

## MOLECULAR AND BIOCHEMICAL CHARACTERIZATION OF RHIZOBIA FROM CHICKPEA (*Cicer arietinum*)

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There are various mechanisms through which plant's growth and yield can be promoted by plant growth promoting rhizobacteria (PGPR) that reside inside the soil. The use of pesticides, chemical fertilizers and other supplements could be replaced by PGPR. There are various substances produced by the rhizosphere microorganisms that are important for growth promotion as they affect morphology of plants indirectly. Their nodulation activity, biofilm formation, root colonization were observed and characterized for solubilization of phosphate, production of indole acetic acid and fixation of nitrogen. It was found that ten out of seventeen isolates from chickpea had positive nitrogen fixing activity but at varying levels and the range of fixed nitrogen was 136.666 to 303.666 nmoles C<sub>2</sub>H<sub>2</sub> reduced/h/mg protein. Bacterial isolate CM1 showed maximum nitrogen fixation ability. Thirteen isolates produced IAA in the range of 3.833-18.100 µg/mL. Bacterial isolate, CM1 produced maximum indole acetic acid. Among chickpea bacterial isolates, sixteen were positive in phosphate solubilizing ability while only one was negative. Range of phosphate solubilization among chickpea isolates was 4.533-12.333µg/mL. Maximum value for phosphate solubilization was observed in CM4. Potential root nodulating bacteria were identified using *16S rRNA* sequence analysis. CM1, CM2, CM3, CM5, CM6, CM11, CM15, CM24 and CM25 were identified as *Mesorhizobium ciceri* while CM4 as *Mesorhizobium mediterraneum*.

**Keywords:** PGPR, Chickpea rhizobia, *Mesorhizobium*, mutualism, Indole Acetic Acid (IAA), Nitrogen fixation

### INTRODUCTION

Chickpea (*Cicer arietinum* L.) being a pulse crop belongs to the family *Leguminosae* and is globally ranked at third position after dry beans (*Phaseolus vulgaris* L.) and dry peas (*Pisum sativum* L.). It is highly self-pollinated rabi crop with less than 1% cross pollination (Smithson *et al.*, 1985; Singh, 1987). Major chickpea producing countries include India, Pakistan, Mexico, Turkey, Canada and Australia. Chickpea contributes more than 20% of world pulse production (Anonymous, 2014). The per capita availability of chickpea per day across the world is 3.4g whereas in Pakistan it is 16.23g. Pakistan ranks second in area and third in chickpea production worldwide (FAO, 2014). The area under cultivation of chickpea is 949.6 thousand hectares with a yield of 399 thousand tons which share approximately 4% of national economy (Anonymous, 2014). Among pulses, chickpea contributes about 70% of the pulse crops, 10.2% in area and 5.8% of crops production in Pakistan (Khan *et al.*, 1991). The nutritive value of chickpea is increased after dehulling *i.e.* 25.3 to 28.9% protein contents (Hulse, 1991; Muehlbauer and Rajesh, 2008). Chickpea also contains 0.3% phosphorus, 3% ash, 4.8-5.5% oil, 0.2% calcium 38-59% carbohydrates and 3% fiber. In chickpea, the digestibility of carbohydrates and proteins ranges from 76 to 78% and from

57 to 60% respectively (Hulse, 1991; Huisman and Vanderpoel, 1994).

Chickpea is considered as the best cool season crop in semi-arid regions of India and Pakistan. The optimum conditions required for the growth and production of chickpea include temperature ranges from 18 to 26°C day and 21 to 29°C night (Duke, 1981; Muehlbauer *et al.*, 1988).

Chickpea is very important to ensure productivity of cropping systems by fixing atmospheric nitrogen. Rhizobia lives on the nodules which are present on the roots of this crop and these bacteria have explicit roles in converting atmospheric nitrogen into a plant accessible form via biological nitrogen fixation (BNF). By this procedure, a significant amount of nitrogen is deposited in the soil free of cost which can be used by the same crop and the subsequent ones. The efficacy of such crops in maximum nitrogen fixation mainly depends upon cultivar, number of nodules and the competent bacterial strain present in root nodules. Major constraints in achieving maximum chickpea production are severe climatic circumstances, poor soils, weeds, insufficient moistness and inadequate or no application of fertilizers (Aslam *et al.*, 1997). Typically, farmers prefer not to use any chemical fertilizers for the growth of chickpea and other grain legumes. Furthermore, the consequences of harsh climate on chickpea results in low

Rhizobial populations which ultimately leads to low yields (Aslam *et al.*, 1997).

Rhizobia are bacteria present in soil and act as small factories to fix the atmospheric nitrogen. They are known by their ability to infect roots of the legume (*Fabaceae*) crops. An outgrowth is produced by rhizobia termed as nodule. Formerly, rhizobia were included in these genera *Allorhizobium*, *Azorhizobium*, *Bradyrhizobium*, *Ensifer*, *Mesorhizobium* and *Rhizobium*. But currently it is discovered that rhizobia is paraphyletic group, and are categorized into proteobacteria classes *i.e.* beta, alpha and gamma (Benhizia *et al.*, 2004; Angus and Hirsch, 2010). The atmospheric nitrogen is fixed by biological nitrogen fixation process and because of this, rhizobia are known as symbionts of legume crops. In initial phases of symbiosis, bacteria produce nod factors and plant releases flavonoids, after recognition, process of nodulation starts (Oldroyd, 2013). The atmospheric nitrogen is metabolized and converted into different nitrogenous compounds by rhizobia so that plants can easily uptake these compounds; frequently through particular structures *i.e.* nodules. The production of nitrogen fertilizers involves large amount of energy from fossil fuels. By the interaction of rhizobia and legume, not only legume crops take benefit but there are subsequent positive effects for other crops as well (Lupwayi *et al.*, 2004).

Rhizobia is symbiotic in nature and belongs to family *Rhizobiaceae*, it fixes 50-100 kg/ ha atmospheric nitrogen in association with legume plants. It is valuable for pulses, pea, chickpea, lentil, groundnut, soybean etc. The successful nodulation of leguminous plants by Rhizobia principally depends on the availability of suitable strains for specific legumes. The roots of particular legumes are colonized by Rhizobia in order to form tumor like progressions termed as root nodules, which serve as workshops for production of ammonia. Rhizobia has capability of nitrogen fixation in mutual association with legume and some non-legume crops like *Parasponia*. Presence or absence of legume crops in field can increase or decrease rhizobia population in the soil. The population of active strains of the rhizobia located close to the rhizosphere can be restored by artificial seed inoculation to accelerate the nitrogen fixation. All legumes require specific species of rhizobia to produce efficient nodules (Venkateswarlu, 2008). Bacteria possess ribosomal RNA (rRNA) cistron comprised of three genes: the small subunit *16S*, and large subunits *23S* and *5S*. Phylogenetic studies can be done by a genetic marker known as *16S rRNA*. Relationship between closely related groups, such as species or genera can be determined by sequence information which is widely regarded as one of the most valid criteria (Litvaitis, 2002; Casamatta *et al.*, 2005; Rajaniemi *et al.*, 2005; Svenning *et al.*, 2005).

The purpose of this study was to isolate and identify the best rhizobial strain to improve growth and yield of chickpea

(*Cicer arietinum*). Characterization was performed to determine the growth promoting attributes of the isolates.

## MATERIALS AND METHODS

The proposed research was conducted at the Center of Agricultural Biochemistry and Biotechnology (CABB) and Institute of Soil and Environmental Sciences, (ISES), University of Agriculture, Faisalabad, Pakistan. A part of the study was also conducted at the Integrative Biology Department, University of California - Berkeley, Berkeley, CA, USA.

**Isolation of bacteria from root nodules:** Chickpea plants were grown in the field and bacteria were isolated four weeks after germination at nodulation period. Extra soil was removed from the roots by extensive washing with sterile distilled water and 10 nodules were removed from the roots using sterilized forceps. For further sterilization, nodules were dipped in ethanol for 1 min and subsequently in 3% calcium hypochlorite solution for 5 min. Nodules were washed five times with sterile water. To ensure nodule's surface sterilization, they were incubated  $28\pm 2^{\circ}\text{C}$  for 72 h on yeast extract mannitol (YEM) agar medium on plates (Vincent, 1970). Nodules were crushed in 20 $\mu\text{L}$  sterile distilled water after surface sterilization and this suspension was spread in a Petri dish and further streaking was done on YEM plates having Congo red and incubated at  $28\pm 2^{\circ}\text{C}$ . Based on the morphology, different colonies were picked and further streaked until the cultures were purified completely (Marsudi *et al.*, 1999). These pure cultures were placed at  $-80^{\circ}\text{C}$  in 50% glycerol. Suspensions of bacterial cultures were prepared in saline solution and a single drop was observed under light microscope to determine the size and shape of cells. Gram's staining was performed following the protocol by Vincent (1970).

**Nodulation assay:** Nodulation assays with slight modifications were conducted using the protocol described by Hameed *et al.* (2004). Bacteria were grown separately in YEM broth and maintained at  $10^6$  cfu mL $^{-1}$ . Chickpea seeds were surface sterilized using 0.1% mercuric chloride for 5 min and thoroughly washed with autoclaved water. Seeds were germinated on moist filter paper in sterile petri plates at  $25\pm 2^{\circ}\text{C}$  under dark. Germinated seedling of 1 cm length was treated with each bacterium inoculum separately. Assemblies of magenta boxes having 1:1 v/v vermiculite-perlite were sterilized and seedlings were transplanted. Day and night temperature was  $30\pm 2^{\circ}\text{C}$  and  $20\pm 2^{\circ}\text{C}$  respectively (Fraile *et al.*, 1988). Nitrogen free Hoagland solution of quarter strength was given to the plants (Arnon and Hoagland, 1940). Plants were examined after 55 days for nodulation and data were collected.

**Characterization of selected strains:** Characterization of bacterial isolates was performed to determine their biochemical characteristics

**Nitrogen fixation:** Nitrogenase activity was assessed using H<sub>2</sub> -flame ionization detector and Porapak Q column on a gas chromatograph (Thermoquest, Trace G.C, Model K, Rodono Milan, Italy), by the procedure of acetylene reduction assay (Hardy *et al.*, 1968). A single colony of each bacterial isolate was inoculated in 15 mL vials of nitrogen free media (5 mL) and incubated at 28±2°C for 48 h to assess the nitrogen fixing ability of bacterial isolates (Okan *et al.*, 1977). Vials were injected with 10% acetylene (v/v). Analysis of gas samples (100 µL) was conducted using gas chromatograph (Thermoquest, Trace G.C, Model K, Rodono Milan, Italy) after 16h incubation at room temperature using H<sub>2</sub> -flame ionization detector and Porapak Q column (Hameed *et al.*, 2004).

**Indole acetic acid production:** As an IAA precursor, tryptophan (100 mg L<sup>-1</sup>) was added as a supplement in YEM broth that contained bacterial isolates in order to detect and quantify production of IAA. Fe-HClO<sub>4</sub> and Fe-H<sub>2</sub>SO<sub>4</sub> reagents that produce pink color were used to assess qualitative IAA production in bacterial cultures after one week (Gordon and Weber, 1951). Ethyl acetate oxidation protocol was followed to quantify IAA (Tien *et al.*, 1979) using HPLC (Perkin Elmer, USA).

**Phosphate solubilization:** Single colony of each tested bacterial isolate was spotted on the center of Pikovskaya's plate containing tricalcium phosphate, (Pikovskaya, 1948) and incubated at 25±2°C for 6-9 days. Clear zone of phosphate solubilization around colonies was observed on the plates. Method of Phospho-molybdate blue color was used for the quantification of phosphate solubilization (Nair *et al.*, 2007) using spectrophotometer (Campec M350 Double Beam, USA).

**Microtiter plate biofilm formation assay (Abiotic surface):** With some modifications, microtiter plate assay described by Fujishige *et al.* (2006) was used to study biofilm formation on abiotic surface. Bacterial isolates were grown in YEM broth to optical density 2.0 at λ600 nm (OD<sub>600</sub>) and centrifuged for 2 min at 8,000 rpm and the pellets were washed with sterile distilled water. Cells were again suspended in YEM broth and their optical density was maintained 0.2 at OD<sub>600</sub>. In polyvinyl chloride plate of 96 wells, 150 µL of bacterial culture were added in separate wells (Fisher, USA) and un-inoculated YEM broth was used as control. For fast growing bacteria, plates were incubated at 28°C for 24 h while slow growing bacteria were incubated for 72 h. Medium was discarded and sterilized distilled water was used to wash the wells after incubation. Plates were dried, and wells were treated with 150 µL of crystal violet (0.001%) for 15 min. Plates were washed with sterile distilled water to remove the excessive dye from the wells. Finally, ethanol 95% (150 µL) was added in the wells and the quantification was done on plate reader by measuring absorbance at 570 nm.

**Root biofilm formation assay (Biotic surface):** Biofilm formation was also conducted on roots. For this purpose plants were harvested 10 days after germination and 1.5 cm pieces of roots were cut and placed in microtiter plate of 96 wells. Bacterial isolates (200µL) maintained at OD<sub>600</sub> = 0.2 were poured in the wells. For fast growing bacteria, plates were incubated at 28°C for 24 h while slow growing bacteria were incubated for 72 h. After incubation, pieces of roots were washed thoroughly and suspended in 1mL sterile distilled water. From the root surface, bacterial biofilms were removed and vigorously shaken in sterile distilled water and 100 µL suspension was cultured on YEM agar plates and colony forming units (cfu) were counted after 5 days as cfu 0.1 mg<sup>-1</sup> root.

**Molecular identification of bacteria:**

**DNA Extraction:** Alkaline lysis method was used to isolate genomic DNA with minor changes (Maniatis *et al.*, 1982). Bacteria were grown at 28±2°C for 4-5 days in YEM broth with constant shaking. Centrifuged for 10 minutes at 5,000 × g in eppendorf tube, 500 µL TE buffer containing 5 µL RNAase (10 mg mL<sup>-1</sup>) and 100 µL lysozyme (15 mg mL<sup>-1</sup>), were added to the pellet. The suspension of cell was incubated at 37°C for 30 min and 10% SDS (30 µL) was added and again incubated at 70°C for 20 min. Proteinase K 10µL (20 mg mL<sup>-1</sup>) was added to this suspension and incubated at 45°C for 2 h. Centrifuged at 13,000 × g for 10 min and extracted twice with (25:24) phenol/ chloroform followed by twice extractions with (24:1) chloroform/ isoamyl alcohol. Na-acetate (3M, pH 5.2) 1/10 (v/v) isopropanol of 0.6 (v/v) were added and incubated for 1h at -20°C. Centrifuged at 13,000 × g for 10 min, washed pellet with 70% ethanol, dried and suspended in 50µL TE. Quantity and quality of isolated DNA was determined by spectrophotometer and gel electrophoresis.

**PCR Primers, amplification and profile:** Gene specific primers fD1-5'-AGAGTTTGATCCTGGCTCAG-3' and rP2-5'-ACGGCTACCTTGTTACGACTT-3' (Weisburg *et al.*, 1991) were used to partially amplify *16S rRNA*. Amplification of gene fragments was done in a PCR thermocycler in 25 µL reaction mixture. The reaction was carried out in 1 X PCR buffer that contained 25ng DNA, 0.4 µM each primer, 10mM of dNTP (Fermentas), 1.5mM of MgCl<sub>2</sub> and 1.25U of *Taq* DNA Polymerase (Fermentas). The reaction was carried out under the following conditions: 5 min denaturation at 95°C; then 30 cycles of 30 sec at 95°C, 30 sec at 56°C and 30 sec at 72°C with a final extension at 72°C for 10 min in a Gene Amp PCR system 9700 cycler (Perkin Elmer, New Jersey, USA).

**Electrophoresis:** Amplified PCR products were resolved in 0.9% agarose gel containing 2µL of ethidium bromide (20 mg L<sup>-1</sup>) in 0.5 X TBE (Trisborate-EDTA) buffer. DNA ladders 1kb (GeneRuler, Fermentas) was used as size marker. Gels were visualized under UV light and photographed.

**Purification of PCR product, sequencing and analysis:** Partial *16S rRNA* fragments amplified were purified using PureLink™ Quick Gel Extraction Kit (Invitrogen) and QIAquick PCR purification kit (QIAGEN) as described in manufacturer's protocol. Purified PCR products were sequenced from UC Berkeley sequencing facility using Big Dye Terminator v 1.1 Cycle Sequencing Kit. The comparison of gene sequences with related sequences was done in GenBank databases using NCBI, BLAST (Altschul *et al.*, 1990) at <http://www.ncbi.nlm.nih.gov/blast/Blast.cgi> and ClustalW2 software.

## RESULTS

**Isolation of bacteria:** Nodules were collected from the research area Institute of Soil and Environmental Sciences (ISES), University of Agriculture, Faisalabad, Pakistan. Based on the colony morphology, seventeen bacterial isolates were selected from chickpea nodules. It was observed that bacterial colonies were different in texture, colors and shapes. Characterization of the colonies based on morphology is presented in (Table 1). It was observed that all bacteria were motile under light microscope with variable movements. Gram's reaction was conducted as well that showed gram negative nature of all isolates (Table 1).

**Biochemical characterization:** Nitrogen fixing ability of isolates based on acetylene reduction assay (ARA) was assessed using gas chromatograph and ten out of seventeen bacteria had positive nitrogen fixing ability at varying levels. Nitrogen fixation range among the bacterial isolates was 136.666 to 303.666 nmoles C<sub>2</sub>H<sub>2</sub> reduced/h/mg protein. Maximum nitrogen fixing efficiency was observed in CM1,

303.666 nmoles C<sub>2</sub>H<sub>2</sub> reduced/h/mg protein followed by CM2 (257.0), CM6 (236.666), CM24 (220.666), CM15 (206.666), CM4 (204.0), CM5 (200.333), CM25 (174.0) while CM11 fixed least nitrogen 136.666 nmoles C<sub>2</sub>H<sub>2</sub> reduced/h/mg protein (Table 2).

Among all isolates, thirteen produced IAA while other four did not. IAA production range was 3.833-18.100 µg/mL. Bacterial isolate, CM1 produced maximum indole acetic acid (18.100 µg/mL) followed by CM2 (15.300 µg/mL), CM3 (12.700 µg/mL), CM4(11.466 µg/mL), CM5 (9.666 µg/mL), CM16 (9.233 µg/mL), CM21 (8.4 µg/mL), CM24 (7.400 µg/mL), CM14 (7.300 µg/mL), CM15 (6.466 µg/mL), CM6 (6.000 µg/mL), CM11 (5.400 µg/mL) while CM9 (3.833 µg/mL) produced least amount of indole acetic acid (Table 2).

Sixteen bacterial isolates had the ability of phosphate solubilization. Phosphate solubilization ranged from 4.533 to 12.333µg/mL. Maximum amount of phosphate solubilization was observed in CM4 (12.333µg/mL) followed by CM2 (11.633 µg/mL), CM3 (10.900 µg/mL), CM1 (10.100 µg/mL), CM5 (10.000 µg/mL), CM11 (9.733 µg/mL), CM21 (9.166 µg/mL), CM9(8.733 µg/mL), CM14 (8 µg/mL), CM25 (7.2 µg/mL), CM13 (6.933 µg/mL), CM29 (6.933 µg/mL), CM15 (6.433 µg/mL), CM16 (6.366 µg/mL), CM24 (5.633 µg/mL) while CM6 showed the lowest level of phosphate solubilization 4.533 µg/mL (Table 2).

**Biofilm formation, root colonization and nodulation assay:** The isolates of bacteria were examined for their ability of root colonization. It was observed that all isolates had good ability of root colonization. Isolates were also observed for the formation of biofilm and all were efficient in their ability

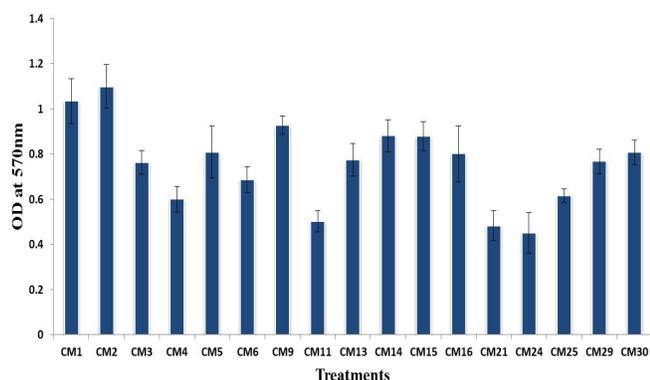
**Table 1. Characterization of bacterial isolates based on morphology from chickpea nodules.**

Sr. No.	Isolate	Colony morphology	Cell morphology	Gram's reaction
1.	CM1	Round medium, white	Small rods	-ve
2.	CM2	Round small, white	Small rods	-ve
3.	CM3	Round small, white	Small rods	-ve
4.	CM4	Round small, white	Small rods	-ve
5.	CM5	Round medium, white	Small rods	-ve
6.	CM6	Round small, white	Small rods	-ve
7.	CM9	Round medium, white	Small rods	-ve
8.	CM11	Round medium, white	Small rods	-ve
9.	CM13	Round large, shiny white	Medium rods	-ve
10.	CM14	Round large, shiny white	Medium rods	-ve
11.	CM15	Round medium, white	small rods	-ve
12.	CM16	Round small, white	Small rods	-ve
13.	CM21	Round medium, white	Small rods	-ve
14.	CM24	Round medium, white	Small rods	-ve
15.	CM25	Round large, white	Small rods	-ve
16.	CM29	Round large, white	Small rods	-ve
17.	CM30	Round large, shiny white	Medium rods	-ve

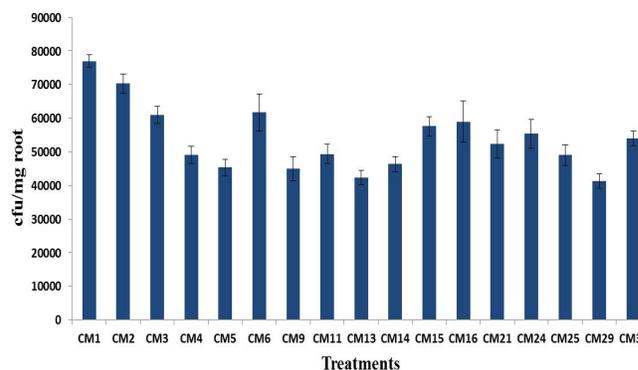
**Table 2. Biochemical characterization of chickpea bacterial isolates. Bars are means ± standard error.**

Sr. No.	Isolates	ARA (n moles C <sub>2</sub> H <sub>2</sub> reduced /h/mg protein)	IAA in culture medium µg/mL	P-solubilized (µg/mL)
1.	CM1	303.666±11.46	18.100±1.231	10.100±0.751
2.	CM2	257.000±8.858	15.300±1.237	11.633±0.659
3.	CM3	231.666±6.196	12.700±0.325	10.900±0.851
4.	CM4	204.000±9.219	11.466±0.904	12.333±1.301
5.	CM5	200.333±7.844	9.666±0.846	10.000±0.922
6.	CM6	236.666±11.46	6.000±0.684	4.533±1.062
7.	CM9	-	3.833±0.655	8.733±0.791
8.	CM11	136.666±10.26	5.400±0.443	9.733±0.578
9.	CM13	-	-	6.933±0.717
10.	CM14	-	7.300±0.618	8.000±0.859
11.	CM15	206.666±10.19	6.466±0.416	6.433±1.376
12.	CM16	-	9.233±1.271	6.366±0.822
13.	CM21	-	8.400±0.922	9.166±0.328
14.	CM24	220.666±14.40	7.400±0.554	5.633±0.670
15.	CM25	174.000±8.055	-	7.200±0.684
16.	CM29	-	-	6.933±0.784
17.	CM30	-	-	-

of biofilm formation. CM2 showed the highest amount of biofilm formation while others showed at different levels (Fig. 1). In comparison with the other isolates, CM1 showed maximum ability of root colonization followed by CM2 (Fig. 2).

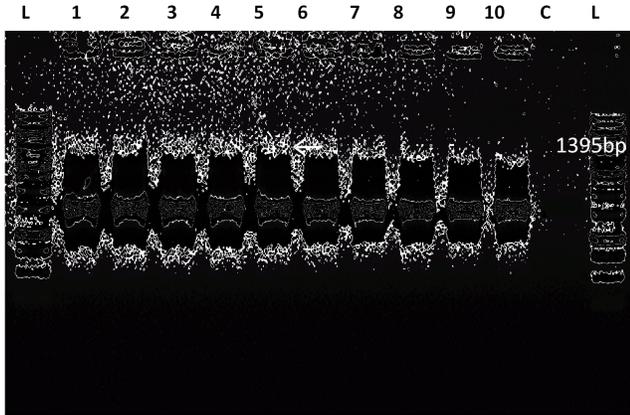


**Figure 1. Comparison of biofilm formation among bacterial treatments of chickpea. Bars are means ± standard error.**



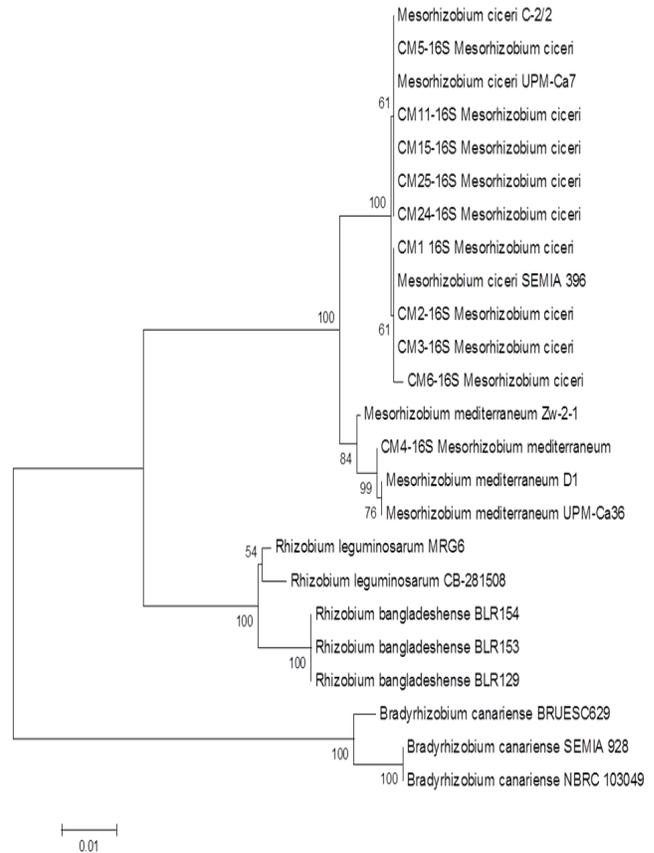
**Figure 2. Comparison of root colonization among bacterial treatments of chickpea. Bars are means ± standard error**

**Partial amplification, cloning, sequencing and Phylogenetic analysis of 16S rRNA:** Bacterial genomic DNA isolated from ten isolated selected on the basis of their characterization was used as template for the partial amplification of 16S rRNA gene using specific primers (Fig. 3). Homology search of gene sequences showed 99% homology with related sequences in the database. Multiple sequences alignment was done using ClustalW2 software. Accession numbers of sequences submitted in Genbank are CM1, KX226345; CM2, KX226346; CM3, KX226347; CM4, KX226348; CM5, KX226349; CM6, KX226350; CM11, KX226351; CM15, KX226352; CM24, KX226353; CM25, KX226354.



**Figure 3. PCR amplification of partial 16S rRNA from chickpea bacterial DNA. DNA 4µL was electrophoresed in 1% agarose gel at 80v for 30min. L=Ladder, 1=CM1, 2=CM2, 3=CM3, 4=CM4, 5=CM5, 6=CM6, 7=CM11, 8=CM15, 9=CM24, 10=CM25, C=Control, L=Ladder.**

Phylogenetic analysis was performed using MEGA5 software. 16S rRNA of *Mesorhizobium mediterraneum* was making another cluster in phylogenetic analysis. *Rhizobium* and *Bradyrhizobium* species were distantly related to *Mesorhizobium* species (Fig. 4).



**Figure 4. Phylogenetic tree of 16S rRNA of chickpea strains with other rhizobial strains. MEGA5 was used for phylogenetic analysis.**

## DISCUSSION

There are several mechanisms for the production of crop and improvement of soil fertility by various processes like solubilization of phosphate, production of indole acetic acid, fixation of nitrogen, biocontrol etc. in which biofertilizers play a major role (Vessey, 2003). Substantial improvement in yield as well as growth of crops as a result of inoculation of microbes has been studied and reported by many researchers (Yadegari *et al.*, 2008; Minaxi *et al.*, 2012). Isolates were selected based on the morphology of colonies isolated from the nodules of chickpea. Bacterial colonies of different shapes (round and rod) colors and texture were observed. Nitrogen fixation efficiency of all the bacterial isolates was estimated based on their acetylene reduction assay (ARA) through gas chromatography. Among the chickpea isolates, range of fixed nitrogen was 136.666 to 303.666 nmoles C<sub>2</sub>H<sub>2</sub> reduced/h/mg protein. Most of the bacterial strains tested were nitrogen fixers as well as phytohormone producers which showed their role in growth

and yield enhancement. Similar findings to these have been reported by scientists (Glick, 1995; Glick *et al.*, 1999).

High Pressure Liquid Chromatography (HPLC) was used for quantification as well as detection and phytohormone (indole acetic acid) secretion by isolates of bacteria in the pure cultures. Analysis of bacterial isolates by HPLC showed their ability of phytohormone production and these hormones affect growth and branching of roots. Hoflich *et al.* (2001) isolated PGPR from mycorrhizosphere of pine that produced auxin (phytohormone), cellulase and pectinase and reported increased seedling growth of the pine. Also many researchers have reported PGPR produces phytohormones (Holl *et al.*, 1988; Haahtela *et al.*, 1990; Turjanista *et al.*, 1995; Zimmer *et al.*, 1995). Auxins have an important role in development of roots, enlargement and division of cells as well as phototropism. All the isolates were evaluated for indole acetic acid production in the medium free of nitrogen with 100 mg/L L-tryptophan and NH<sub>4</sub>Cl 1g/L. In the synthesis of indole acetic acid, tryptophan is a precursor. Among chickpea isolates, thirteen out of seventeen produced IAA. Range of IAA production among chickpea isolates was 3.833-18.100 µg/mL. Similar findings have been recorded by Verma *et al.* (2010) in *Rhizobium*, *Pseudomonas* and *Azotobacter*. In *Azospirillum* and *Pseudomonas*, production of cytokinins, auxins and gibberellins in the chemically defined media has also been widely studied and reported (Gonzalez-Lopez *et al.*, 1986).

Among Chickpea isolates, sixteen bacterial isolates were positive in phosphate solubilizing ability while only one isolate was negative. Range of phosphate solubilization among chickpea isolates was 4.533-12.333µg/ml. Similar research has been reported by Forchetti *et al.* (2007) on biochemical characterization and sequencing of sunflower isolates using advanced bioinformatics tools. Ahmad *et al.* (2008) reported that *Mesorhizobium*, *Azotobacter*, *Bacillus* and *Pseudomonas* were good for the solubilization of phosphate. Maximum solubilization was observed in *P. aeruginosa* followed by *Mesorhizobium*, *Azotobacter* when compared with *Trichoderma*. The phosphate solubilizing microorganisms have the ability to enhance phosphorus availability to plant via organic phosphorus mineralization and through the conversion of inorganic phosphorus into a form which is more available to the plant (Kazempour, 2004).

For bacterial species identification, *16S rRNA* is extensively used in the scientific investigations and clinical practice (Clarridge, 2004; Petti *et al.*, 2005). Traditional methods of identification of microbes frequently failed in isolate's identification within species. This type of identification was misguided and lead towards incorrect interpretation of data so the exact identification of bacteria was important in clinical microbiology (Mignard and Flandrois, 2006). Phenotypic methods were laborious, also entirely failed to identify few bacteria, for example Gram-positive rods.

Amplification of nucleic acid for microbial identification and bioinformatics for DNA Sequence-based analysis were reported to be more authentic (Rudi *et al.*, 2007). Since the discovery of DNA sequencing and PCR, comparison of species of bacterial gene sequences indicated that within species and among same genus species, *16S rRNA* is conserved. Sequence information could be used for the construction of phylogenetic trees on base difference (Olsen and Woese, 1993). Zhang *et al.* (2002) used similar tools who quoted that bacterial phylogenetic structure had been widely under study by *16S rRNA* sequence comparison.

For the analysis of *16S rRNA*, identification of species is easy when whole gene or partial gene is sequenced (Chakravorty *et al.*, 2007). The purpose of study was characterization of *16S rRNA* 10 rhizobial strains. Multiple sequence alignment was and phylogenetic analysis of these bacterial isolates was conducted using MEGA5 software (Clarridge, 2004). The utility of the *16S rRNA* for the 13 species of bacterial detection has been reported by Schmalenberger *et al.* (2001).

Based on the morphological and biochemical characterization, isolates of chickpea were selected for their identification using *16S rRNA* sequencing. Among chickpea isolates, CM1 and CM2 showed 99% homology with *Mesorhizobium ciceri* and excellent properties of plant growth promotion. These selected strains were able to fix nitrogen, solubilized phosphate and produced indole acetic acid. They were also capable of root colonization and biofilm formation indicating potential rhizobia for use as biofertilizers.

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*Genetic diversity in chestnuts of Kashmir valley*