International Journal of Agriculture, Environment & Biotechnology
Citation: IJAEB: 7(3): 491-498 September 2014
DOI Number: 10.5958/2230-732X.2014.01353.9

Microbiology

Biochemical Characterization and Molecular Fingerprinting of Plant Growth Promoting Rhizobacteria

Abdul Ahad[1], Md. Nadeem Akhtar[2], Pashupat Vasmatkar*[3] and Pratibha Baral[3]

[1]Department of Molecular Biology and Genetic Engineering, College of Basic Sciences and Humanities.
[2]Department of Plant Pathology, College of Agriculture.
[3]Department of Biochemistry, CBSH, G.B. Pant University of Agriculture and Technology, Uttarakhand, INDIA.

*Corresponding author: vpashupat@gmail.com

Paper No. 234 Received: May 9, 2014 Accepted: July 19, 2014 Published: September 20, 2014

Abstract

Plant growth promoting rhizobacteria (PGPR) are a heterogeneous group of bacteria that can be found in the rhizosphere, which can improve the extent or quality of plant growth directly or indirectly. However, screening strategies for selecting the best rhizobacterial strain for rhizosphere competence with other microbial species in the plant rhizosphere will require more comprehensive knowledge. In present investigation nine different strains were tested for their PGPR properties by using RFLP analysis on 16S rRNA gene or amplified rDNA restriction analysis (ARDRA). 16 S rDNA amplification was done and restriction profiling was done using two endonuclease i.e. msp1 and taq1. Depending upon banding pattern of all the nine strains dendogram was created using NTsys software. A clear cut difference was seen in genetic diversity among the strains. Pseudomonas was found to be the most effective strain among all. Depending upon the outcome we can conclude that ARDRA can be effective tool for analyzing the genetic diversity among different bacteria and PGPR starin e.g. Pseudomonas, Bacillus can be used as a potent biofertilizer.

Highlights

- Nine Bacterial strains were obtained from Rhizospheric lab and were molecular characterized and sequenced.
- ARDRA analysis of the diversity of strains revealed that strains are contained in two clusters,
- Isolate P3 (Pseudomonas fluorescence) was found to be most efficient PGPR which solubilized insoluble phosphorus, produced IAA, siderophore, amylase, lipase and catalase.
- A dendogram was constructed based on the restriction profile of nine strain restricted with Msp I and Taq I, revealed 14B, 8B, 9F and 13B had 100% similarity while strain 1B was placed distinctly in the tree (exhibiting less than 65% similarity).

***Keywords*:** *Pseudomonas*, PGPR, Siderophore, Phosphate Solubilisation.

Rhizospheric bacterial communities have efficient systems for uptake and catabolism of organic compounds present in root exudates. The Rhizospheric bacterium which is associated with growth promotion of plants called Plant growth promoting Rhizobacteria (PGPR) (Tilak *et al.,* 2005). PGPR are able to enhance the availability of

nutrients including N, P and other micronutrients. For example, Rhizobium spp. in symbiosis with their legume host plant, and Azospirillum in non-symbiotic association with their host plant, can fix atmospheric N2 (Miransari and Smith, 2008, Arzanesh *et al.,* 2010). PGPR including Bacillus spp. Pseudomonas fluorescence and P. putida are able to enhance P availability, by production of organic acids and phosphatase enzymes through producing siderophores, PGPR can also increase Fe solubility to the plant (Glick *et al.,* 1998; Haas and Defago, 2005; Jalili *et al.,* 2009; Zabihi *et al.,* 2010). The genetic diversity studies using methods such as enzyme electrophoresis (Young, 1985; Leung *et al.,* 1994), restriction fragment length polymorphism (RFLP) (Young and Wexler, 1988; Kaijalainen and Lindstroem, 1989; Demezas *et al.,* 1991), or polymerase chain reaction (PCR) based techniques (Harrison *et al.,* 1992; Amarger *et al.,* 1994; Leung *et al.,* 1994; Laguerre *et al.,* 1994Turner *et al.,* 1996) shows that PGPR have many common genetic and biochemical characteristics. The 16S ribosomal RNA genes are useful for such studies since these genes are present in all bacteria and contains both conserve regions which can be used for primer design for polymerse chain reaction (PCR) amplification and variable region which can be used to distinguish sequences from each other (Smith and Goodman, 1999). Restriction fragment length polymorphism (RFLP) analysis of amplified 16S rDNA gene for identification of plant growth promoting rhizobacteria (PGPR) has also been utilized by several workers for the identification of several novel species (Vanechoutte *et al.,* 1992). RFLP analysis on 16S rRNA gene or amplified rDNA restriction analysis (ARDRA) is a useful technique for genotype identification, to infer genetic variability and similarity of microorganisms (Yang *et al.,* 2007). Present study was conducted on 9 strains of Plant Growth Promoting Rhizobacteria (PGPR) with a focus on the analysis of their biochemical and functional assay as well as genetic diversity based on ARDRA and 16S rRNA sequences analysis, which work as molecular fingerprinting of a particular strain.

Materials and Methods

Isolation, Purification and maintenance of cultures

Nine bacterial strains were obtained from Rhizospheric lab (College of Basic Science and Humanatics Department) Gobind ballabh Pant University of agriculture and technology, Pantnagar, Uttarakhand by replica plating technique. On the basis of sequencing they were designated and presented in table 1. The technique involved the serial dilution and spreading on nutrient agar and incubated at 35ºC for 48h. Morphologically distinct and isolated bacterial colonies of rhizobacterial strain were pinched up and purified by repeated streaking on Nutrient Agar medium.

The pure cultures of each isolates was transferred on nutrient agar and stored at 4°C in refrigerator. For long-term preservation, glycerol stocks were prepared by adding 4.5ml of nutrient broth (full strength) into 10ml of half strength nutrient broth with log phase bacterial culture. One ml of which was preserved at -20°C as glycerol stock in cryovials.

Table 1. Name and designation of bacterial strain

Sr. No.	Designation	Name of bacterial strain
1	1B	*Bacillus cereus* 5507
2	4	*Bacillus subtilis*
3	14B	Bacillus cereus BSB 38
4	9F	Bacillus cereus JDM2^{-1}
5	8B	Bacillus cereus SND
6	13B	Bacillus cereus RSR
7	P3	Pseudomonas Fluorescence
8	8	Bacillus cereus PMV21
9	92	Bacillus cereus

Morphological characterization

An isolated bacterial colony was studied for margin, shape, elevation, surface, pigmentation, size, cell size, cell shape and arrangement.

Biochemical assay for plant Growth promoting traits

Indole acetic acid (IAA) production test

IAA production was detected by the modified method as descrbed by Bric *et al.,* (1991). Bacterial cultures were grown for 72h on their respective media at 35ºC. Fully grown cultures were centrifuged at 3000 rpm for 30 min. The supernatant (2ml) was mixed with two drops

Table 2. Morphology of bacterial isolates used in the study

Characters	Bacterial Strain								
	14B	**8**	**8B**	**1B**	**13B**	**P3**	**9F**	**92**	**4**
Colony Morphology									
Colony size	M	M	L	L	S	M	S	L	M
Margin	U	E	U	U	E	E	E	E	U
Surface	R	Sm	Wr	R	Sm	Sm	Sm	Sm	R
Elevation	Um	C	Um	Um	C	C	C	C	C
Chromogenesis	Cr	W	Cr	Cr	Cr	F	W	W	W
Form	I	Cir	I	I	Cir	Cir	Cir	Cir	I
Cell Morphology									
Gram reaction	+	+	+	+	+	-	+	+	+
Shape	Rod	Rod	Long Rod	Rod	Rod	Long Rod	Rod	Rod	Rod
Size	S	Vs	L	M	Vs	L	Vs	S	S
Arrangement	Sc	Sc	chain	Sc	Sc	Chain	Sc	Sc	Sc

+= Present, -= Absent, M= Medium, S= Small, L= Large, E = Entire, U= Undulated, R= Rough, Sm= Smooth, VS= Very Small, F= Florescent, Wr= Wrinkled, Cir= Circular, I= Irregular, W= white, Um= Umbonate, C= Convex, Sc= Scattered, Cr= cream, F= Florescent.

of orthophosphoric acid and 4 ml of the Salkowski agent, 35% perchloric acid, 1 ml of 0.5M Ferric chloride solution ($FeCl_3$ solution). Development of pink color indicates IAA production. Optical density was observed at 530 nm and compared with standard curve of IAA.

Siderophore production

The siderophore production was determined by Chrome Azurol S (CAS) assay and quantitatively in liquid medium as described by Schwyn and Neilands, (1987). Development of yellow-orange halo around the growth was considered as positive for siderophore production. In case of quantitative estimation absorbance was measured at 630 nm.

Phosphate solubilization

Bacterial isolates were first screened on Pikovskaya's agar plates for phosphate solubilization index as described by Gaur, (1990). On modified Pikovskaya agar with insoluble tricalcium phosphate (TCP), a loop full of each culture was placed on the centre of agar plates and incubated at 30±0.1°C for 5 days. The solubilization zone was determined by subtracting the diameter of bacterial colony from the diameter of total zone.

Catalase activity

Catalase activity test was performed by adding a drop of 3% hydrogen peroxide to 48 hr old bacterial colony on a clean glass slide and mixed using a sterile tooth-pick. The effervescence indicated catalase activity.

Lipase production

Lipase medium was used to check the lipase production, which was indicated by clear zones surrounding the colonies (Smibert and Krieg, 1994).

Extraction of Bacterial genomic DNA

The DNA of test isolates was isolated by using CTAB (Cetyl Trimethyl Ammonium Bromide) method. 5 µ of extracted DNA was electrophoresed on 0.8% agarose gel, with quantitative marker in one lane (Low DNA Mass Ladder,MBI Fermentas), stained with ethidium

bromide (0.5mg/ml) and the gel was examined on a Gel documentation system. The quantity and purity of DNA was checked by measuring the absorbance of purified DNA sample at 260 nm using spectrophotometer.

Amplification of 16S rDNA

Genomic DNA was used for 16S rDNA amplification. The PCR primers used were:

Gm 3f 5' AGA GTT TGA TCM 3' (8 to 23 *E.coli*)
Gm 4r 5' TAC CTT GTT ACG ACT T3' (1492 to 1507 E.coli)

Phylogenetic Analysis

The bands generated by digestion of PCR amplified 16S rDNA with MspI and Taq I were used to construct a UPGMA dendrogram using NTSYS pc version 2.02i computer programme calculating Euclidean coefficient.

Results and Discussion

Morphological characterization

Out of nine test isolates eight isolates were gram positive and one was gram negative. Colony morphology of bacterial strain was studied by growing them on Nutrient Agar plate medium at 30°C. After 24 hr of incubation, observations on colony morphology, margin, surface, elevation, pigmentation etc. were reported (Table-2).

Biochemical assay for plant Growth promoting traits

Among the nine PGPR strains grown in culture medium amended with tryptophan, strains produced IAA as detected by the Salkowski reagent. The highest concentration of IAA was observed from PGPR strain P3 (33µg/ml) followed by 8B (16µg/ml). Lowest concentration of IAA was produced in 13B (5µg/ml). (Fig. 1 and Table 3).

All Strain showed siderophore production by producing orange halo zones around the colonies against blue background except strain 92 when grown on CAS agar plates (Fig.2). All strains of bacillus were found to be positive with the exception of strain 8 which show negative test for P soliblization and one pseudomonas strain was also found to be positive (Fig.3).

Bacterial strain 14B, 13B, 1B and P3 in the present study were able to produce catalase. Bacterial strains showing catalase activity must be highly resistant to environmental, mechanical and abiotic stress stress. Air bubbles are produced after adding H2O2 in actively growing bacterial culture in tryptone broth in a positive catalase test. Only P3 showed positive results while others were negative for catalase enzyme.

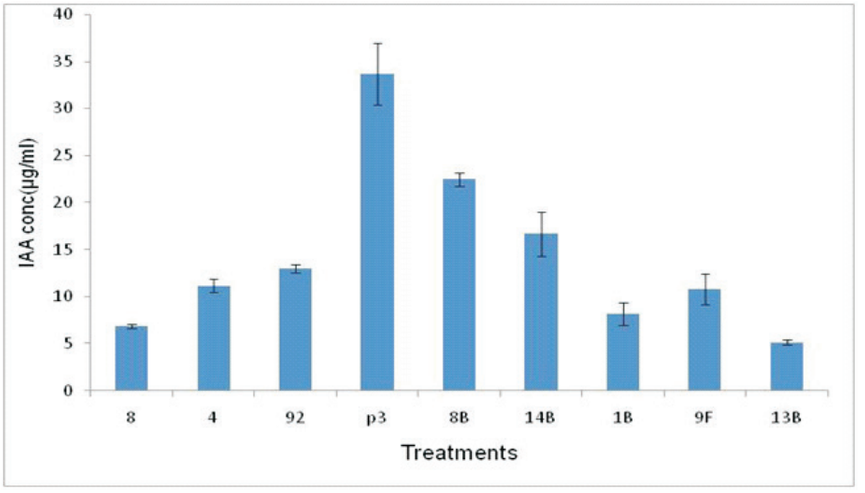

Fig. 1. IAA Production

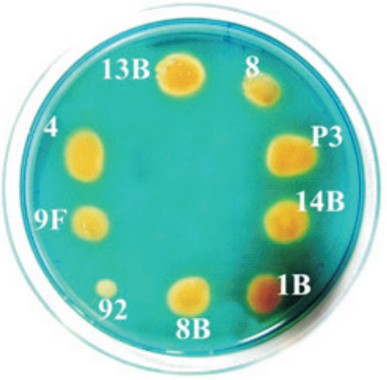

Fig. 2. Siderophore production

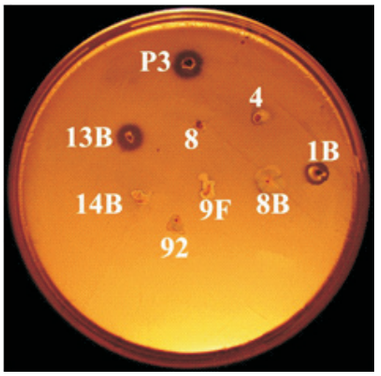

Fig. 3. Phosphate Solubilization

Table 3. Biochemical assay of the bacterial strain Properties

Sr. No	Assay	Bacterial Strain								
		14B	13B	1B	P3	92	9F	8B	8	4
1)	IAA Production	+	+	+	+	+	+	+	+	+
2)	Siderophore production	+	+	+	+	–	+	+	+	+
3)	P-Solubilization	+	+	+	–	–	–	–	–	–
4)	Catalase	–	–	–	+	–	–	–	–	–
5)	Lipase	–	–	–	+	–	+	–	–	+

+= Present,–= Absent

Strain 9F, P3 and 4 showed positive test for lipase production. Rest all showed negative test for lipase production and did not show white zone around growth.

Molecular Phylogenetic characterization

Phylogenetic analysis using ARDRA

Amplified 16S rDNA was restricted with enzyme Msp I and Taq I. Restriction profile was interpreted on the basis of bands developed (Fig. 4a and b). DNA bands were scored as 1 (present) and 0 (absent). All intense and reproducible bands were considered. Further this pattern was used to form similarity indices by UPGMA using NTSYpc software.

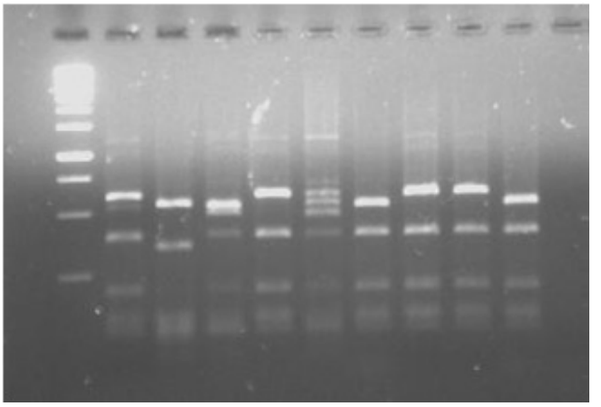

Fig. 4a. Restriction profile of strains with Msp I

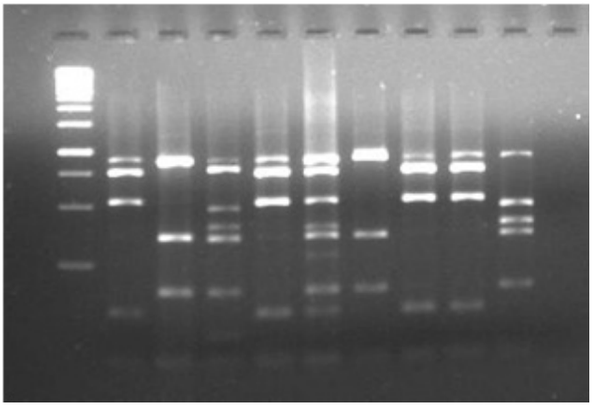

Fig. 4b. Restriction profile of strains with Taq I.

A dendogram was constructed based on the restriction profile of nine strain restricted with Msp I and Taq I (Fig.5a and b). Bacterial strains were grouped, according to the similarities of the restriction patterns obtained with the two endonucleases (TaqI andMspI), into 9 ARDRA types. Data analysis showed that the isolates could be assigned to two major groups (i) ARDRA types 14B 8B 9F 13B 1B (ii) ARDRA types P3 92 4 8. Starins, 14B, 8B, 9F and 13B showed 100% similarity while strain 1B was placed distinctly in the tree (exhibiting less than 65% similarity).In second ARDRA type, P3 and 92 showed 100% similarity and placed in the same cluster while strain 4 showed less than 90% similarity in this cluster and strain 8 was placed distinctly compared to this cluster and showed less than 70% similarity.

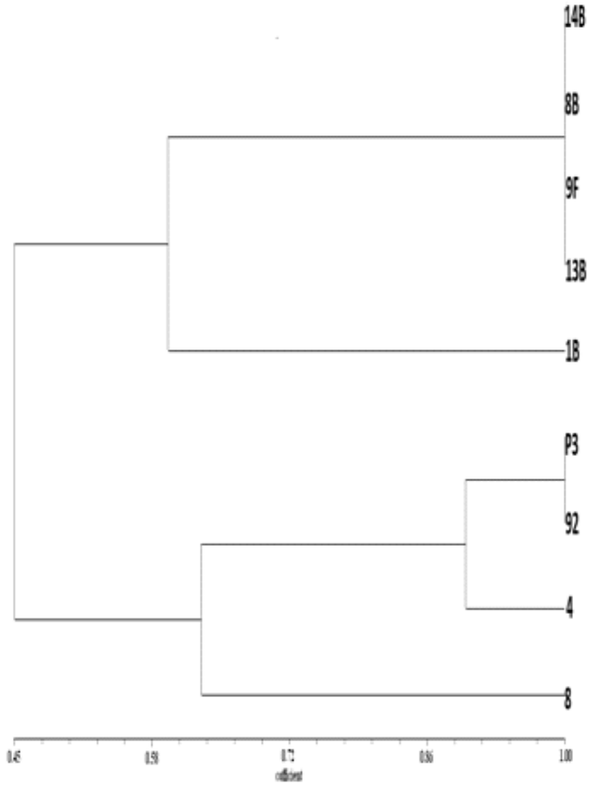

Fig. 5a. Dendogram of strains Basd on Msp I

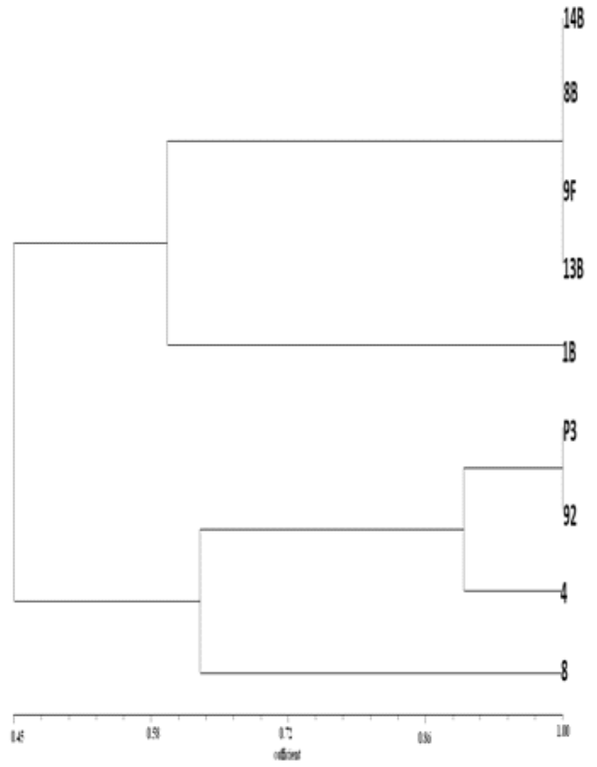

Fig. 5b. Dendogram of strains Basd on Taq I

PGPR colonize plant roots and exert beneficial effects on plant growth and development by a wide variety of mechanisms. To be an effective PGPR, bacteria must be able to colonize roots because bacteria need to establish itself in the rhizosphere at population densities sufficient to produce the beneficial effects. The exact mechanism by which PGPR stimulate plant growth is not clearly established, although several hypotheses such as production of phytohormones (e.g IAA), suppression of deleterious organisms(e.g HCN,siderophores), activation of phosphate solubilization and promotion of the mineral nutrient uptake are usually believed to be involved in plant growth promotion (Glick, 1995; Lalande *et al.,* 1989). There are many papers related to the advantages and screening of PGPR from crop plants. Bacterial strain were screened for different plant growth promotion activities and characterized by biochemical tests. Bacterial strain 14B, 13B, 1B and P3 in the present study were able to produce catalase. Bacterial strains showing catalase activity must be highly resistant to environmental,

mechanical and abiotic stress stress. A number of studies suggest that PGPR enhances the growth, seed emergence, crop yield, and contribute to the protection of plants against certain pathogens and pests (Kloepper, 1980; Ryu and S. Zhang, 2004; Minorsky, 2008). In the present study isolate P3 was found to be most efficient PGPR which solubilized insoluble phosphorus, produced IAA, siderophore, amylase, lipase and catalase. Such type of study is necessary as it advocates that use of PGPR as inoculants or biofertilizers is an efficient approach to replace chemical fertilizers

ARDRA analysis of the diversity of strains revealed that strains are contained in two clusters, in one cluster strains 14B, 8B, 9F and 13B are placed together which show 100% similarity at molecular level even though they exhibit variability in their biochemical and functional characterization, and 1B strain in this group showed less than 65% similarity as compared to these strains and showed the genetic diversity, exhibit some common characterization. In other cluster strains P3 (pseudomonas spp.), 92 (bacillus spp.) showed 100% similarity, it may be happen because in this investigation only two endonuclease enzymes are used which may be find same restriction site at rDNA in both different species. In second group strain 8 showed diversity while exhibit some common characterization regarding strains.

Conclusion

It is becoming increasingly apparent that most of the PGPR strains can promote plant growth by several mechanisms, though most studies currently focus on individual mechanisms and have not yet been able to sort out the relative contributions of different processes that are also responsible for successful plant growth promotion. However, carefully controlled field trials of crop plants inoculated along with rhizobacteria are necessary for maximum commercial exploitation of PGPR strains.

Acknowledgements

We are extremely thankful to the Department of Science and Technology for providing necessary financial assistance to carry out this research work.

References

Amarger, N., Bours, M. Revoy, F.M. Allard, R. and Leguerre, G. 1994. Rhizobium tropici nodulates field grown Phaseolus vulgaris in france. *Plant Soil* **161**: 147-156.

Arzanesh, M.H., Alikhani, H.A. Khavazi, K. Rahimian H.A. and Miransari, M. 2010. Wheat (Triticum aestivum L.) growth enhancement by Azospirillum spp. under drought stress. *World Journal of Microbiology and Biotechnology* in press.

Demezas, D. H., Reardon, T. B., Watson, J. M. and Gibson, A. H. 1991. Genetic diversity among Rhizobium leguminisarum bv. Trifolli strains revealed by allozyme and restriction fragment length polymorphism analysis. *Applied Environmental Microbiology* **57**: 3489-3495.

Glick, B. R. 1995. The enhancement of plant growth by free living bacteria. *Microbiology* **41:**109-117.

Glick, B.R., Penrose, D.M. and Li, J. 1998. A model for the lowering of plant ethylene concentrations by plant growth promoting bacteria. *Journal of Theoretical Biology* **190**: 63 -68.

Haas, D. and Défago, G. 2005. Biological Control of soil borne pathogens by fluorescent pseudomonads. *Nature Reviews Microbiology* **3**: 307-319.

Harrisom, S. P., Mytton, L. R., Skot, L., Dye., M. and Cresswell, A. 1992. Characterization of rhizobium isolates by amplification of DNA polymorphism using random primer. *Canadian Journal of Microbial Ecology* **38**: 1009-1051.

Jalili, F., Khavazi, K., Pazira, E. and Nejati, A. Asadi Rahmani, H., Rasuli Sadaghiani, H. and Miransari, M. 2009. Isolation and characterization of ACC deaminase producing fluorescent pseudomonads, to alleviate salinity stress on canola (Brassica napus L.) growth. *Journal of Plant Physiology* **166**: 667-674.

Kaijalainen, S. and Lindstrom, K 1989. Restriction fragment length polymorphism analysis of rhizobium galegae strains. *Journal of Bacteriology* **171**: 5561-5566.

Kloepper, J. W., Ryu, C. M. and Zhang S. 2004. Induced systemic resistance and promotion of plant growth by Bacillus spp. *Phytopathology* **94**: 1259-1266.

Laguerre, G., Allard, M. R., Revoy F. and Amerger, N. 1994. Rapid identification of rhizobia by restriction fragment length polymorphism analysis of PCR-Amplified 16S as ribosomal-RNA gene. *Applied Environmental Microbiology* **60**: 56-63.

Lalande, R., N. Bissonnette, D. Coutlée, H. Antoun. 1989. Identification of rhizobacteria from maize and determination of their plant-growth promoting potential. *Plant Soil* **115**: 7-11.

Leung, K., Strain, S. R., De Bruijn, F. J. and Bottomley, P. J. 1994. Genotypic and phenotypic comparison of chromosomal types within an indigenous soil population of Rhizobium leguminosarum bv. Trifolii. *Applied Environmental Microbiology* **60**: 416-426.

Miransari, M., and Smith, D.L. 2008. Using signal molecule genistein to alleviate the stress of suboptimal root zone temperature on soybean-Bradyrhizobium symbiosis under different soil textures. *Journal of Plant Interactions* **3**: 287-295.

Smith KP, Goodman RM, 1999. Host variation for interactions with beneficial plant-associated microbes. Annual Review of Phytopathology **37**: 473-491.

Tilak, KVBR., Ranganayaki, N. Pal, KK. De R, Saxena, AK. Nautiyal, CS. Mittal, S. Tripathi, AK. Johri, BN. 2005. Diversity of plant growth and soil health supporting bacteria. *Current Science* **89** (Suppl 1): 136-150.

Turner, S. L., Rigottier-gois, L., Power, R. S., Amarger, N. and Young, J. P. W. 1996. Diversity of repC plasmid-replications sequence in Rhizobium legumenosarum. *Micribiology* **142**: 1705-1713.

Vaneechoutte, M., Possau, R., Devos, P., Gills, M., Janssens, d., Paepe, J., Rouck, A., Fiers, T., Clacys, G. and Kersters, K. 1992. Rapid Identification of the bacteria of the Comanonadacea with Amplified Ribosomal DNA restriction analysis (ARDRA). FEMS Microbiology Letter **93**: 227-234.

Yang, JH., Liu, HX., Zhu, GM., Pan, YL., Xu, LD. and Guo, JH. 2007. Diversity analysis of antagonists from rice-associated bacteria and their application in biocontrol of rice diseases. *Journal of Applied Microbiology* **104**: 91-104.

Young, J. P. W. 1985. *Rhizobium* population genetics –enzyme polymorphism in isolates from pea's clover, beans and Lucerne grown at the same site. *Journal General Microbiology* **131**: 2399-2408.

Young, J. P. W. and Wexler, M. 1988. Sym plasmid and chromosomal genotype are correlated in population of Rhozobium leguminosarum. *Journal General Microbiology* **134**: 2731-2739.

Zabihi, H.R., Savaghebi, G.R., Khavazi, K., Ganjali, A. and Miransari, M. 2010. Pseudomonas bacteria and phosphorous fertilization, affecting wheat (Triticum aestivum L.) yield and P uptake under greenhouse and field conditions. Acta Physiologiae Plantarum in press.

Ryu, C-M., Farag, M.A., Hu, C-H., Reddy. M.S., Wei, H.X., Pare, P.W. and Kloepper, J.W. 2003. Bacterial volatiles promote growth in Arabidopsis. *Proceedings of the National Academic of Sciences* **100**: 4927–4932.

Minorsky, P.V. 2008. On the inside. *Plant Physiology* **146**: 323-324.

Gordon, S.A. and Paleg, L.G. 1957. Observations on the quantitative determination of indoleacetic acid. *Physiologia Plantarum* **10**: 39-47.

Schwyn, B., and Nellands, J.B. 1987. Universal chemical assay for the detection and determination of siderophores. *Analytical Biochemistry* **160**: 47-56.

Gaur, A.C. 1990. Phosphate solubilizing microorganisms as biofertilizers. *Omega Scientific Farmer's Digest* **35**(5): 32.

Smibert, RM. And Krieg, NR. 1994. Phenotypic characterization. In: Methods for General and Molecular Biology, ed. by P.R.G. Gerhardt, E. Murray, W.A. Wood, and N.R. Krieg, pp. 607–654. Washington, DC: American Society for Microbiology.