequences of the genes also in three positions 289, 319 and 322.**

Keywords: **Endophytic, Isolates, Klebsiella, Rhizobia, Sequences, Strain.**

I. INTRODUCTION

The taxonomy of nitrogen-fixing bacteria is undergoing substantial revisions due to the advent of molecular methods for phylogenetic analysis, and in certain cases this has proved useful in unraveling ecological relationships among confusing groups. Molecular methods are also proving useful in studies of biodiversity within populations of rhizobial species (Kahindi *et al*., 1997)[1]. Studies correlating genetic with phenetic data are essential for determining the taxonomic significance of results obtained with the various techniques (Schlegel, 1992)[2].

Bacterial classification can be based on phenotypic and/or genotypic features. Phenotyping can be based on morphological, physiological, or biochemical aspects and, in the case of the family *Rhizobiaceae*, also on symbiotic compatibility with legume host plants. Genotyping can be done by various methods such as DNA (rRNA) nucleotide sequence analysis, amino acid sequence analysis, DNA:DNA hybridization, DNA: rRNA hybridization, randomly amplified polymorphic DNA (RAPD) fingerprinting, restriction fragment length polymorphism (RFLP), fingerprinting for repetitive sequences in the genome, RNA oligonucleotide cataloguing and mol% guanine plus cytosine (G+C) of total DNA (Van Rossum *et al*., 1995)[3].

Restriction fragment length polymorphism (RFLP) and direct sequence of 16SrRNA genes have been used at both species and genera level (Romdhane *et al*., 2006, Laguerre *et al*., 1994, Lafay and Burdon, 2001)[4, 5, 6]. One of the most advantages of 16SrRNA region is that, it is highly conservative, hence supports the well-established subdivision of rhizobia into species and genera (Sun *et al*., 2001)[7]. The 16SrRNA gene sequencing previously used to detect potential novel taxa of new isolates (Heyndrickx *et al*., 1996)[8]. Moreover, a longer fragment of the 16SrRNA gene (e.g, 800 bp) contain a conserved region that is sufficient to show the variation within groups of root nodule bacteria (Wolde-meskel *et al*., 2005)[9]. Additionally, the DNA sequence analysis of 16SrRNA region is a powerful tool in discriminating among strains and also known to exhibit a great deal of sequence and length variation (Mutch and Young, 2004)[10].

As the genetic diversity in tropical rhizobial species is still poorly known (Germano *et al*., 2006)[11], this study was designed to characterize three strains of bacteria isolated from groundnut grown in different soils in the Sudan using 16SrRNA.

II. MATERIALS AND METHODS

Isolation of bacteria:

Nodules were collected in August 2011 from three different regions in the Sudan, El-Obied (with sandy soil (Arenosols) in western Sudan, El-Gezira (central Sudan) and El-Gadarif (eastern Sudan) with heavy clay soil (Vertisols). To collect the groundnut nodules the whole plants were pulled up carefully without detaching the nodules. Collected nodules were washed with sterile water and then surface sterilization was done using 70% ethanol and 0.1% $HgCl_2$ and repeatedly washed with sterile water. After surface sterilization, nodules were crushed and then the resulting suspension was streaked onto yeast extract mannitol agar (YEMA) at pH 6.8, the medium contains (g / l): mannitol, 10; K_2HPO_4, 0.5; $MgSO_4.7H_2O$, 0.2; NaCl, 0.4; yeast extract, 1; agar, 20 with Congo Red or Bromothymole Blue. Purity was assured by routine plating on YEMA supplemented with Congo red and the uniform colonies were selected. The basal growth medium is that described by Vincent (1970)[12]. After repeated sub-culturing, pure culture was obtained from a single cell**.** The characters of the isolates were compared with reference strain which was obtained kindly from biofertilization department, Ministry of Sciences and Technology, Sudan.

DNA extraction:

For molecular characterization all experiments were done in Molecular Genetics lab - Institute of Genetics – Technical University of Dresden – Germany. Genomic DNA was isolated similarly as described by Dhaese *et al*. (1979)[13]. Strains were grown in AG broth medium in incubator shaker (150 rev/minutes) at 28°C for 2 days. About 20 ml of the bacterial culture were collected by centrifugation. After washing the bacterial biomass once with TE buffer (10 mM tris, 1 mM EDTA, pH 8), bacteria were resuspended in 300 µl TE buffer. 100 µl of 5% SDS (Sodium dodecyl sulfate**)** and 100 µl pronase E (2.5 mg/ml in TE buffer pre-incubated for 90 minutes at 37℃) were added. After mixing, the solution was incubated for overnight. Then the DNA was thoroughly sheared using a syringe. The DNA was purified by two extractions with 300 µl of Tris-buffered phenol and one extraction with methylene chloride. DNA was precipitated with 2.5 volumes of ethanol. The quality and quantity of the DNA were assessed using a NanoDrop ND-1000 device (Spectrophotometer, USA) and agarose gel electrophoresis stained with ethidium bromide, using a marker ladder as reference.

Amplification and sequencing of *16SrRNA* gene:

For amplification of the *16SrRNA* genes, the forward primer 16Sa (5`-CGCTGGCGGCAGGCTTAACA-3`) and the reverse primer 16Sb (5`-CCAGCCGCAGGTTCCCCT-3`) were used (van Berkum and Fuhrmann, 2000)[14] and amplified near 1500 base pairs of *16SrRNA* from the isolates and the reference strain. The PCR was performed using 5 µl of 10x *pfu* buffer, 1 µl dNTPs, 1 µl forward primer, 1 µl reverse primer, 1 µl template DNA, 1 µl *pfu* DNA polymerase, 1.5 µl DMSO (Dimethyl sulfoxide) and 38.5 µl double distilled water total volume of 50 µl. PCR conditions were: an initial denaturation at 95℃ for 3 minutes, 34 cycles of denaturation at 95℃ for 30 seconds, annealing at 55℃ for 30seconds, extentsion at 72℃ for 2 minutes and final extension at 72℃ for 15 minutes. PCR products were purified by MEGAquick-spin Total fragment DNA purification kit according to manufacture instructions. For visualization PCR products were separated on 1% agarose gel and stained with ethidium bromide, using a marker ladder as reference. The PCR product was sequenced with the above mentioned primers and sequenced again with the primers Kleb16Sf (5`-CCCTGGTAGTCCACGCTGTAAACG-3`) and Kleb16Sr (5`-TTCGCACCTGAGCGTCAGTCTTTG-3`). Sequencing was done by GATC Biotech (Konstanz, Germany).

Data analysis:

The sequence analyses of *16SrRNA* genes were performed by using the algorithm BLASTN (Altschul *et al*., 1997) to identify similarities, the software programs APE (A Plasmid Editor) was used for sequences alignments and translation and the program EMBOSS (European Molecular Biology Open Software Siute) was used for merging DNA sequences.

Nucleotide sequences accession numbers:

The 16SrRNA sequences obtained in this study were deposited in the Gen Bank data base under the following numbers: KJ940119, KJ940120, and KJ940121 for El-Gadarif, El-Gezira and El-Obied strains, respectively.

III. RESULTS AND DISCUSSION

DNA of about 21226bp (base pairs) was extracted for all isolates and the reference strain with concentrations of 164, 263, 72.4 and 850.4 ng / µl for El-Gadarif, El-Gezira, El-Obied and the reference strains, respectively (Fig. 1).

The PCR amplification resulted in about 1500 bp (base pairs) bands of *16SrRNA* gene for the three isolates and the reference strain (Fig. 2). The concentrations of PCR product used for *16SrRNA* gene sequencing were 35.4, 133.7, 33.2 and 74.5 ng / µl for El-Gadarif, El-Gezira, El-Obied and the reference strains, respectively.

For the reference strain about 1039 and 1153 bp for forward and reverse primers, respectively were sequenced and for the isolated strains the sequencing results for forward and reverse primers were (1101 and 1150 bp), (796 and 1213 bp) and (1155 and 1013 bp) for El-Gadarif, El-Gezira and El-Obied strains, respectively The sequence analysis of *16SrRNA* gene for the three isolates and the reference strain revealed that all strains are *Klebsiella spp* like including the reference with similarity 99% for El-Gadarif, El-Gezira and the reference strains and 98% for El-Obied isolate. It was reported previously that bacteria with >97% 16S rRNA gene homology are regarded as the same species (Stackebrandt and Goebel, 1994)[15]. The same result was obtained when sequencing was performed using Kleb16S primers and analyzed which resulted in sequencing (forward and reverse primers) of (702 and 681 bp), (699 and 684 bp), (701 and 686 bp) and (704 and 687 bp) for the reference, El-Gadarif, El-Gezira and El-Obied strains, respectively. The *16SrRNA* sequences merging resulted in 1457 nucleotides for all strains. There were clear differences between the isolates themselves and the isolates and the reference strain, in some nucleotides positions. The differences were concentrated in the region between 801 to 1000 bp namely in three positions 867, 955 and 964 of the sequences as shown in Fig. (3). The reference and El-Gezira strains were found differ in three positions one of them was position 867 in which the nucleotide was (G) for the reference strain and (T) for El-Gezira strain, the second position was 955 in which the nucleotide of interest was unknown for the reference strain and it was (C) for El-Gezira strain and the third position was 964 in which the nucleotide was (C) for the reference and (G) for El-Gezira strain. The reference and El-Obied strains differ only in one position (964), the nucleotide in this position was (C) for the first and (G) for the second strain. The reference strain also differ from El-Gadarif strain in one position (955) the nucleotide in this position was unknown for the reference strain and it was (C) for El-Gadarif strain. Between El-Gezira and El-Gadarif strains there were two positions of differences (867 and 964), the nucleotides of differences were (T and G) and (G and C), respectively. El-Gadarif and El-Obied strains also differ in two positions (955 and 964), for the first position, the nucleotide was (C) for the first strain, and it was unknown for the second strain, and in the other position the nucleotides of difference were (C) and (G) for El-Gadarif and El-Obied strains, respectively. Finally El-Gezira and El-Obied strains also differ in two positions (867 and 955), the nucleotide of difference in the first position was (T) for El-Gezira and (G) for El-Obied strains and it was (C) for El-Gezira strain and unknown for El-Obied strain. The translation of the sequences of *16SrRNA* gene resulted in 485 amino acids for all strains used in this study. However due to the differences in nucleotides positions mentioned above, the amino acids 289, 319 and 322 were found differ from strain to another. In the first position the amino acid was Leucine for the Reference, El-Gadarif and El-Obied strains and it was Phenylalanine for El-Gezira strain. In the second position the amino acid was unknown for both the Reference and El-Obied strain and was Proline for both El-Gezira and El-Gadarif strains. In the third position the amino acids in position 322 was Histidine for both the Reference and El-Gadarif strains and Asprtate for El-Gezira and El-Obied strains. The concentration of the differences in the region lays between 801 to 1000 bp of the sequences may indicate that, these isolates are of the same ancestor and changes occur in this region of *16SrRNA* gene because of different environment and different soil type from which the strains were obtained. Hence the soil type has a direct effect on the genetic characters of the isolates and the changes in this region enable these isolates to adapt themselves to live in the different soil type. Although the analysis of *16SrRNA* genes confirmed that our isolates are *Klebsiella spp.* like, the isolates induce nodule formation in groundnut and failed to nodulate *Phaseolus valgaris* and *Vigna spp.* this result was in consistence with Ibanez *et al*. (2009)[16] who reported that in spite of the fact that nodulation ability of bacteria was confirmed directly after isolation, several months later some strains failed to nodulate peanut, probably due to the loss of symbiotic genes. They also stated that the *16SrDNA* sequences of seven fast-growing strains the phylogenetic analysis showed that these isolates belonged to the Phylum Proteobacteria, class Gammaproteo-bacteria,

ISSN 2348-313X (Print)
International Journal of Life Sciences Research ISSN 2348-3148 (online)
Vol. 3, Issue 1, pp: (140-148), Month: January - March 2015, Available at: www.researchpublish.com

and included *Pseudomonas spp*., *Enterobacter spp*., and *Klebsiella spp*. After storage, these strains became unable to induce nodule formation in *Arachis hypogaea* plants, but they enhanced plant yield. When the isolates were co-inoculated with an infective *Bradyrhizobium* strain, they were even found colonizing pre-formed nodules. They found that the alignments of their nearly full-length *16SrDNA* sequences indicated that all of them were highly similar (99% similarity) to those of *16S* ribosomal genes belonging to members of the Class Gammaproteobacteria, genera *Pseudomonas* (for isolates NCHA33, NCHA35, and NVAM24), *Klebsiella* (for isolates NTI31, TT001) and *Enterobacter* (for isolates NMAN11 and NONC13). They further showed that, the plants inoculated with only the fast-growing strains did not show any nodule structure on their roots, but the shoot dry weights were significantly higher than those from non-inoculated plants, indicating the ability of these isolates to promote plant growth. Since external contamination was excluded, these fast-growing strains were effectively recovered from inside the nodules (Ibanez *et al*., 2009)[16]. They further stated that considering the above results, the Gammaproteobacteria analyzed in this study are best described as opportunistic bacteria and not genuine peanut symbionts, with the cooperation of host– endophyte –rhizobia being necessary for a peanut nodule occupation.

Although there was no direct evidence for the contribution of these peanut fast-growing strains to nitrogen fixation, at least the increase in the shoot dry weight of plants inoculated only with the Klebsiella-like isolates could be related to their ability to fix nitrogen. Other mechanisms, such as the secretion of phytohormones could also have contributed to the plant growth promotion observed (Ibanez et al., 2009) [16]. They also stated that a possible explanation for the presence of Gamma- proteobacteria in peanut nodules is that these strains may colonize root nodules after or during their formation.

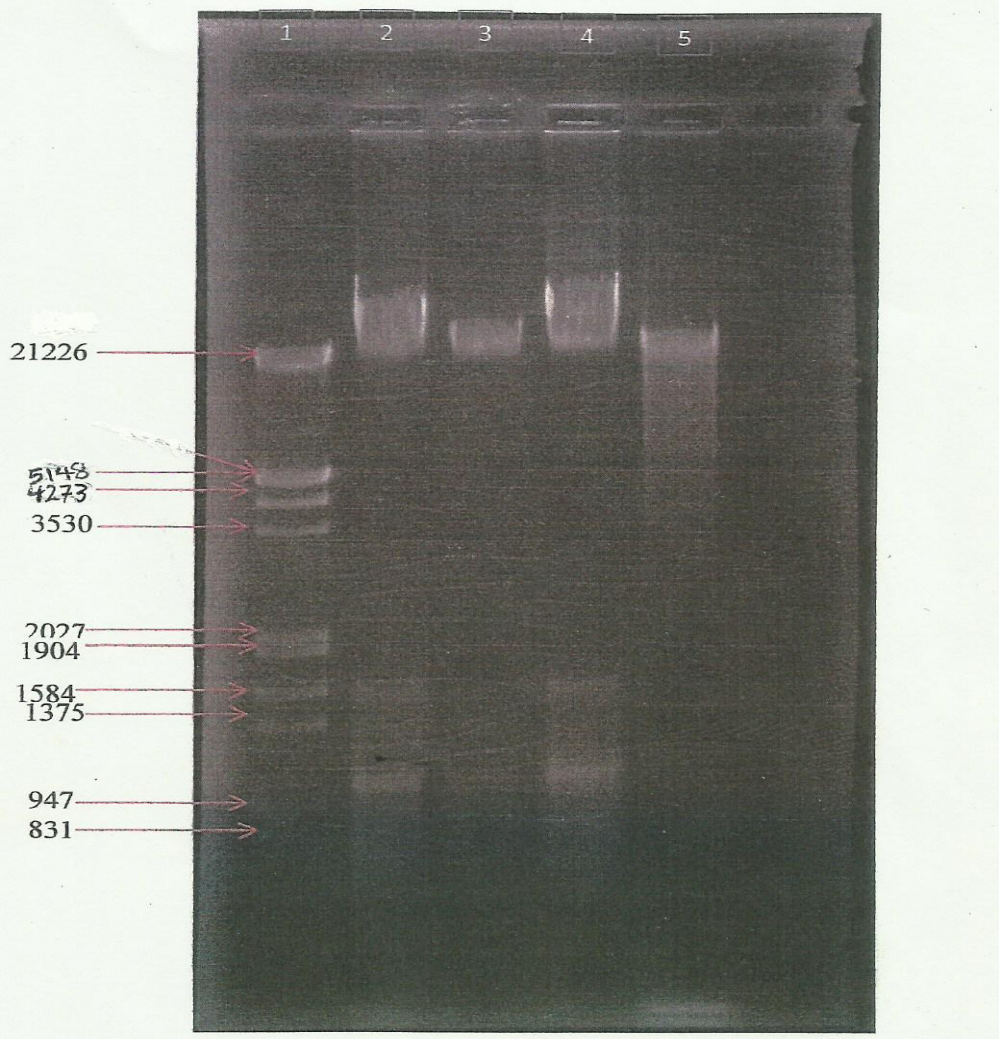

Fig: 1 Genomic DNA for the isolates and the reference strain. Lane (1) = Ladder, Lane (2) = El-Gezira, Lane (3) = El-Obied, Lane (4) = El-Gadarif and Lane (5) = Reference strain.

ISSN 2348-313X (Print)
International Journal of Life Sciences Research ISSN 2348-3148 (online)
Vol. 3, Issue 1, pp: (140-148), Month: January - March 2015, Available at: www.researchpublish.com

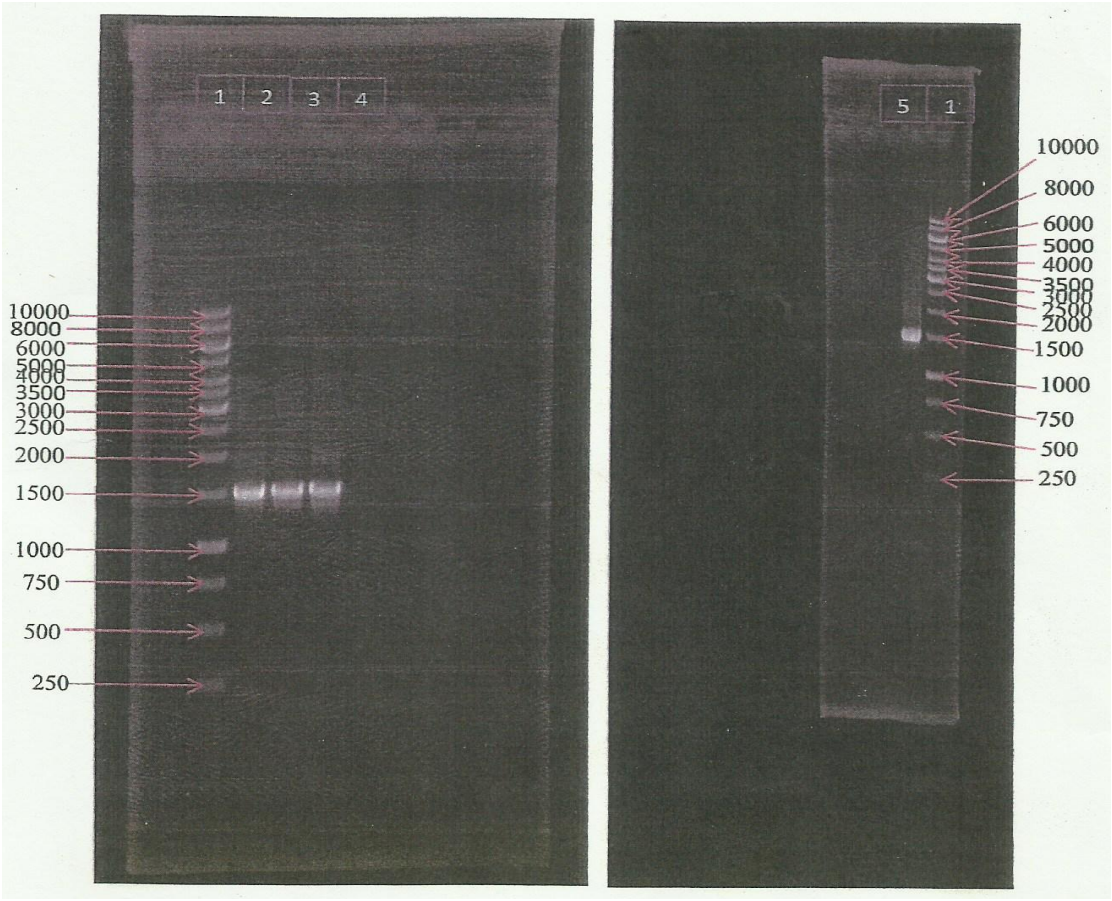

Fig: 2 Amplification of *16SrRNA* gene for the isolates and the reference strain. Lane (1) = Ladder, Lane (2) = El-Obied, Lane (3) = El-Gadarif, Lane (4) = El-Gezira and Lane (5) = Reference strain.

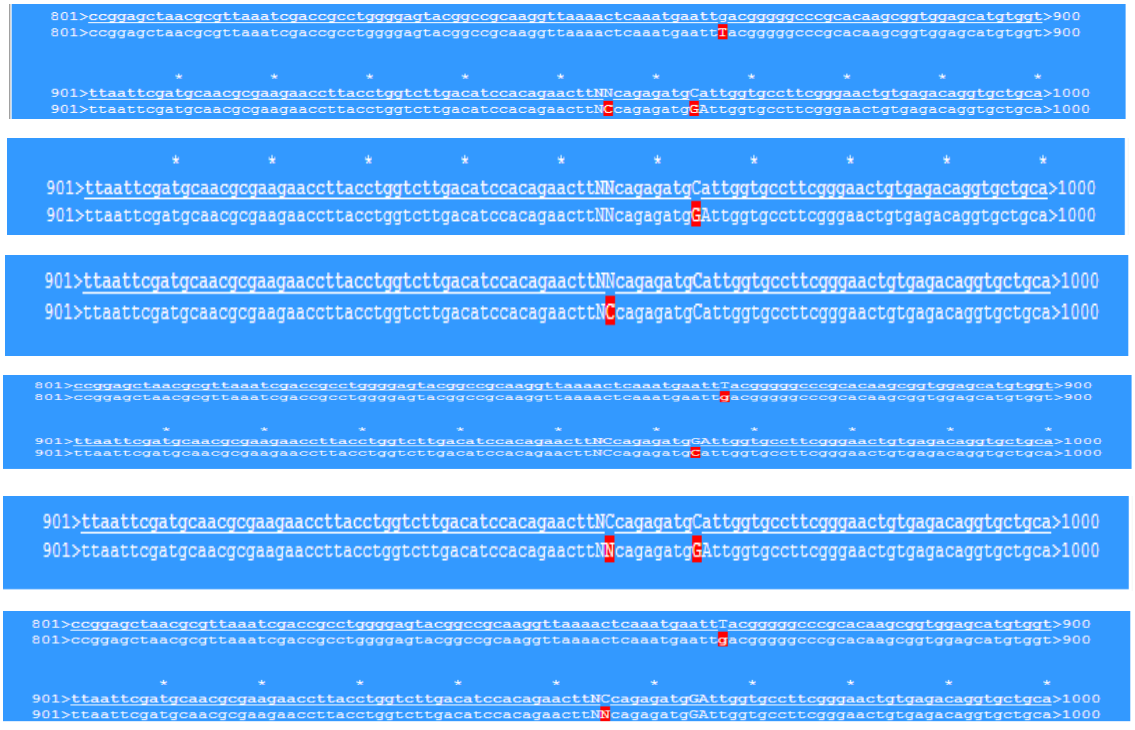

Fig. 3: The nucleotide differences in *16SrRNA* gene between the reference and El-Gezira, the reference and El-Obied, the reference and El-Gadarif, El-Gezira and El-Gadarif, El-Gadarif and El-Obied and El-Gezira and El-Obied strains, respectively.

Also it was reported that as well as the rhizobia, some non-symbiotic bacteria have also been isolated from the root nodule of a wide range of legumes (de Lajudie *et al*., 1999; Gao *et al*., 2001; Kan *et al*., 2007; Zakhia *et al*., 2006)[17, 18, 19, 20]. The presence of bacteria other than rhizobia in legumes was well documented (Sturz *et al.*, 1997)[21]. Also Philipson and Blair (1957)[22] found diverse species, including Gram positive bacteria, in nodules of health clover. The implications of this discovery, would be significant, especially if it is confirmed that the association between legume nodules and Gammaproteobacteria is common in nature and that this interaction is beneficial for plant growth. The results obtained in this work also strengthen the proposition formulated by Sturz *et al*. (1997)[21] that the term ''root nodule bacteria'', often used to refer to the exclusive presence of rhizobia in nodules, should be redefined. *Arachis hypogaea* thus be considered to be promiscuous in nature since root nodules of a single plant are not induced by a predominant genetic variant enriched from the rhizosphere population. On this basis of this finding, peanut nodules may be considered a reservoir for different rhizobial lineage (Angelini *et al*., 2011)[23]. Despite of high specificity of the legume–Rhizobium interaction and the selective nodule environment, non-rhizobial nodule rhizobacteria have been reported. Root nodules which traditionally considered the exclusive niche of rhizobia are being colonized by several free-loaders unrelated to symbiotic nitrogen fixation. Evidence that the healthy nodule interior can contain endophytes not necessarily related to symbiotic or diazotrophic context has been documented i.e. *Klebsiella* (groundnut, clover and bean) as was reported by Ozawa *et al*. (2003)[24]. As in the biochemical tests in this study, isolates were classified as fast growing. This result was typically stated by Silva *et al*. (2012)[25] that the *16SrRNA* sequences of others fast-growing isolates showed similarity with *Enterobacter* sp., *Rhizobium* sp. and *Klebsiella* sp. and it confirmed the results obtained before (Ibanez *et al*., 2009)[16] which showed similarity of the *16SrDNA* sequences of fast-growing isolates from root nodules of *Arachis hypogaea* with *Pseudomonas* spp., *Enterobacter* spp. and *Klebsiella* spp. The authors defended the idea that, these isolates were opportunistic bacteria that colonize nodules induced by rhizobia. Until 2001, all bacteria known to be isolated from nodules were restricted to genera within the Alpha proteobacteria. However, this began to change when isolates belonging to the Beta proteobacteria were discovered as nodule-forming or nodule-associated bacteria (Chen *et al*., 2001, Chen *et al*., 2005, Moulin *et al*., 2001, Valverde *et al*., 2003)[26, 27, 28, 29]. Although we used 16SrRNA gene to characterize our isolates, Other studies revealed that despite the *16SrRNA* gene is efficient to define genera, because it is conserved, but have variable regions; it has also limitations to identify species, due to possible occurrence of genetic recombination and horizontal gene transfer (Neto *et al*., 2010)[30]. Studies on the diversity of rhizobia often depend on the analysis of *16SrRNA* gene sequences for species-level identification. Moreover, several studies have shown that *16SrRNA* genes may undergo recombination and horizontal transfer resulting in sequence mosaicism (van Berkum *et al*., 2003; Vinuesa *et al*., 2005)[31, 32]. Another disadvantage of bacterial identification based on the analysis of *16SrRNA* genes is that, closely related species cannot always be differentiated because of high levels of sequence conservation (Martens *et al*., 2007)[33]. To surmount these difficulties, the use of other genes such as protein coding (house keeping) genes with greater sequence divergence than *16SrRNA* genes, are recommended as supplementary genetic markers for identification of the rhizobia (Martens *et al*., 2007 and 2008)[33, 34].

It has been reported that the endophytic bacteria may have two main effects. They may increase the ability of plants to absorb nutrients from the soil by increasing root development and by assisting in solubilizing phosphorus (Kuklinsky-Sobral *et al*., 2004)[35]. They may also control soil borne pathogens. The inoculation of endophytic bacteria has shown a positive effect on plant growth in contaminated soil (Taghavi *et al*., 2005)[36]. Also, the nodule endophytic bacteria might evolve into symbiotic bacteria by acquiring symbiotic genes from the rhizobia by lateral gene transfer inside the nodules, as reported in rhizobia (Trinick *et al*., 1989)[37] and in endophytic bacteria (Taghavi *et al*., 2005)[36]. However inconsistence with our results there are many studies which reported that endophytes only coexist with symbiotic bacteria in nodules and they do not induce nodules (Wang *et al*., 2006)[38]. Also Pandya *et al*., (2013)[39] demonstrated in an *in vivo* study that rhizobacterial (nonrhizobial) infection within the root hairs of *Vigna radiate* where the presence of native *E. adhaerens* allowed *Pseudomonas* and *Klebsiella* spp. to enter root hairs. The ability of native *E. adhaerens* to infect root hair remained unaltered irrespective of the presence of other rhizobacteria. *Pseudomonas fluorescens* IAM 12022 and *Klebsiella pneumonia subsp. ozaenae* adhered to the surface and base of root hairs but failed to enter IT (infection thread) in the absence of *E. adhaerens*, which confirmed their inability to form an infection thread and invade root hairs independently. However, when co-inoculated with *E. adhaerens*, *P. fluorescens* IAM 12022 and *K. pneumoniae subsp. Ozaenae* successfully colonized root hairs. The infection process was initiated by host-specific *E. adhaerens*, forming IT which was invaded by test rhizobacteria. Ibanez *et al*. (2009)[16] revealed that these isolates were able to colonize the nodules even after their formation. The inability of *P. fluorescens* and *K. pneumoniae* to enter root hairs may be attributed

to their inability to secrete cellulase and pectinase. The role of cellulase the key cell wall-degrading enzyme, in facilitating the primary infection process is reported to be essential in *Rhizobium* (Robledo *et al*., 2008)[40] and degradation of pectin layers by pectate lyase in favoring the entry of *Klebsiella* strains in plant tissues is documented (Kovtunovych *et al*., 1999)[41]. Some endophytes may enter independently from other bacteria through cracks formed at the emergence of lateral roots in the zone of elongation and differentiation of the root (Rosenblueth and Martinez-Romero, 2006)[42] but their localized movement within plant cells requires controlled release of pectinase and/or cellulase (Bekri *et al*., 1999)[43]. Therefore, even if *Pseudomonas* and *Klebsiella* entered roots through cracks, their movement from one cell to the other would be restricted. However when Fouts *et al*. (2008)[44] achieved complete genome sequence of the *Klebsiella pneumonia* 342; they found gene complement capable of hydrolyzing α-linked glucans of starches and pectin and another capable of splitting 1,4-b-glucosidic bonds of cellulosic components and long chain polymers of beta-glucose such as chitin. Most importantly, known strains of *Pseudomonas* and *Klebsiella* lack Nod factors which allow rhizobia to attach to the tip of growing root hair and signal the plant to form nodules. The entry of host-specific rhizobia alone is facilitated through release of Nod factor in response to plant flavonoids (Perret *et al*., 2000)[45].

IV. CONCLUSIONS

Although the tested strains, (the isolated and the reference strains) were classified as fast growing Rhizobia by biochemical characterization, the *16SrRNA* genes sequencing revealed that they are *Klebsiella spp*. and all the isolated strain may belong to the same species. There is a slight difference in the *16SrRNA* gene nucleotides sequences of the three isolates and the reference strain and the using of molecular method was found useful in classification of root nodule bacteria. The using of *Klebsiella* like bacteria in inoculation will resolve the competitiveness problem in different soils and environments and may enhance plant growth in the field better than Rhizobia.

REFERENCES

[1] Kahindi JHP, Woomer P, George T, de Souza Moreira FM,Karanja, N. K. and Giller, K. E "Agricultural intensification, soil biodiversity and ecosystem function in the tropics: the role of nitrogen-fixing bacteria". Applied Soil Ecology., 6(1): 55-76. (1997).

[2] Schlegel HG. "Degradation of natural substances, p. 406–437. In H. G. Schlegel, General microbiology". Cambridge University Press, Cambridge. (1992).

[3] Van Rossum DA, Schuurmans FP, Gilles M, Muyotcha A, van Verseveld HW, Stouthamer AH. and Boogaard FC. "Genetics and genetic analyses of Bradyrhizobium strain modulating peanut (Arachis hypogaea L.) roots". Appl. Environ. Microbiol., 61: 1599-1609. (1995).

[4] Romdhane SB, Nasr H, Samba-Mbaye R. and Neyra M. "Genetic diversity of Acacia tortilis ssp. raddiana in Tunisia assessed by 16S and 16S-23SrDNA genes analysis". J. Appl. Microbiol., 100: 436 – 445. (2006).

[5] Laguerre G, Allard MR, Revoy F. and Amarger N. "Rapid identification of rhizobia by restriction fragment genes". Appl. Environ. Microbiol., 60: 56 – 63. (1994).

[6] Lafay B. and Burdon JJ. "Small-subunit rRNA genotyping of rhizobia nodulating Australian Acacia spp". Appl. Environ. Microbiol., 67: 396 – 402. (2001).

[7] Sun GL, Willian M, Liu J, Kasha KJ. and Pauls KP. "Microsatellite and RAPD polymorphisms in Ontario corn hybrids are related to the commercial sources and maturity rating". Mol. Breed., 7: 13 – 24. (2001)

[8] Heyndrickx M, Vauterin L, Vandamme P, Kersters K. "Application of combined amplified ribosomal DNA restriction analysis (ARDRA) patterns in bacterial phylogeny and taxonomy". J. Microbiol. Methods., 26: 247 – 259. (1996).

[9] Wolde-meskel E, Terefework Z, Frostegard A. and Lindstrom K. "Genetic diversity and phylogeny of rhizobia isolated from agro forestry legume species in southern Ethiopia". Int. J. Syst. Evol. Microbiol., 55: 1439 – 1452. (2005).

[10] Mutch LA. and Young JPW. "Diversity and specificity of Rhizobium leguminosarum biovar viciaeon wild and cultivated legumes". Mol. Ecol., 13: 2435 – 2444. (2004).

[11] Germano MG, Paˆmela M, Fabio LM. and Mariangela H. "RFLP analysis of the rRNA operon of a Brazilian collection of bradyrhizobial strains from 33 legume species". International Journal of Systematic and Evolutionary Microbiology, 56: 217–229. (2006).

[12] Vincent JL. "A manual for the practical study of root- nodule bacteria". IBP hand book NO. 75. Blackwells, Oxford. (1970).

[13] Dhaese P, De Greve H, Decraemer H, Schell J. and Van Mongatu M. "Rapid mapping of transposon insertion and deletion mutations in the large Ti-plasmids of Agrobacterium tumefaciens". Nucliec Acid Research., 7: 1837-1849. (1979).

[14] Van Berkum P. and Fuhrmann JJ. "Evolutionary relationships among the soybean bradyrhizobia reconstructed from 16S rRNA gene and internally transcribed spacer region sequence divergence". Int J Syst Evol Microbiol., 50: 2165-2172. (2000).

[15] Stackebrandt E, Goebel B. "Taxonomic note: A place for DNA-DNA reassociation and 16S rRNA sequence analysis in the present species definition in bacteriology". Int J Syst Bacteriol., 44:846-849. (1994).

[16] Ibanez F, Jorge A, Tania T, Marı´a LT. and Adriana F. "Endophytic occupation of peanut root nodules by opportunistic Gammaproteobacteria". Systematic and Applied Microbiology., 32: 49-55. (2009).

[17] de Lajudie P, Willems A, Nick G, Mohamed TS, Torck U, Filali-Maltouf A, Kersters K, Dreyfus B, Lindström K. and Gillis M. "Agrobacterium bv. 1 strains isolated from nodules of tropical legumes". Systematic and Applied Microbiology, 22: 119–132. (1999).

[18] Gao Z, Terefework W. and Chen KL."Genetic diversity of rhizobia isolated from Astragalus adsurgens growing in different geographical regions of China". Journal of Biotechnology., 91: 155–168. (2001).

[19] Kan FL, Chen ZY, Wang ET, Tian CF, Sui XH, and Chen WX."Characterization of symbiotic and endophytic bacteria isolated from root nodules of herbaceous legumes grown in Qinghai–Tibet Plateau and in other zones of China". Archives of Microbiology. 188: 103-115. (2007).

[20] Zakhia F, Jeder H, Willems A, Gillis M, Dreyfus B. and de Lajudie P."Diverse bacteria associated with root nodules of spontaneous legumes in Tunisia and first report for nifH-like gene within the genera Microbacterium and Starkeya". Microbial Ecology. 51: 375-393. (2006).

[21] Sturz A.V, Carter MR. and Johnston H.W."A review of plant disease, pathogen interactions and microbial antagonism under conservation tillage in temperature humid agriculture" . Soil Till Res., 41: 169 -189. (1997).

[22] Philipson MN. And Blair ID." Bacteria in clover root tissue". Can J Microbial., 3: 125-129. (1957).

[23] Angelini J, Ibáñez F, Taurian T, Tonelli ML, Valetti L. and Fabra A."A study on the prevalence of bacteria that occupy nodules within single peanut plants". Curr. Microbiol., 62:1752-1759. (2011).

[24] Ozawa T, Ohwaki A. and Okumura K."Isolation and characterization of diazotrophic bacteria from the surface-sterilized roots of some legumes". Sci Rep Grad Sch Agric & Biol Sci., 55: 29-36. (2003).

[25] Silva FV, Simões-Araújo JL, Silva Júnior JP, Xavier GR and Rumjanek NG."Genetic diversity of Rhizobia isolates from Amazon soils using Cowpea (Vigna unguiculata) as trap plant". Brazilian Journal of Microbiology., 682-691. (2012).

[26] Chen WM, deFaria S. and Straliotto R."Proof that Burkholderia strains form effective symbioses with legumes: a study of novel Mimosa-nodulating strains from South America". Appl. Environ. Microbiol., 71: 7461-7471. (2005).

[27] Chen WM, Laevens S, Lee, TM, Coenye T, deVos P, Mergeay M. and Vandamme P."Ralstonia taiwanensis sp. nov. Isolated from root nodules of Mimosa species and sputum of a cystic fibrosis patient". Int. J. Syst. Evol. Microbiol., 51: 1729-1735. (2001).

[28] Moulin L, Munive A, Dreyfus B. and Boivin-Masson C." Nodulation of legumes by members of b-class of Proteobacteria". Nature., 411: 948-950. (2001).

[29] Valverde A, Velazquez E, Gutierrez C, Cervantes E, Ventosa A. and Igual JM. "Herbaspirillum lusitanum sp. nov., a novel nitrogen-fixing bacterium associated with root nodules of Phaseolus vulgaris". Int. J. Syst. Evol. Microbiol., 53: 1979-1983. (2003).

[30] Neto IVR, Ribeiro RA. and Hungria M."Genetic diversity of elite rhizobial strains of subtropical and tropical legumes based on the 16S rRNA and glnII genes". World J Microbiol Biotechnol., 26: 1291-1302. (2010).

[31] van Berkum P, Terefework Z, Paulin L, Suomalainen S, Lindstro¨ m K. and Eardly BD. "Discordant phylogenies within the rrn loci of rhizobia". J Bacteriol., 185: 2988–2998. (2003).

[32] Vinuesa P, Silva C, Werner D. and Martinez-Romero E."Population genetics and phylogenetic inference in bacterial molecular systematics: the roles of migration and recombination in Bradyrhizobium species cohesion and delineation". Mol Phylogenet Evol., 34: 29-54. (2005).

[33] Martens M, Delaere M, Coopman R, De Vos P, Gillis M. and Willems A."Multilocus sequence analysis of Ensifer and related species". Int J Syst Evol Microbiol., 57: 489-503. (2007).

[34] Martens M, Dawyndt P, Coopman R, De Vos P, Gillis M. and Willems A."Advantages of multilocus sequence analysis for taxonomic studies: a case study using 10 housekeeping genes in the genus Ensifer (including former Sinorhizobium)". Int J Syst Evol Microbiol., 58: 200–214. (2008).

[35] Kuklinsky-Sobral J, Araujo WLm Mendes R, Geraldi IO, Pizzirani- Kleiner AA. and Azevedo JL."Isolation and characterization of soybean-associated bacteria and their potential for plant growth promotion". Environmental Microbiology, 6: 1244-1251. (2004).

[36] Taghavi S, Barac T, Greenberg B, Borremans B, Vangronsveld J. and van der Lelie D."Horizontal gene transfer to endogenous endophytic bacteria from poplar improves phytoremediation of toluene". Applied and Environmental Microbiology, 71: 8500-8505. (2005).

[37] Trinick MJ, Goodchild DJ. And Miller C."Localization of bacteria and hemoglobin in root nodules of Parasponia andersonii containing both Bradyrhizobium strains and Rhizobium leguminosarum biovar trifolii". Applied and Environmental Microbiology, 55: 2046-2055. (1989).

[38] Wang LL, Wang ET, Liu J, Li Y. and Chen WX."Endophytic occupation of root nodules and roots of Melilotus dentatus by Agrobacterium tumefaciens". Microbial Ecology., 52: 436-443. (2006).

[39] Pandya M, Gattupalli NK. And Shalini R."Invasion of rhizobial infection thread by non-rhizobia for colonization of Vigna radiata root nodules". FEMS Microbiol Lett., 348: 58-65. (2013).

[40] Robledo M, Jimenez-Zurdo JI. and Velazquez E."Rhizobium cellulase CelC2 is essential for primary symbiotic infection of legume host roots". P Natl Acad Sci USA. 105: 7064-7069. (2008).

[41] Kovtunovych G, Lar O, Kamalova S, Kordyum V, Kleiner D. and Kozyrovska N. "Correlation between pectate lyase activity and ability of diazotrophic Klebsiella oxytoca VN 13 to penetrate into plant tissues". Plant Soil., 215: 1–6. (1999).

[42] Rosenblueth M. and Martinez-Romero E."Bacterial endophytes and their interactions with hosts". Mol Plant Microbe Interact, 19: 827-837. (2006).

[43] Bekri MA, Desair J, Keijers V, Proost P, Searle-van Leeuwen M, Vanderleyden J. and vande Broek A."Azospirillum irakense produces a novel type of pectate lyase". J. Bacteriol. 181: 2440–2447. (1999).

[44] Fouts DE,Tyler HL, DeBoy RT, Daugherty S, Ren Q, Badger JH, Durkin AS, Huot H, Shrivastava S, Kothari S, Dodson RJ, Mohamoud Y, Khouri H, Roesch LF, Krogfelt KA, Struve C, Triplett EW. and Methé B.A."Complete genome sequence of the N2-fixing broad host range endophyte Klebsiella pneumoniae 342 and virulence predictions verified in mice". PLoS Genet., 4(7): 1-18. (2008).

[45] Perret X, Staehelin C. and Broughton WJ." Molecular basis of symbiotic promiscuity". Microbiol Mol Biol Rev., 64: 180-201. (2000).