Int.J.Curr.Microbiol.App.Sci (2017) *6*(9): 3149-3162

International Journal of Current Microbiology and Applied Sciences
ISSN: 2319-7706 **Volume 6 Number 9 (2017) pp. 3149-3162**
Journal homepage: http://www.ijcmas.com

Original Research Article https://doi.org/10.20546/ijcmas.2017.609.389

Phenotypic and Genotypic Analysis of Summer Mungbean Rhizobia

Moumita Ghosh[1], Poonam Sharma[2], Asmita Sirari[2], Navprabhjot Kaur[1] and Kailash Chand[1*]

[1]Department of Microbiology, Punjab Agricultural University, Ludhiana (141004), Punjab, India
[2]Department of Plant Breeding and Genetics, Punjab Agricultural University, Ludhiana-141004, Punjab, India
**Corresponding author*

Keywords

Bradyrhizobium sp., Indole acetic acid (IAA), P-solubilization, RAPD, *Rhizobium* sp., summer mungbean, UPGMA software

Article Info

Accepted:
28 August 2017
Available Online:
10 September 2017

A B S T R A C T

Phenotypic and genotypic diversity of rhizobia isolated from root and soil rhizosphere of summer mungbean was assessed on the basis of plant growth promoting traits and using RAPD primers. In present studies, isolates of rhizobia from nodules were purified on Congo red Yeast Mannitol Agar (CRYEMA) medium from summer mungbean growing areas of Punjab state, India. Slow and fast growing colonies of rhizobial isolates were selected on Bromo Thymol Blue (BTB) supplemented with YEMA medium. Among five RAPD primers used in present study, two of the primers RAPD-4 and RAPD-5 showed maximum number of detectable bands. Highest polymorphism detected with RAPD-5 during PCR amplification. Cluster analysis divided rhizobial isolates into five distinct groups. The data depicts that although the growth promoting parameter i.e. Indole acetic acid (IAA) production, phosphate solubilization were very promising in LSMR-1, LSMR-5, LSMR-8, LSMR-9, LSMR-19 and LSMR-23 but they were genetically diverse and fall under different groups of cluster analysis. Whereas, LSMR-1 and LSMR-5 showed a very close relation isolated from same native rhizosphere. The assessment of genetic diversity is a prerequisite and important step for the improvement of any legume crop. It is concluded that significant genetic diversity in rhizobial strains exists with respect to their efficiency for IAA production and P-solubilization.

Introduction

Summer Mungbean (*Vigna radiata* L. Wilczek.), like most legumes is able to fix atmospheric nitrogen through symbiotic association with compatible *Rhizobium*. Summer mungbean occupies an estimated area of about 50.0 thousand hectares with an average yield of 4.45 q/acre (Anonymous, 2014) in Punjab as summer season crop. Inclusion of short duration legumes like summer mungbean in rice-wheat system can play an important role in sustaining soil fertility and crop productivity. Symbiotic nitrogen fixation (SNF) is a result of intimate mutualistic relationship between the root nodule bacteria and host plant. *Rhizobium* inoculation can be demonstrated in summer mungbean as sustainable, environment friendly agro-technological practice. Most of these bacterial species are in the Rhizobiace family in the alpha- proteobacteria and are in either the *Rhizobium*, *Mesorhizobium* and *Bradyrhizobium* genera (Weir, 2012). To

obtain efficient nodulation and nitrogen fixation, it is important to know the predominant types of rhizobial strains present in soil and assess their functionality traits for improvement in symbiotic efficiency and yield improvement in summer mungbean.

Native legumes are potential sources of diverse indigenous rhizobial population. Various phenotypic and genotypic methodologies are being used to characterize bacteria (Rai *et al.,* 2012). Although phenotypic methods play a significant role in identification but the molecular methods are more reliable and authenticated to study genetic diversity of bacterial isolates (Fetyan and Mansour, 2012). Negligible reports on the molecular diversity of rhizobia infecting legumes of Indian origin and particularly summer mungbean are available (McInnes *et al.,* 2004). Studies on the molecular diversity of root nodule bacteria in rhizosphere, soil or nodules have been conducted to properly classify rhizobia and also to correlate with promiscuity, symbiotic properties, effectiveness and competitiveness of these bacteria in different legumes but no conclusion has been drawn finally (Chen *et al.,* 2003; Rosenblueth *et al.,* 2004; Safronova *et al.,* 2004). Molecular techniques based on the polymerase chain reaction (PCR) are very convenient for characterization, because they are rapid, simple and discriminative. PCR has been found useful for rhizobial strain differentiation using GC-rich oligonucleotide RAPD primers (Sajjad *et al.,* 2008).

Earlier workers (El- Hady *et al.,* 2010; Bhuyan *et al.,* 2014) have reported that DNA markers have many advantages over morphological markers. For simple, efficient and economic way of strains identification and diversity analysis RAPD-PCR based DNA fingerprinting has widely used (Gherardi *et al.,* 1998). RAPD markers have advantage that they are random and do not require any prior sequence information for implementation. RAPDs are generated by PCR amplification using single, short, synthetic, random oligonucleotide as primers that acts both as forward as well as reverse primer (Yadev *et al.,* 2014). RAPD analysis can be mostly used to reveal genetic relationship between genotypes of a species (Bhuyan *et al.,* 2014). DNA markers such as RAPD provide a direct measure of rhizobial genetic diversity.

This work aimed to analyze the diversity of rhizobia collected from different locations of Punjab using morphological, biochemical and functional tests and to characterize the genetic relationship among twenty three rhizobia of summer mungbean with respect to RAPD markers. In order to measure of genetic diversity of rhizobia and go beyond diversity based on the optimization of SNF associated with multifarious PGP traits for better management and develop more efficient strategies for crop improvement in different areas of Punjab, India.

Materials and Methods

The present study was conducted in the Pulses Research Microbiology Laboratory, Department of Plant Breeding and Genetics, Punjab Agricultural University, Ludhiana with reference culture M1.

Isolation of Rhizobial cultures

Rhizobial cultures were isolated from rhizospheric soil and root nodule samples collected from summer mungbean (*Vigna radiata* L. Wilzek.) growing areas of Punjab (Table 1). Isolation of rhizobial cultures was done according the method of Vincent, 1970. Pure culture of single white, opaque, gummy and translucent colonies with entire margin were obtained and maintained by repeated streaking on Yeast Extract Mannitol Agar

medium (YEMA) at 15 days intervals for further studies.

Growth on Bromothymol blue (BTB) agar medium for differentiation

To differentiate *Bradyrhizobium* from *Rhizobium*, cultures were streaked on BTB agar plates made by adding 5 ml of 0.5% BTB in ethanol (0.5g $100ml^{-1}$) to 1 litre of YEMA medium with incubation of 2-10 days at 28±2ºC. Change in the colour of medium (blue) for rhizobial isolates classified as slow growers and yellow for the fast growers (Somasegaran and Hoben., 1994).

Morphological and biochemical studies of rhizobial isolates

Morphological characterization was done according to Somasegaran and Hoben, 1994 on the basis of colony morphology including shape, colour, surface margin and gram-staining reaction. Collected strains were also biochemically screened using following tests: Oxidase, Catalase, Citrate utilization, Methyl Red (MR), Voges-Proskauer (VP), Urease and Nitrate reduction tests as described by Cappuccino and Sherman (1992).

Multifunctional Plant Growth Promoting (PGP) traits of summer mungbean rhizobia

IAA production

Characterization of rhizobial cultures for the production of IAA was carried out as described by Gordon and Weber (1951). *Rhizobium* cultures were grown in YEM broth supplemented with 0.01% tryptophan separately and incubated for 3 days at 28±2ºC.

Exponential phase cultures were centrifuged at 10,000 rpm for 20 minutes. Two drops of orthophosphoric acid were added to the supernatant (2 ml). Further 4 ml of the Salkowski reagent (1 ml of 0.5 M $FeCl_3$ in 50 ml of 35% $HClO_4$) were added to 2 ml of supernatant and incubated for 20 min at room temperature in order to develop pink colour. Absorbance was measured at 535 nm. IAA concentrations were determined by comparison with standard curve.

Assay for Phosphate (P) solubilization in summer mungbean rhizobia

Qualitative estimation

P solubilization ability of different *Rhizobium* and *Bradyrhizobium* isolates were determined qualitatively by streaking the strains on Pikovaskaya's and NBRIP (National Botanical Research Institute's Phosphate growth medium) media (Arora 2007). Presence of yellow clear halo zone around bacterial growth after 5-7 days incubation period at 28±2°C was used as indicator for positive P solubilization (Nautiyal, 1999). Phosphate solubilization index (PSI) was calculated by using following formula:

PSI Index= A/B

Whereas, A= Total diameter (colony + halo zone); B= Diameter of colony.

Promising P solubilizers were further tested for quantitative phosphate solubilization.

Quantitative measurement

For this study sterilization of 100 ml of Pikovaskaya's broth dispensed in each 250 ml conical flasks with 0.1g P_2O_5 of tri-calcium phosphate (TCP) was done. The flasks were inoculated with 1 ml suspension of overnight grown culture and incubated at 28±2°C for 15 days. Presence of yellow colour after addition of 1ml of ammonium molybdate (AM) and ammonium vandate (AV) in equal ratio to

culture supernatant confirmed phosphate solubilizing activity and measured spectroscopically (Elico UV-VIS spectrophotometer) at 420 nm after 25 minutes incubation for quantitative estimation of phosphate solubilization (Jackson., 1973). The values were calculated with the help of standard curve made of 5 ppm KH_2PO_4.

Molecular characterization of rhizobial isolates using RAPD primers

Rhizobium and *Bradyrhizobium* sp. isolated from summer mungbean rhizosphere was characterized at molecular level by using RAPD primers. Genomic DNA of different *Rhizobium* sp. and *Bradyrhizobium* sp. was extracted by alkaline lyses method of Sambrook *et al.,* 1989.

PCR amplification and gel electrophoresis

Quantification of DNA was carried out by using agarose gel electrophoresis. DNA was visualized under UV light and diluted in appropriate amount of TE buffer to yield a working solution and stored at -20°C.

PCR amplification of the rhizobial genomic DNA was carried out using five RAPD primers. The PCR reaction was performed in 20 µl reaction mixture containing 4 µl of taq polymerase buffer, 2 µl MgCl, 0.8 µl dNTP mix, 2 µl RAPD primer, 0.3 µl taq DNA polymerase enzyme, 2.5 µl of template DNA (40 ngμl^{-1}) and 8.4 µl of double distilled water and reaction mixture was overlaid with two drops of mineral oil, incubated for 5 min at 94ºC for initial denaturation and then amplified for 35 cycles consisting of 1 min at 94ºC, 1 min annealing temperature at 31ºC for [RAPD-1(*5'GGTGCGGGAA'3*) and RAPD-5(*5'AAGGCGGCAG'3*)] or 29°C for [RAPD-2 (*5'GTTTCGCTCC'3*), RAPD-3 (*5'AA GAGCCCGT'3*) and RAPD-4 (*5'AAG GCTCGAC'3*)] was found to be optimized for generating clear and reproducible bands for RAPD primers and 72°C for 2 min followed by 10 min incubation at 72ºC with a 4ºC holding temperature. DNA ladder of 1kb size was loaded on both or either side of the gel to calculate the size/molecular weight of the polymorphism DNA fragments. Samples were run for electrophoresis in agarose gel tank for approximately 2h at 50 volts.

After electrophoresis, the amplified products were viewed under ultraviolet transilluminator and photographed using the Alpha-Innotech Gel Documentation System. Out of five primers two primers RAPD-4 and RAPD-5 have maximum number of scorable bands. All visible and un-ambiguously scorable fragments amplified by primers were scored under the heading of total scorable fragments.

Statistical data analysis

Multivariate analysis was conducted to generate a dissimilarity matrix using DAR win software(17), version 5.0.158 based on un-weight paired group of arithmetic means averages (UPGMA) to estimate genetic distance and relatedness of rhizobial strains.

Results and Discussion

Morphological and Biochemical studies of isolated summer mungbean rhizobia

Twenty three *Rhizobium* sp. were isolated from different soil and nodule samples of summer mungbean rhizosphere (Table 1). Out of 23 rhizobial isolates, 18 *viz.* LSMR-1, LSMR-2, LSMR-3, LSMR-4, LSMR-5, LSMR-6, LSMR-7, LSMR-8, LSMR-9, LSMR-11, LSMR-13, LSMR-16, LSMR-17, LSMR-19, LSMR-20, LSMR-21, LSMR-22, LSMR-23 and M1(reference) turned the yeast extract mannitol agar medium supplemented with bromothymol blue (BTB) dye from green to yellow showing as acid producers

(Plate 1) *i.e.* fast growing rhizobial isolates showed sufficient growth and more gum production having a mean generation time of 24-36 h. Other 5 rhizobial isolates (LSMR-10, LSMR-12, LSMR-13, LSMR-14 and LSMR-18) changed the colour from green to medium blue due to alkali production and categorized as slow grower rhizobial isolates with sufficient growth but less gum production, having a mean generation time of 36-48 h (Plate 1). The colonies obtained were gummy, translucent and circular with entire or smooth margins and Gram negative, rod shaped as revealed by Gram's staining technique.

YEM-BTB medium used for categorizing indigenous legume root nodulating as fast and slow growing rhizobia based on acid/ alkali production supported by Harpreet *et al.,* (2012) and Deka and Azad, (2006). Similarly Maruekarajtinplenga *et al.,* (2012) reported about two types of slow growing rhizobia with respect to duration of incubation period.

On the basis of biochemical test, all the rhizobial isolates were found to be positive for Oxidase, Urease, Citrate utilization, Nitrate reduction and Catalase activities and negative for Methyl Red (MR), Voges-Proskauer (VP) and Urea hydrolysis tests. Reliable identification of specific rhizobial isolates is necessary for the study of their symbiotic association with legumes.

The results of present studies are well coherent with findings of Shahzad *et al.,* (2012) observed that *Rhizobium meliloti* isolated from root nodules of alfa alfa showed negative tests for Methyl Red (MR), Voges-Proskauer (VP), Indole, Citrate utilization test and Urea hydrolysis tests.

Moreover, Zohra *et al.,* (2016) found positive Oxidase and Catalase test for fast growing rhizobial strains isolated from wild chickpea.

Screening of rhizobial isolates for multifunctional Plant Growth Promoting (PGP) traits

Indole acetic acid production (IAA)

All the *Rhizobium* and *Bradyrhizobium* isolates were tested for their ability to produce IAA both in the presence and absence of precursor L- tryptophan (Table 2). Significant differences were found among isolates in their ability to produce IAA. A low amount of IAA was produced by all the isolates in the absence of L-tryptophan, which ranged from 1.04-8.25 μgml^{-1}. In the presence of L-tryptophan the amount of IAA produced by all rhizobial isolates ranged from 1.35-16.04 μgml^{-1}. Significantly high IAA was produced by fast growing *Rhizobium sp*. LSMR-19(16.04± 0.231μgml^{-1}) followed by LSMR-5(15.85±0.352μgml^{-1}), LSMR-1 (14.65±0.629μgml^{-1}) and LSMR-23 (13.70±0.289 μgml^{-1}). Minimum amount of IAA was produced by *Bradyrhizobium* sp. LSMR-12(1.35±0.058 µgml-1 of IAA) in the presence of L-tryptophan.

Our work is well supported by the work of Kumar and Ram, (2012) who showed that *Rhizobium* strain isolated from root nodules of five cultivars of *Vigna trilobata* produced a maximum of 80.96 μgml^{-1} of IAA. Appunu *et al.,* (2009) also reported that in presence of L-tryptophan nearly 9% of slow-growers and 10% of fast growers synthesized IAA. Study of Zahir *et al.,* (2010) in mungbean showed that rhizobial isolates varied widely in increasing level of L-tyrptaphan for improving the growth and yield of mungbean.

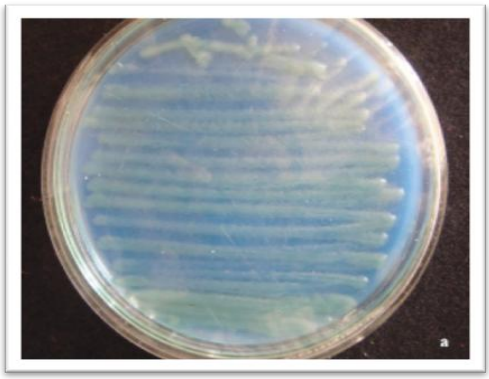
P- solubilization by summer mungbean rhizobial isolates

Phosphorous is major growth limiting nutrient and unlike nitrogen, there is no large atmospheric reservoir that can be made biologically available. Data depicts that, 65%

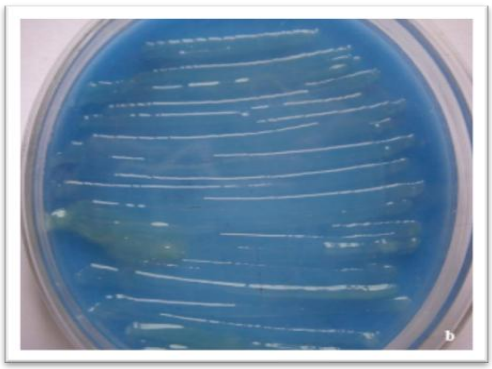
of isolates were P-solubilizers whereas reference culture M-1 showed negative P-solubilizing activity. P- solubilization ability of rhizobial isolates varied as evidence by the size of halo zone ranged from 3.5mm to 11.2 mm on Pikovskaya's and NBRIP agar media. According to diameter size, further one *Bradyrhizobium* sp. (LSMR-10) and six *Rhizobium* sp. (LSMR-1, LSMR-5, LSMR-8, LSMR-9, LSMR-11 and LSMR-22) were selected for further quantitative measurement.

The relative efficiency of the seven potential isolates of *Rhizobium* and *Bradyrhizobium* strains was studied for solubilizing TCP at different intervals of days (3,6,9,12 and 15 days). Increasing amount of P was released by different isolates with increasing period of incubation till 12th day with decreasing pH due to organic acid productions (Fig. 1). The phosphate solubilizing activity was observed upto15th day. The maximum phosphate solubilization was observed on 12th day, which ranged from 2.26 to 6.11 mg100ml^{-1}. Significantly high phosphate was solubilized by LSMR-1(6.11±0.087 mg100ml^{-1}) followed by LSMR-5(5.57±0.179 mg100ml^{-1}). Whereas amount of low phosphate solubilizing activity was recorded in LSMR-9(2.75±0.231 mg100ml^{-1}) followed by LSMR-8(2.33±0.133 mg100ml^{-1}) at 12th day.

Plate.1 Differentiation of slow growing Rhizobia on BTB agar medium



a) Fast growing (*Rhizobium* sp.)



b) Slow growing (*Bradyrhizobium* sp.)

Plate.2a RAPD-PCR amplification of DNA isolated from mungbean rhizobia using RAPD-5(In L= 1kb DNA Ladder, 1= LSMR-1, 2= LSMR-2, 3=LSMR-3, 4=LSMR-4, 5=LSMR-5, 6=LSMR-6, 7=LSMR-7, 8=LSMR-8, 9=LSMR-9, 10=LSMR-10, 11=LSMR-11, 12=LSMR-12, 13=LSMR-13 and 14= LSMR-14

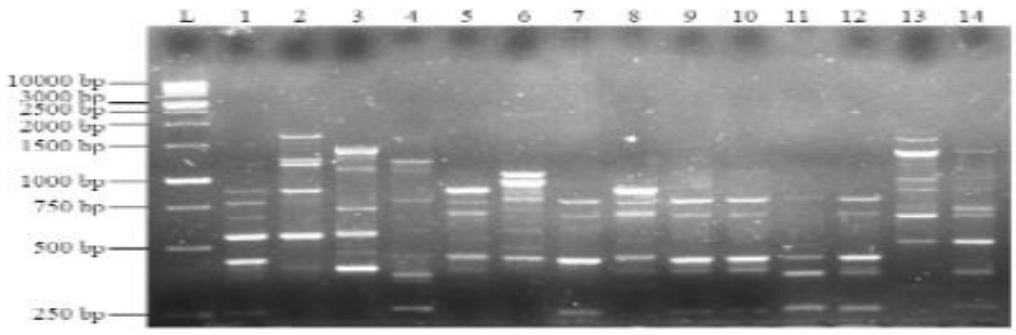


Plate.2b RAPD-PCR amplification of DNA isolated from mungbean rhizobia using RAPD-5(In fig. L= 1kb DNA Ladder, 15= LSMR-15, 16= LSMR-16, 17=LSMR-17, 18=LSMR-18, 19=LSMR-19, 20=LSMR-20, 21=LSMR-21, 22=LSMR-22, 23=LSMR-23 and 24=M-1(Reference culture)

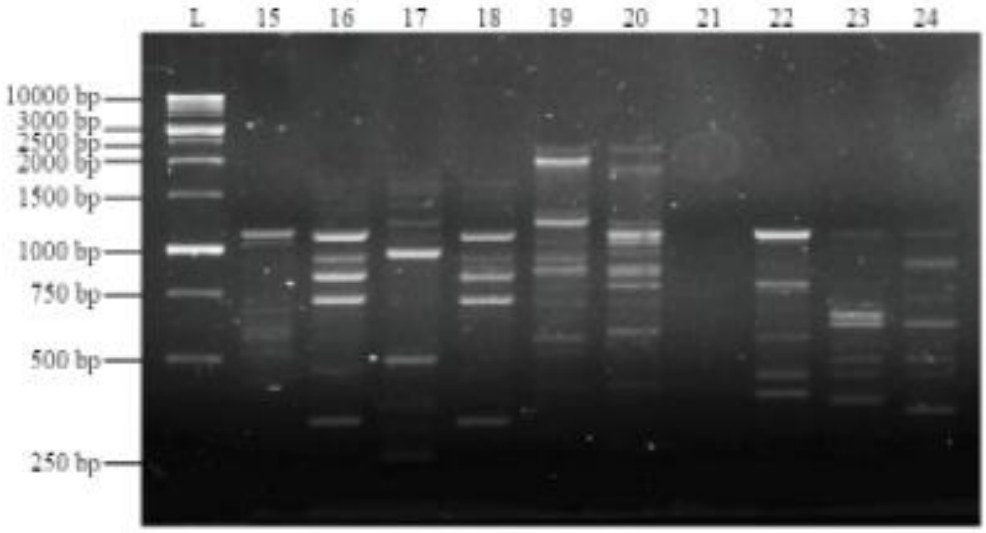


Fig.1 Quantitative measurement of P-solubilization by *Rhizobium* sp. and *Bradyrhizobium* sp. of summer mungbean on Pikovaskaya's medium as a function of time

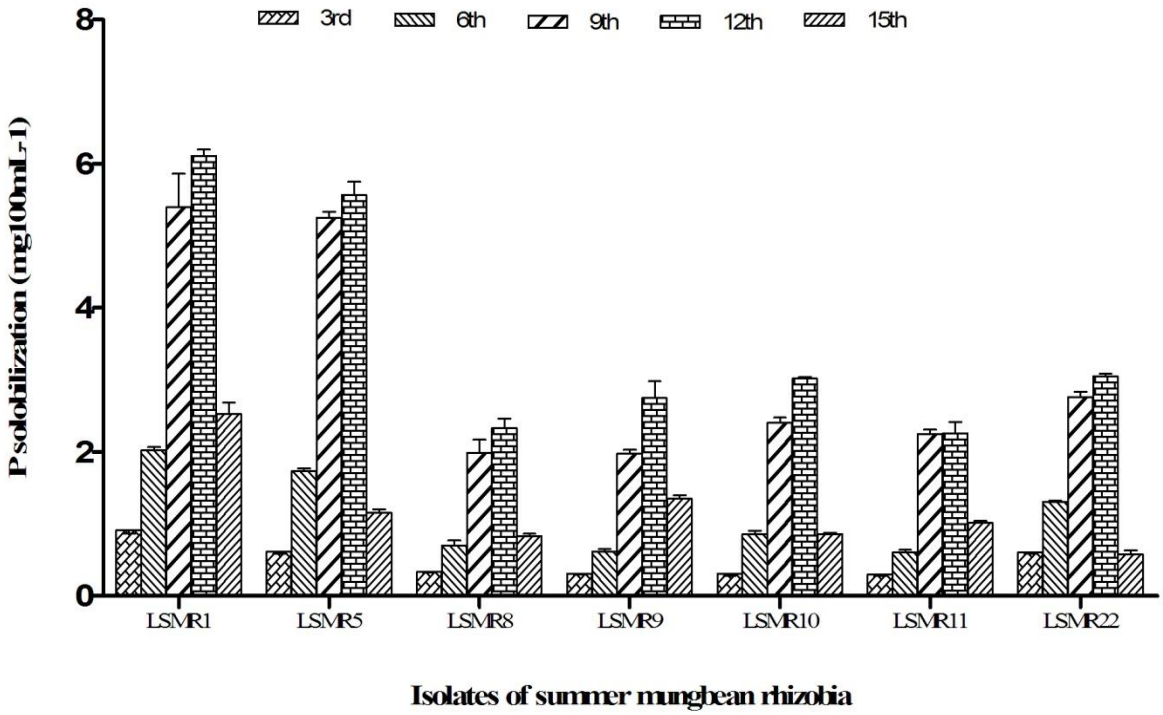


Fig.2 Dendrogram of 24 summer mungbean rhizobia obtained from dissimilarity matrix (DARwin – UPGMA software)

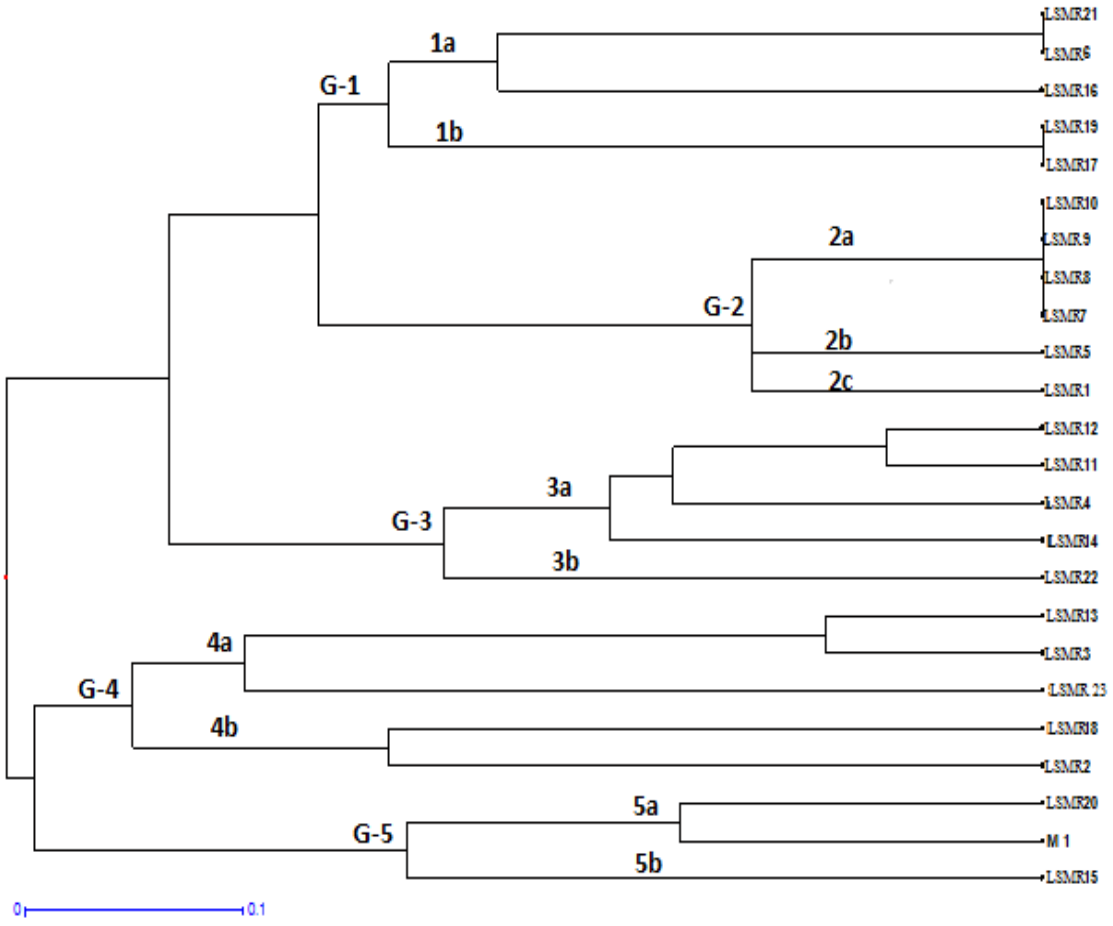


Table.1 Location of rhizospheric soil and root nodule samples of summer mungbean

Rhizospheric soil samples	**Location of sample**
1- 9	Manuke I, Manuke II, Manuke III, Manuke IV, Manuke V, Manuke VI, Manuke VII, Rauni I, Rauni I
Root nodule samples	**Location of sample**
10 -23	Narinagarh I, Narinagarh II, Narinagarh III, Ladhowal I, Ladhowal II, Ladhowal III, Manuke VIII, PAU Farm I, PAU Farm II, PAU Farm III PAU Farm IV, PAU Farm V, Adilabad Sangrur

Table.2 Quantitative estimation of Indole acetic acid (IAA) production by *Rhizobium* sp. and *Bradyrhizobium* sp. of summer mungbean in presence and absence of L-tryptophan

Isolates of *Rhizobium* sp./*Bradyrhizobium* sp. isolates	IAA production (µlmL^{-1})	
	IAA (Trp-)	IAA (Trp +)
LSMR 1	**6.20±0.127**	**14.65±0.629**
LSMR 2	6.90±0.208	7.10±0.292
LSMR 3	5.30±0.462	8.70±0.260
LSMR 4	4.65±0.277	5.60±0.375
LSMR 5	**5.80±0.404**	**15.85±0.352**
LSMR 6	3.15±0.035	3.85±0.115
LSMR 7	3.55±0.248	3.90±0.254
LSMR 8	3.70±0.312	8.35±0.231
LSMR 9	5.05±0.029	10.35±0.404
LSMR 10	4.15±0.006	8.75±0.289
LSMR 11	2.15±0.023	9.65±0.589
LSMR 12	1.04±0.017	1.35±0.058
LSMR 13	3.85±0.859	4.50±0.115
LSMR 14	3.05±0.029	6.35±0.058
LSMR 15	4.30±0.462	5.30±0.491
LSMR 16	2.70±0.231	6.20±0.144
LSMR 17	3.21±0.052	5.05±0.231
LSMR 18	1.65±0.058	3.85±0.144
LSMR 19	**5.60±0.167**	**16.04±0.231**
LSMR 20	4.10±0.040	5.45±0.404
LSMR 21	3.60±0.058	4.41±0.139
LSMR 22	5.70±0.271	6.50±0.225
LSMR 23	**8.25±0.208**	**13.70±0.289**
M 1(Reference culture)	5.35±0.214	11.65±0.404
CD at 5%	**0.73**	**1.43**

Table.3 Dissimilarity matrix of *Rhizobium* sp. and *Bradyrhizobium* sp. of summer mungbean rhizobial isolates

Isolates	LSMR 1	LSMR 2	LSMR 3	LSMR 4	LSMR 5	LSMR 6	LSMR 7	LSMR 8	LSMR 9	LSMR 10	LSMR 11	LSMR 12	LSMR 13	LSMR 14	LSMR 15	LSMR 16	LSMR 17	LSMR 18	LSMR 19	LSMR 20	LSMR 21	LSMR 22	LSMR 23	M 1
LSMR 1	0.00																							
LSMR 2	0.50																							
LSMR 3	0.80	0.80																						
LSMR 4	0.71	0.75	1.00																					
LSMR 5	0.40	0.60	1.00	0.71																				
LSMR 6	0.56	1.00	1.00	1.00	0.71																			
LSMR 7	0.20	1.00	1.00	0.67	0.20	0.50																		
LSMR 8	0.20	1.00	1.00	0.67	0.20	0.50	0.00																	
LSMR 9	0.20	1.00	1.00	0.67	0.20	0.50	0.00	0.00																
LSMR 10	0.20	1.00	1.00	0.67	0.20	0.50	0.00	0.00	0.00															
LSMR 11	0.67	1.00	1.00	0.43	0.67	0.60	0.60	0.60	0.60	0.60														
LSMR 12	0.43	1.00	1.00	0.25	0.43	0.67	0.33	0.33	0.33	0.33	0.14													
LSMR 13	0.67	0.43	0.20	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00												
LSMR 14	0.67	0.71	0.60	0.43	1.00	1.00	1.00	1.00	1.00	1.00	0.33	0.43	0.67											
LSMR 15	0.75	0.75	1.00	1.00	0.67	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00										
LSMR 16	1.00	1.00	1.00	1.00	1.00	0.50	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00									
LSMR 17	0.67	1.00	1.00	0.71	0.67	0.60	0.60	0.60	0.60	0.60	1.00	0.71	1.00	1.00	1.00	0.60								
LSMR 18	1.00	0.60	1.00	1.00	0.50	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00							
LSMR 19	0.67	1.00	1.00	0.71	0.67	0.60	0.60	0.60	0.60	0.60	1.00	0.71	1.00	1.00	1.00	0.60	0.00	1.00						
LSMR 20	1.00	0.78	0.71	0.78	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	0.75	1.00	1.00	0.71	1.00	1.00	1.00					
LSMR 21	1.00	1.00	0.75	1.00	1.00	0.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	0.67	0.50	0.60	1.00	0.60	0.71				
LSMR 22	1.00	1.00	0.67	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	0.50	1.00	1.00	1.00	1.00	1.00	0.33			
LSMR 23	0.60	1.00	1.00	1.00	0.60	0.00	0.50	0.50	0.50	0.50	0.60	0.67	1.00	1.00	1.00	0.50	0.60	1.00	0.60	1.00	0.50	1.00		
M 1	1.00	1.00	1.00	0.60	1.00	0.71	1.00	1.00	1.00	1.00	0.50	0.60	1.00	0.50	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	0.00

Table.4 Grouping of isolates according to the dendrogram produced by DARwin software

Group	Sub-group	Isolates of *Rhizobium* sp. and *Bradyrhizobium* sp.	Location
G-1	1a	LSMR-21 LSMR-6 LSMR-16	Adilabad Rauni Ladhowal
	1b	LSMR-19 LSMR-17	Ladhowal PAU farm
G-2	2a	LSMR-10 LSMR-9 LSMR-8 LSMR-7	Narinagarh Manuke Manuke Rauni
	2b	LSMR-5	Manuke
	2c	LSMR-1	Manuke
G-3	3a	LSMR-12 LSMR-11 LSMR-4 LSMR14	PAU farm PAU farm Manuke Narinagarh
	3b	LSMR-22	Manuke
G-4	4a	LSMR-13 LSMR-3 LSMR-23	Narinagarh Manuke Sangrur
	4b	LSMR-18 LSMR-2	PAU farm Manuke
G-5	5a	LSMR-20 M-1	Parbhani Reference culture
	5b	LSMR-15	Ladhowal

Results are similar with the findings of Belal *et al.,* (2013) who observed that fast growing *Rhizobium leguminosarum* isolated from faba bean showed 7 mm zone of solubilization and 640 µgml^{-1} of P-solubilization at 7 days of incubation. These observations suggest that slow grower *Bradyrhizobium* sp. are poor inorganic P-solubilizers as compared to fast grower *Rhizobium* sp. of summer mungbean.

Kumar and Ram, (2014) also reported, maximum solubilization efficiency of 125% was recorded with the strain MRR104 while it is between 40-75% in rest of strains.

Genetic diversity analysis with RAPD-PCR technique

Traditional methods used for distinguishing morphological and functional traits of rhizobial strains are frequently fail to identify the strains within a species. So, it is necessary to obtain a better understanding of microbial diversity with strain identification at molecular level. In present study, analyses of twenty three rhizobial strains were done by using 5 oligonucleotide RAPD primers. PCR amplification with some primers indicated that each primer- template yielded distinct,

easily detectable bands of variable intensities (Plate 2a and 2b). The bands used for polymorphism study were those reproducible over repeated runs with sufficient intensity to detect presence or absence with confidence.

Out of five, two primers amplified the genomic DNA of all the rhizobial strains, giving 22 alleles, ranging from 250bp to 3.0 kb size. The additional alleles generated by other three primers could not be included in the study as amplification was not observed in most of the strains. From these 22 alleles, 21 alleles were polymorphic between one and other strains. Further Euclidian similarity coefficient was estimated which ranged from 0.00 to 1.00 (Table 3).

Genotypes based on RAPD markers (Fig. 2) revealed that all the rhizobial strains converged into 5 distinct groups viz. G-1, G-2, G-3, G-4 and G-5 (Table 4). Group G-1, was further divided into two subgroups 1a (LSMR-6, LSMR-16, LSMR-21) and 1b (LSMR-17 and LSMR-19). Similarly G-2 sub- divided into 3 groups, 2a (LSMR-10, LSMR-9, LSMR-8, LSMR-7), 2b (LSMR-5) and 2c (LSMR-1); again G-3 sub-divided into 2 groups, 3a (LSMR-12, LSMR-11, LSMR-4, LSMR-14) and 3b (LSMR-22). G-4 sub-divided into 2 groups, 4a (LSMR-13, LSMR-3, LSMR-23) and 4b (LSMR-18, LSMR-2) and G-5 subdivided into 2 groups, 5a (LSMR-20, M-1) and 5b (LSMR-15).

There was low degree of similarity detected between *Rhizobium* sp. indicated high genetic diversity between the strains (G-2: LSMR 1, LSMR 5, LSMR 8, LSMR 9; G-3: LSMR 4, LSMR 22 and G-4: LSMR 3) isolated from Manuke location with three different groups of clusters. Similarly, Naz *et al.*, (2009) reported lowest similarity coefficient (0.273) between isolates Rkh 2 and Rkh 3, collected from Khewra salt range and they also belong to two different groups of clusters.

Good level of genetic diversity was observed among rhizobial strains in present study. Although, LSMR-21 and LSMR-6, LSMR-19 and LSMR-17; LSMR-7, LSMR-8, LSMR-9 and LSMR-10 strains of rhizobia found genetically identical as revealed by 100% similarity. Sajjad *et al.,* 2008 also found highest similarity (0.8162) between the rhizobial strains of F-1 and L-3, lowest similarity (0.5946) was present between L-3 and L-29. Two isolates each from PAU (LSMR 12 and LSMR 18) and Narinagarh farms (LSMR 10 and LSMR 14) and 1 from Ladhowal farm (LSMR 15) were found to be *Bradyrhizobium* sp. The PCR results indicated the presence of specific bands between 1000-1500 bp in all the isolates of *Bradyrhizobium* sp. of summer mungbean.

The development of RAPD-PCR analysis provided a new tool for investigating genetic polymorphisms in many different organisms and recently has been used for *Rhizobium* identification and *Bradyrhizobium* genetic analyses (Sajjad *et al.,* 2008, Kang *et al.,* 2012).

Three *Rhizobium* strains (LSMR-1, LSMR-19 and LSMR-23) were found very promising based on biochemical and multifunctional PGP traits and were genetically diverse showing 33 to 60% similarity. Thus the preliminary information generated in present study could be useful for selection of single strain representing one genetic group for extensive field evaluation.

References

Anonymous, 2014. *Package of practices for Rabi crops.* Pp. 39-41. Punjab Agricultural University, Ludhiana.

Appunu, C., Sasirekha, N., Ramalingam, V., Prabavathy and Nair, S. 2009. A significant proportion of indigenous rhizobia from India associated with soybean (*Glycine max* L.) distinctly

belong to *Bradyrhizobium* and *Ensifer* genera. *Biology and Fertility of Soils* 47:56-63.

Arora, D. K., 2007. Microbial identification modules for some agriculturally important micro-organism pp 97-107. *National Bureau of Agriculturally important micro-organism* (NBAIM) Mau.U.P.

Belal, E. S., Hassan, M. M. and El-Ramady, H. R. 2013. Phylogenetic and characterization of salt-tolerant rhizobial strain nodulating faba bean plants. *African Journal of Biotechnology* 12: 4324-37.

Bhuyan, S.I., Hossain, M.S., Islam, M.M., Begum, S.N., Urbi, Z. and Hossain, M.S. 2014. Molecular assessment of Genetic Diversity and Relationship in selected Green gram Germplasms. *Biotechnology*, 13: 126-134.

Cappuccino, J. C., and Sherman, N. 1992. *Microbiology: A Laboratory Manual*. Pp. 125-79. New York.

Chen, W. M., Moulin, L., Bontemps, C., Vandamme, P., Bena, G. and Boivin-Masson, C. 2003. Legume symbiotic nitrogen fixation by beta-proteobacteria is widespread in nature. *Journal of Bacteriology* 185:7266–72.

Deka, A. K., and Azad, P. 2006. Screening for efficient strains of *Bradyrhizobium*. *Indian Journal of Pulses Research 19*: 79-82.

El-Hady. E.A.A.A., Haiba, A.A.A., El-Hamid, N.R.A., Al-Ansary, A.E.R.M.F. and Mohamed, A.Y. 2010. Assessment of Genetic Variations in Some *Vigna* Species by RAPD and ISSR Analysis. *New York Science Journal* 3: 120-128.

Fetyan, N. A. H., and Mansour, A. A. 2012. Molecular, Biochemical and Physiological characterization of symbiotic bacteria isolated from saline soil in Saudi Arabia. *Australian Journal of Basic and Applied Sciences* 6: 229-39.

Gherardi, M., Mangin, B., Goffinet, B., Bonnet, D. and Huguet, T. 1998. A method to measure genetic distance between allogamous populations of alfalfa (*Medicago sativa*) using RAPD molecular markers. *Theory Application Geneticia*, 96: 406-412.

Gordon, S. N., and Weber, R. P. 1951. Colorimetric estimation of Indole Acetic Acid. *Journal of Plant Physiology* 26:192-195.

Jackson, M. L., 1973. Estimation of phosphorus content. Soil chemical analysis, Printer Hall, New Delhi (India).

Kang, J.W., Song, J., Doty, S. L. and Lee, D. K. 2012. Diversity of rhizobia associated with leguminous trees growing in South Korea. *Journal of Basic Microbiology* 53: 291-98.

Kaur, H., Poonam Sharma, P., Kaur, N. and B S Gill, B.S. 2012. Phenotypic and Biochemical Characterization of *Bradyrhizobium* and *Ensifer* spp. isolated from soybean rhizosphere. *Bioscience Discovery* 3:40-46.

Kumar, G. K. M., and Ram, R. 2014. Phosphate Solubilizing Rhizobia Isolated from *Vigna trilobata*. *African Journal of Microbiology Research* 2(3):105-109.

Kumar, P. R., and Ram, M. R. 2012. Production of indole acetic acid by *Rhizobium* isolates from *Vigna trilobata* (L.) Verdc. *African Journal of Microbiology Research* 6: 5536-41.

Maruekarajtinplenga, S., Homhaulb, W. and Chansa-ngaveja, K. 2012. Presence of natural variants of *Bradyrhizobium elkanii* and *Bradyrhizobium japonicum* and detection of *Bradyrhizobium yuanmingense* in Phitsanulok province, Thailand. *Science Asia* 38: 24–29.

McInnes, A., Thies, J. E., Abbott, L.K. and Howieson, J.G. 2004. Structure and diversity among rhizobial strains, populations and communities–a review. *Soil Biology and Biochemistry* 36: 1295–1308.

Nautiyal, C. S., 1999. An efficient microbiological growth medium for screening phosphate solubilizing microorganisms. *FEMS Microbiology Letters* 170: 265-70.

Naz, I., Bano, A. and Hassan, T. U. 2009.

Morphological, biochemical and molecular characterization of rhizobia from halophytes of khewra salt range and attock. *Pakistan Journal Botany* 41: 3159-68.

Rai, R., Dash, P.K., Mohapatra, T. and Singh, A. 2012. Phenotypic and molecular characterization of indigenous rhizobia nodulating chickpea in India. *Indian Journal of Experimental Biology* 50:340-350.

Rosenblueth, M., and Martinez-Romero, E. 2004. *Rhizobium etli* maize populations and their competitiveness for root colonization. *Archieve of Microbiology* 181:337–344.

Safronova, V. I., Piluzza, G., Belimov, A. A. and Bullitta, S. 2004. Phenotypic and genotypic analysis of rhizobia isolated from pasture legumes native of Sardinia and Asinara Island. *Antonie van Leeuwenhoek* 85:115–127.

Sajjad, M., Tanwir, A. M., Arshad, M., Zahir, A. Z., Yusuf, F. and Rahman, S. U. 2008. PCR studies on genetic diversity of Rhizobial strains. *International Journal of Agricuture and Boilogy* 10: 505–510.

Sambrook, J., Fritsch, E.F. and Maniatis, T., 1989. *Molecular Cloning*. Cold Spring Harbor, New York: *Cold Spring Harbor Laboratory*, U.S.A.

Shahzad, F., Shafee, M., Abbas, F., Babar, S., Tariq, M. M. and Ahmad, Z. 2012. Isolation and Biochemical Characterization of *Rhizobium Meliloti* From Root Nodules Of Alfalfa (*Medico Sativa). Journal of Animal and Plant Sciences* 22(2): 522-524.

Somasegaran, P., and Hoben, H. J. 1994. *Handbook for Rhizobia: Methods in legume-Rhizobium Technology*. Pp 1-450. Springer-Verlag, New York.

Vincent, J. M., 1970. *A manual for the practical study of root nodule bacteria*. In I.B.P. Handbook No. 15. *Blackwell Scientific Publications*, Oxford, England. Pp. 73–97.

Weir, B. S., 2012. The current taxonomy of rhizobia. NZ Rhizobia website. http://www.rhizobia.co.nz/taxonomy/rhizobia.

Yadav, K., Yadav, S.K., Yadav, A., Pandey, V.P. and Dwivedi, U.N. 2014. Comparative Analysis of Genetic Diversity among Cultivated Pigeonpea (*Cajanus cajan* (L) Millsp.) and Its Wild Relatives(*C. albicans* and *C. lineatus*) Using Randomly Amplified Polymorphic DNA(RAPD) and Inter Simple Sequence Repeat(ISSR) Fingerprinting. *American Journal of Plant Sciences*, 5:1665-1678.

Zahir, Z. A., Shah, M. K., Naveed, M. and Akhtar, M. J. 2010. Substrate dependent auxin production by *Rhizobium phaseoli* improve the growth and yield of *Vigna radiata* L. under salt stress conditions. *Journal of Microbiology and Biotechnology* 20: 1288-1294.

Zohra, H.F., Mourad, K. and Meriem, K.H. 2016. Preliminary characterization of slow growing rhizobial strains isolated from *Retama monosperma* (L.) Boiss root nodules from Northwest coast of Algeria. *African Journal of Biotechnology* 15: 854-867.

How to cite this article:

Moumita Ghosh, Poonam Sharma, Asmita Sirari, Navprabhjot Kaur and Kailash Chand. 2017. Phenotypic and Genotypic Analysis of Summer Mungbean Rhizobia. *Int.J.Curr.Microbiol.App.Sci.* 6(9): 3149-3162. doi: https://doi.org/10.20546/ijcmas.2017.609.389