

Full Length Research Paper

Phylogenetic analysis of 23S rRNA gene sequences of some *Rhizobium leguminosarum* isolates and their tolerance to drought

Khalid S. Abdel-Lateif^{1,2*}, Omar A. Hewedy¹ and Abdel Fattah M. El-Zanaty¹

¹Department of Genetics, Faculty of Agriculture, Menoufia University, Egypt.

²High Altitude Center, Taif University, Saudi Arabia.

Received 4 May, 2016; Accepted 3 August, 2016

The phylogenetic relationships among thirteen *Rhizobium leguminosarum* bv. *viciae* isolates collected from various geographical regions were studied by analysis of the 23S rRNA sequences. The average of genetic distance among the studied isolates was very narrow (ranged from 0.00 to 0.04) and the studied isolates formed two main groups based on cluster analysis. The isolates were tested for their growth on YMA medium supplemented with concentrations 10, 20 and 30% of polyethylene glycol plus control. All isolates exhibited good drought-tolerant efficiencies at 10% PEG. While most of the isolates could not tolerate up to 20% PEG, isolates of Rlv6, Rlv9, Rlv12 and Rlv13 tolerated up to 20% PEG.

Key words: *Rhizobium leguminosarum*, 23S rRNA gene, phylogenetic tree, diversity and drought tolerance.

INTRODUCTION

Rhizobia are gram-negative bacteria that can establish a symbiotic relationship with the roots of leguminous plants and form root organ called nodule, wherein bacteria is hosted and nitrogen is fixed. The classification of the bacteria belonging to the genus of *Rhizobium* was based on plant infection abilities for a long time (Van Berkum and Eardly, 1998). Recently, The rRNA genes are very useful for investigating the phylogenetic relationships among bacteria since their evolution are slow and their gene products performs central functions in the bacterial cell (Pulawska et al., 2000). 16S rRNA gene was the most frequently used in microbial phylogeny studies (Kolbert and Persing, 1999, Ismail et al., 2013; El-Zanaty

et al., 2014). Previous studies showed that the 23S rRNA gene contains more genetic information and better diagnostic sequence stretches than the 16S rRNA gene. Moreover, the progress in sequencing tools and the decrease in their costs make the 23S rRNA powerful tool to elucidate the taxonomic relationships (Pulawska et al., 2000; reviewed in Hunt et al. (2006) and Pei et al. (2009). Wolde-meskel et al. (2005) investigated the genetic diversity among 195 rhizobial strains isolated from root nodules of 18 agroforestry species growing in diverse zones in southern Ethiopia using PCR-RFLP of the ribosomal operon (16S rRNA gene, 23S rRNA gene and the internal transcribed spacer (ITS) region between

*Corresponding author. E-mail: k_dein2001@yahoo.com.

them).

The environmental stresses such as salinity, drought and heavy metals can suppress the growth and symbiotic characteristics of most rhizobia (Duzan et al., 2004; Gálvez, 2005). Drought is the main abiotic factor that affects survival and growth of soil microorganisms. Moreover, drought stress has negative effects on plant growth and metabolisms (Ehsanpour and Amini, 2003; Aydi et al., 2008; Fall et al., 2011). It was suggested that drought limits nodulation through its effects on persistence and survival of rhizobia in the soil, root-hair colonization and infection by rhizobia (Zahran, 1999; Vriezen et al., 2007; Mhadhbi et al., 2008). Therefore, isolation of rhizobia strains tolerant to stresses like drought is necessary for efficient nitrogen fixation and improving plant productivity especially in the water limited areas (Abd El-Halim et al., 2001; Abdel-Salam et al., 2002; Diouf et al., 2007). Athar and Johnson (1996) reported that nodulation, growth and nitrogen fixation in alfalfa can be improved by inoculating plants with competitive and drought tolerant rhizobia. In addition, it was demonstrated that, selecting stress-tolerant cultivars and stress-tolerant rhizobia could be a rational strategy to improve the yield of legumes in stressed environments (Ben Rhomdhane et al., 2007; Meuelenberg and Dakora, 2007; Mhadhbi et al., 2008). Therefore, the objectives of this study were to: 1) analyze the phylogenetic relationships among thirteen isolates of *Rhizobium leguminosarum* bv. *viciae* obtained from various geographical regions in Egypt by analysis of the 23S rRNA sequences; 2) characterize the isolates of *R. leguminosarum symbiovar vicia faba* according to their tolerance to drought.

MATERIALS AND METHODS

Rhizobium isolation

Rhizobial isolates used in this study were isolated from nodules of *Faba bean* plants nodules growing in different Egyptian regions (Table 1) as described previously by Hewedy et al. (2014).

DNA extraction

The bacterial cultures of isolates were grown in Yeast Extract Manitol medium (YEM) and extraction of total DNA was performed using GeneJET™ Genomic DNA Purification kit (Fermentas, Vilnius, Lithuania) following manufacturer's instructions. Samples were kept at -20°C until using in PCR reactions.

PCR amplification of 23S rRNA

The amplification of 23S rRNA partial gene(s) of tested isolates was done using 23S forward and reverse primers designated from NCBI site (<http://www.ncbi.nlm.nih.gov/>). The forward primer was 5'TGG GCA CTG TCT CAA CGA '3, while the reverse primer was 5'GGA TAG GGA CCG AAC TGT CTC 3. The polymerase chain reaction (PCR) was performed in 50-µL reaction volume containing 100 ng DNA, 50 µL PCR Master Mix (Fermentas, Lithuania) and 20 µM of

Table 1. Nomination and location of studied *Rhizobium* isolates.

Isolate	Geographical origin
Rlv1	Quesna City, Menoufia Governorate
Rlv2	Banha City, Qalyoubia Governorate
Rlv3	Sadat City, Menoufia Governorate
Rlv4	Zefta City, Gharbia Governorate
Rlv5	Beni-Suef City, Beni-Suef Governorate
Rlv6	Suez City, Suez Governorate
Rlv7	El-Tor city, South Sinai Governorate
Rlv8	Al Arish City, North Sinai Governorate
Rlv9	Rafah City, North Sinai Governorate
Rlv10	New Al Menia City, Menia Governorate
Rlv11	Moshtohor City, Qalyoubia Governorate
Rlv12	Cairo City, Cairo Governorate
Rlv13	Ismailia City, Ismailia Governorate

forward and reverse primers. Amplifications were performed with the following PCR conditions: i) initial denaturation at 95°C for 5 min, ii) 40 cycles of 95°C for 30 s, 58°C for 30 s, 72°C for 30 s and 10 min final extension at 72°C.

23S rRNA partial gene sequencing

The PCR products for the thirteen isolates were used in sequencing of the 23S rRNA gene from both strands using the same primers used in PCR amplification and Big Dye Terminator v3.1 cycle sequencing kit at Bioneer (Daejeon, Korea).

Drought tolerance

The effect of drought on rhizobia-growth was examined in YMA medium supplemented with concentrations 10, 20 and 30% of polyethylene glycol (PEG 6000). One hundred microliters of suspension (1×10^8 cfu/ml) of overnight grown cultures of studied isolates were transferred to petri dishes containing YMA medium supplemented with the previous PEG concentrations. After incubation at 28°C for three days, the bacterial growth was determined by counting the number of colonies formed.

Data analysis

The reference 23S rRNA gene of *R. leguminosarum* (AF207785.1) was retrieved from NCBI site (<http://www.ncbi.nlm.nih.gov/>). The alignment, genetic diversity and phylogenetic analyses were conducted using MEGA version 6 (Tamura et al., 2013).

Two ways ANOVA was used to analyze the data of drought tolerance at 0.05 levels. Descriptive statistics, GLM and Duncan test at 0.05 were calculated by SAS computer program (SAS, 2004).

RESULTS AND DISCUSSION

Amplification and sequencing of 23S rRNA gene

This work presents study on the genetic diversity of

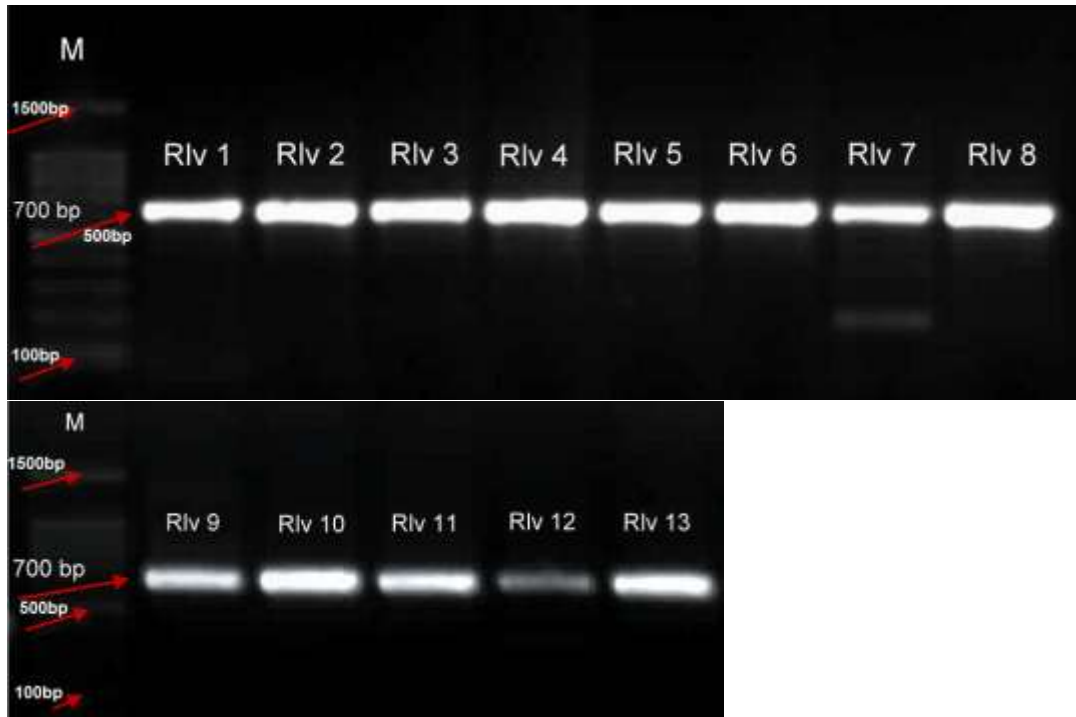


Figure 1. Partial amplification of 23S rRNA gene in *Rhizobium leguminosarum* symbiovar. *Viciae* Isolates (From Rlv1 to Rlv13); M, GelPilot 100 bp Plus Ladder (Qiagen, cat. no. 239045).

thirteen isolates of *R. leguminosarum* bv. *Viciae* collected from different *Vicia faba* fields and representing several governorates in Egypt (Table 1). Since, the structure of ribosomes is largely conserved among the three kingdoms of life organisms and the horizontal gene transfer events are unlikely to occur in the highly constrained rRNA genes, these advantages make the phylogenetic analysis and taxonomic classification of cellular organisms based on rRNA genes very ideal (Pei et al., 2009). The 23S rRNA partial gene was amplified using 23S rRNA primers which were designed on NCBI site. All of isolates yielded a single-fragment about 700 bp as indicated in Figure 1. After amplification, the same primers were used for partial sequencing of 23S rRNA gene. The alignment was done for all isolates sequences with the reference 23S rRNA gene of *R. leguminosarum* (AF207785.1) published in NCBI (<http://www.ncbi.nlm.nih.gov/>). The genetic diversity was estimated based on the number of base pairs substitution per site among all isolates using mega program version 6 (Tamura et al., 2013) as shown in Table 2. The phylogenetic relationship analysis using UPGMA method, divided the isolates into two principal groups (Figure 2); the first group included the isolates Rlv6, Rlv8 and Rlv9, while the second group contained isolates of Rlv1, Rlv2, Rlv3, Rlv4, Rlv5, Rlv7, Rlv10, Rlv11, Rlv12 and Rlv13. Although, the tested isolates were collected from different geographic zones in Egypt, the values of genetic distances among these isolates were very low and

ranged from 0.00 to 0.04 (Table 2). This may be due to the high conserved nature of the 23S rRNA gene sequences (Pei et al., 2009), so the sequences variability is limited and, second, it is possible that these isolates have originated from the same genetic background and the human activities like soil and plant transfer limited the genetic diversity of these isolates (Ismail et al., 2013).

Analysis of drought tolerance

The rhizobial isolates were tested for their drought tolerance in YMA medium supplemented with increasing concentrations of PEG 6000 (10, 20 and 30%) and evaluated as tolerant and sensitive based on the number of colonies formed. In general, all isolates showed good drought-tolerant efficiencies at 10% PEG, while most of them could not tolerate up to 20% PEG (Table 3). These results are in agreement with a previous study which showed that most rhizobial strains, which nodulate important crops, are very sensitive to drought and abiotic factors such as high salt, pH, and temperature (Rehman and Nautiyal, 2002). The results showed that nine isolates (Rlv1, Rlv2, Rlv3, Rlv4, Rlv5, Rlv7, Rlv8, Rlv10 and Rlv11) could not tolerate 20% PEG concentration and were considered as sensitive to drought stress. In contrast, the isolates Rlv6 of Suez, Rlv9 of Rafah, Rlv12 of Cairo and Rlv13 of Ismailia succeeded to grow on 20% of PEG and are considered as tolerant. It was shown that

Table 2. Genetic distance divergence between isolates sequences based on base substitution, analysis conducted using the Maximum Composite Likelihood model using MEGA6 program (Tamura et al., 2013).

Isolate	Riv1	Riv2	Riv3	Riv4	Riv5	Riv6	Riv7	Riv8	Riv9	Riv10	Riv11	Riv12	Riv13
Riv1	0.00												
Riv2	0.01	0.00											
Riv3	0.00	0.01	0.00										
Riv4	0.01	0.01	0.01	0.00									
Riv5	0.00	0.01	0.00	0.01	0.00								
Riv6	0.04	0.04	0.04	0.03	0.04	0.00							
Riv7	0.00	0.01	0.00	0.01	0.00	0.04	0.00						
Riv8	0.04	0.04	0.04	0.03	0.04	0.00	0.04	0.00					
Riv9	0.04	0.03	0.04	0.02	0.04	0.01	0.04	0.01	0.00				
Riv10	0.00	0.01	0.00	0.01	0.00	0.04	0.00	0.04	0.04	0.00			
Riv11	0.01	0.00	0.01	0.01	0.01	0.04	0.01	0.04	0.03	0.01	0.00		
Riv12	0.01	0.02	0.01	0.02	0.01	0.03	0.02	0.03	0.02	0.01	0.01	0.00	
Riv13	0.00	0.01	0.00	0.01	0.00	0.04	0.00	0.04	0.04	0.00	0.01	0.01	0.00

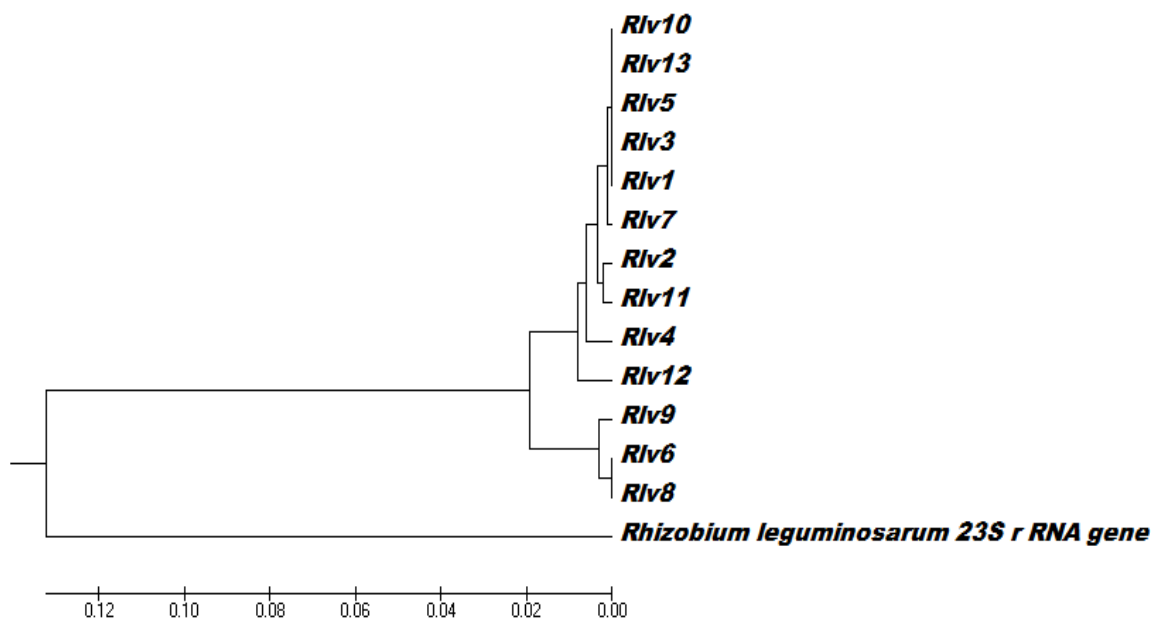


Figure 2. Phylogenetic relationship between 13 rhizobial isolates using UPGMA method and MEGA6 program based on 23S rRNA gene sequences data.

Table 3. Rhizobial isolates growth (Number of colonies) in YMA medium supplemented with increasing doses of PEG 6000 ranged between 10 to 30%.

Isolate	YMA medium supplemented with PEG 6000			
	Control (0%)	10%	20%	30%
Riv1	72.66 ^{de}	36.33 ^c	0.00 ^c	0.00
Riv2	111.33 ^{ab}	63.33 ^{ab}	0.00 ^c	0.00
Riv3	70.66 ^{de}	50.00 ^{bc}	0.00 ^c	0.00
Riv4	99.33 ^{bc}	54.00 ^{bc}	0.00 ^c	0.00
Riv5	92.33 ^{bcd}	59.66 ^{abc}	0.00 ^c	0.00
Riv6	112.66 ^{ab}	67.00 ^{ab}	2.33 ^b	0.00

Table 3. Contd.

Rlv7	57.66 ^e	47.00 ^{bc}	0.00 ^c	0.00
Rlv8	84.66 ^{cd}	56.00 ^{abc}	0.00 ^c	0.00
Rlv9	96.66 ^{bc}	56.33 ^{abc}	3.66 ^b	0.00
Rlv10	98.66 ^{bc}	68.00 ^{ab}	0.00 ^c	0.00
Rlv11	82.00 ^{cd}	53.66 ^{bc}	0.00 ^c	0.00
Rlv12	96.00 ^{bc}	63.33 ^{ab}	3.33 ^b	0.00
Rlv 13	131.00 ^a	80.00 ^a	8.00 ^a	0.00

* Means within classification followed by different letters are significantly different (Duncan, 0.05 level).

the isolate RLV13 of Ismailia gave the best growth in presence of PEG then the isolates; Rlv6 of Suez, Rlv9 of Rafah, Rlv12 of Cairo respectively Table 3. The failure of rhizobia isolates to grow at the highest doses of PEG is due to PEG reduces water availability by binding water molecules without penetrating the cell wall (Rehman and Nautiyal, 2002; Rasanen et al., 2004; Cytryn et al., 2007; Abdel-Salam et al., 2010). Moreover, the osmotic stress can affect negatively on Rhizobia by changing their morphology, survival in soil, root-hair colonization, infection and dehydration of cells (Niste et al., 2013). On the other hand, the success of isolates Rlv6, Rlv9, Rlv12 and Rlv13 to grow on 20% of PEG may be due to acquiring some mechanisms or pathways for drought tolerance through their growth in water limited soils. These results are supported with previous review suggesting that Rhizobia having some key tolerance mechanism/pathways against certain abiotic stresses (Gopalakrishnan et al., 2015) and with other observations illustrated the roles of rhizobia in adaptation of crops to various abiotic stresses (Yang et al., 2009; reviewed by Grover et al., 2010).

Conflict of interests

The authors have not declared any conflict of interest.

REFERENCES

- Abd El-Halim MM, Abdel-Salam MS, Ibrahim SA, Abd-El-Aal SK, Attallah AG (2001). Genetic improvement of *Bradyrhizobium japonicum* to tolerate abiotic stresses via protoplast fusion. *J. Agric. Sci. Mansoura Univ.* 26(9):5427-5436.
- Abdel-Salam MS, Abd El-Halim MM, Ibrahim SA, Bahy-Edin A, Abo-Aba SE (2002). Improvement of *Rhizobium leguminosarum* biov. *trifolii* competency via rhizobiotoxin - gene transfer. In: *Proceeding of the Second International Conference on Plants & Environmental Pollution*, 4-9 February, Luknow, India, pp. 153-160.
- Abdel-Salam MS, Ibrahim SA, Abd El-Halim MM MM, Badawy FM, Abo-aba SEM (2010). Phenotypic characterization of indigenous Egyptian Rhizobial strains for abiotic stresses performance. *J. Am. Sci.* 9:498-503.
- Athar M, Johnson DA (1996). Nodulation biomass production and nitrogen fixation in alfalfa under drought. *J. Plant. Nutr.* 19:185-199.
- Aydi SS, Aydi S, Gonzalez E, Abdely C (2008). Osmotic stress affects water relations, growth, and nitrogen fixation in *Phaseolus vulgaris* plants. *Acta Physiol. Plant* 30:441-449.
- Ben Romdhane S, Tajini F, Trabelsi M, Aouani ME, Mhamdi R (2007). Competition for nodule formation between introduced strains of *Mesorhizobium ciceri* and native populations of rhizobia nodulating chickpea (*Cicer arietinum*) in Tunisia. *World J. Microbiol. Biotechnol.* 23:1195-1201.
- Cytryn EJ, Sangurdekar DP, Streeter JG, Franck WL, Chang WS, Stacey G, Emerich DW, Joshi T, Xu D, Sadowsky MJ (2007). Transcriptional and physiological responses of *Bradyrhizobium japonicum* to desiccation-induced stress. *J. Bacteriol.* 89(19):6751-6762.
- Diouf D, Samba-Mbaye R, Lesueur D, Ba AT, Dreyfus B, de Lajudie P, Neyra M (2007). Genetic diversity of *Acacia seyal* Del. rhizobial populations indigenous to Senegalese soils in relation to salinity and pH of the sampling sites. *Microb. Ecol.* 3:553-566.
- Duzan HM, Zhou X, Souleimanov A, Smith DL (2004). Perception of *Bradyrhizobium japonicum* Nod factor by soybean [*Glycine max* (L.) Merr.] Root hairs under abiotic stress conditions. *J. Exp. Bot.* 408:2641-2646.
- Ehsanpour AA, Amini F (2003). Effect of salt and drought stress on acid phosphatase activities in alfalfa (*Medicago sativa* L.) explants under in vitro culture. *Afr. J. Biotechnol.* 5:133-135.
- El-Zanaty AF, Abdel-lateif K, Elsobky M (2014). Molecular identification of *Rhizobium* isolates nodulating Faba in Egyptian soils. *J. Bioprocess. Biotech.* 5:194.
- Fall DM, Ourarhi M, Missbah EN, Zoubeyrou BAM, Abelmoumem H (2011). The efficiency and competitiveness of three *Mesorhizobium* sp. strain nodulating *Acacia Senegal* (L.) Willd under water deficiency conditions in greenhouse. *Symbiosis* 54:87-94.
- Gálvez MD (2005). Nodule metabolism in *Pisum sativum* L. in response to water stress: carbon/nitrogen interactions and the possible molecules involved in the modulation of the response, Ph.D. thesis, Public University of Navarre.
- Gopalakrishnan S, Sathya A, Vijayabharathi R, Varshney RK, Gowda CLL, Krishnamurthy L System (2015). Plant growth promoting rhizobia: challenges and opportunities. *3 Biotech* 5(4):355-377.
- Grover M, Ali SZ, Sandhya V, Rasul A, Venkateswarlu B (2010). Role of microorganisms in adaptation of agriculture crops to abiotic stresses. *World J. Microbiol. Biotechnol.* 27:1231-1240
- Hewedy OA, Eissa RA, Elzanaty AM, Nagaty HH, Abd Elbary MI (2014). Phenotypic and Genotypic Diversity of *Rhizobia* Nodulating *Faba Bean* from Various Egyptian Locations. *J. Bioprocess. Biotech.* 4:170-178.
- Hunt DE, Klepac-Ceraj V, Acinas SG, Gautier C, Bertilsson S, Polz MF (2006). Evaluation of 23S rRNA PCR Primers for Use in Phylogenetic Studies of Bacterial Diversity. *Appl. Environ. Microbiol.* 72(3):2221-2225.
- Ismail M, El-Zanaty AM, Eissa RA, Hewedy OA (2013). Genetic Diversity of *Rhizobium leguminosarum* as Revealed by 16S rRNA Gene Sequence. *Am. Eurasian J. Agric. Environ. Sci.* 13:797-801.
- Kolbert CP, Persing DH (1999). Ribosomal DNA sequencing as a tool for identification of bacterial pathogens. *Curr. Opin. Microbiol.* 2:299-305.
- Meuelenberg F, Dakora FD (2007). Assessing the biological potential of N2-fixing leguminosae in Botswana for increased crop yields and commercial exploitation. *Afr. J. Biotechnol.* 4:325-334.

- Mhadhbi H, Jebara M, Zitoun A, Limam F, ME Aouani (2008). Symbiotic effectiveness and response to mannitol-mediated osmotic stress of various chickpea-rhizobia associations. *World J. Microbiol. Biotechnol.* 7:1027-1035.
- Niste M, Vidican R, POP R, Rotar I (2013). Stress Factors Affecting Symbiosis Activity and Nitrogen Fixation by Rhizobium Cultured *in vitro*. *ProEnvironment/ProMediu* 6:42-45.
- Pei A, Nossa CW, Chokshi P, Blaser MJ, Yang L, et al. (2009). Diversity of 23S rRNA Genes within Individual Prokaryotic Genomes. *PLoS. One* 4(5):e5437.
- Pulawska J, Maes M, Willems A, Sobiczewski P (2000). Phylogenetic Analysis of 23S rRNA Gene Sequences of *Agrobacterium*, *Rhizobium* and *Sinorhizobium* Strain. *Syst. Appl. Microbiol.* 23:238-244.
- Rasanen LA, Saijets S, Jokinen K, Lindstrom K (2004). Evaluation of the roles of two compatible solutes, glycine betaine and trehalose, for the *Acacia senegal*-*Sinorhizobium* symbiosis exposed to drought stress. *Plant Soil* 260(1-2):237-251.
- Rehman A, Nautiyal CS (2002). Effect of Drought on the Growth and Survival of the Stress-Tolerant Bacterium *Rhizobium* sp. NBR12505 *sesbania* and its Drought-Sensitive Transposon Tn 5 Mutant. *Curr. Microbiol.* 5:368-377.
- SAS (2004). User's guide: Statistics, version 9. 4th Ed. SAS Ins., Inc., Cary., NC, USA.
- Tamura K, Stecher G, Peterson D, Filipowski A, Kumar S (2013). MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. *Mol. Biol. Evol.* 30:2725-2729.
- Van Berkum P, Eardly BD (1998). Molecular evolutionary systematics of the *Rhizobiaceae*. In: *The Rhizobiaceae molecular biology of model plant-associated bacteria*. (H.P. Spaink, A. Kondorosi, P.J.J. Hooykaas, eds.) Kluwer Academic Publishers. pp. 1-24.
- Vriezen JAC, de Bruijn FJ, Nusslein K (2007). Responses of rhizobia to desiccation in relation to osmotic stress, oxygen, and temperature. *Appl. Environ. Microbiol.* 73:3451-3459.
- Wolde-meskel E, Terefe-work Z, Frostega A, Lindstro K (2005). Genetic diversity and phylogeny of rhizobia isolated from agroforestry legume species in southern Ethiopia. *Int. J. Syst. Evol. Microbiol.* 55:1439-1452.
- Yang J, Kloepper JW, Ryu CM (2009). Rhizosphere bacteria help plants tolerate abiotic stress. *Trends Plant Sci.* 14(1):1-4.
- Zahrán HH (1999). *Rhizobium*-legume symbiosis and nitrogen fixation under severe conditions and in an arid climate. *Microbiol. Mol. Biol. Rev.* 12:968-998.