

Original Research Article

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## Isolation and Characterization of Nodule Associated Bacteria from Chickpea and their Potential for Plant Growth Promotion

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### ABSTRACT

A total of seventy three NAB were isolated from surface sterilized nodules of different chickpea (*Cicer arietinum*) cultivars grown in the field from IARI, New Delhi using various kinds of media. Screening for the improved seedling growth showed that 91.78% of them enhanced radical length, and 87.67% of them increased plumule length under *in-vitro* conditions. About twenty four isolates were selected from preliminary screening and were subjected to their growth promoting potential under pot culture conditions using selected cultivar (PUSA 372). The promising nodule associated bacteria (NAB 69) showed a significant increase in plant growth regarding shoot dry weight (40.63%) and root dry weight (45.09%) in comparison to control. Its interactive effect was evaluated with *Mesorhizobium ciceri* for growth attributes and nodulation potential under pot culture conditions. The highest nitrogenase activity (74.12  $\mu\text{moles C}_2\text{H}_4/\text{g}$  of fresh weight of nodules/hr) was recorded in the treatment involving NAB 69 along with *Mesorhizobium ciceri* at 45 DAS (Vegetative stage). *In-vitro* screening for the functional potential of NAB showed a positive result with P solubilization. However, nitrogenase activity and siderophore production were not detected in the selected bacterial isolate. The 16S rDNA sequencing revealed that the bacterial isolate was closely related to *Enterobacter* sp. with 99% maximum identity. This investigation led to identifying the natural associations between the most promising nodule associated bacterium and *Mesorhizobium ciceri* in chickpea and the positive influence of their interactions.

### Keywords

Chickpea,  
Endophytes,  
Root nodules,  
Growth promoting  
traits, Rhizobium,  
Nitrogen fixation,  
16S rRNA

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### Introduction

Chickpea (*Cicer arietinum*) is an important legume crop grown under rainfed agriculture in India. It's economic success relies on symbiotic nitrogen fixation (SNF) with root nodulating bacterium *Mesorhizobium ciceri*. These bacteria regularly interact with other rhizospheric microorganisms as well as host endophytic microorganisms. The endophytic bacteria reside latently or colonize the plant tissues actively without causing any apparent harm (Samish *et al.*, 1963).

Endophytic bacteria, in addition to colonizing roots, shoots, leaves, seeds, and fruits, etc., these are also reported to colonize nodules of legume plants. The nodule endophytes have been isolated from alfalfa, clover, pea, bean, chickpea, soybean and lotus, etc. (Hung and Annapurna, 2004; Muresu *et al.*, 2008; Dudeja *et al.*, 2012), and a great diversity of these has been reported depending on the host genotypes and environmental conditions. The endophytic bacteria mainly belong to the

members of *Methylobacterium*, *Devosia*, *Blastobacter*, *Ochrobactrum*, *Shinella*, *Burkholderia*, *Cupriavidus*, *Bacillus*, *Pseudomonas* and enterobacterial species (Li *et al.*, 2008; Zhao *et al.*, 2011). Some of these are known to stimulate plant growth, nitrogen fixation and also may induce of resistance to plant pathogens.

Endophytic symbiotic association between root nodulating *Rhizobium* and legumes is well documented where the microsymbiont fixes nitrogen in exchange of carbon from the host plant. Members of endosymbiotic Rhizobiaceae include the genera of *Rhizobium*, *Bradyrhizobium*, *Sinorhizobium*, *Mesorhizobium*, *Azorhizobium* and *Allorhizobium*, which nodulate different legume crops. Different plant species are reported to harbour endophytes in the range of  $10^3 - 10^6$  CFU (Colony Forming Unit)/g tissue. The presence of endophytic bacteria depends on the plant genotype, plant age, tissue sampled, and also the season of isolation (Kuklinsky-Sobral *et al.*, 2004). Further, nutrient availability, as well as soil type, may also determine their abundance and diversity. Non-rhizobial plant growth promoting endophytes such as *Arthrobacter*, *Bacillus*, *Burkholderia*, *Dyella*, *Methylobacterium*, *Microbacterium*, *Staphylococcus*, *Streptomyces* and *Bacillus* can also colonize nodules of a wide range of legumes (Tokala *et al.*, 2002; Bai *et al.*, 2002). Co-inoculation of *Rhizobium* with nodule endophytes improved plant growth, nodulation and yield in different legume crops (Sturz *et al.*, 1997; Pandey *et al.*, 2005; Rajendran *et al.*, 2008). There is limited information available on the interactive effect between rhizobia and other Nodule associate bacteria (NAB) on nodulation efficiency and associated crop response in chickpea. Therefore, the present study was focused to identify and characterize the nodule associated bacterium and its interaction with

*Mesorhizobium* for their functional role as well as growth and productivity of chickpea.

## **Materials and Methods**

### **Sampling procedure for chickpea crop**

Healthy and undamaged plant samples of different chickpea cultivars (BGD 72, PUSA 372, GNG 1581, PUSA 547, K 850, PUSA 256, ICC 5335, BGD 1005, SUBHRA) grown at IARI research field were uprooted at vegetative stages (45 DAS) of crop growth.

### **Nodule associated bacteria in chickpea**

#### **Isolation and purification**

Nodule associated bacteria (nodule endophytes) were isolated from chickpea root nodules. Nodule tissues were surface sterilized using 4% sodium hypochlorite (NaOCl) for 4 min. followed by washing with sterile distilled water several times. It was then treated with 70% ethanol and washed at least five times with sterile distilled water (Gagne *et al.*, 1987). Surface sterilized nodule tissue was then macerated in surface sterilized mortar and pestle and serially diluted using water blanks. After authenticating surface sterilization procedure, suitable dilutions (100 µl) were spread plated on different media *viz* Yeast Extract Mannitol Agar (YEMA), Trypticase Soy Agar (TSA), Pikovskaya Medium, Nutrient Agar, Kings B Medium, R2A Agar and Jensen's Medium. The plates were incubated at  $28 \pm 2^\circ\text{C}$  for 24 hr and the isolates were purified by quadrant streaking on respective growth medium.

#### **Growth and maintenance**

Single and purified nodule associated bacterial colony was grown on nutrient agar medium. The working cultures were grown on nutrient agar slants and maintained at  $4^\circ\text{C}$ .

Sub culturing was done as and when required. Stock culture of each isolate was also maintained as 15% glycerol stock at -20°C. The isolates were named as NAB and suffixed with arabic numeral to specify the isolate number.

### **Preliminary screening of nodule associated bacteria by seed bioassay under *in-vitro* conditions and experimental observations**

Preliminary screening of nodule associated bacterial isolates for the growth of chickpea was performed by seed bioassay. Healthy chickpea seeds (cultivar PUSA 372) were sterilized by treating with 70% ethanol for 30s, followed by 0.1% mercuric chloride for 3 min and then rinsing several times with sterile distilled water. Seed inoculation was performed by soaking surface sterilized seeds in bacterial suspension from exponential growth stage for 30 min. These inoculated seeds were transferred to 0.8% water agar plates, incubated at 24±2°C for 4 days and observed for seedling growth. Uninoculated control was also maintained. Observations on radical and plumule length, as well as germination percentage were measured and compared with appropriate control. The percent germination (%) was calculated by dividing number of seed germinated to the total number of seeds sown. Seedling growth (cm) was measured in terms of radical and plumule length per germinated seed and average was calculated. Promising endophytes were selected for further screening. The selected bacterial isolates from preliminary screening were further reconfirmed by seed bioassay.

### **Secondary screening of nodule associated bacteria for growth of chickpea under pot culture conditions**

About twenty four selected bacterial isolates screened after seed bioassays were assessed for their influence on the growth of chickpea

plants under pot culture conditions. Pots of size 4" were filled with 2.5 kg of soil taken from IARI fields. Surface sterilized chickpea seeds (PUSA 372) were subjected to seed bacterization. A total of twenty five treatments with the completely randomized design were replicated three times, and three treated seeds were sown at 5 cm depth with equal spacing. Seeds without bacterial inoculation were used as the controls. Plants were harvested after 45 days and observations were scored on nodule numbers, root and shoot dry weight per plant. Root and shoot samples were dried in an oven at 60°C till constant weight was achieved and each treatment, average root and shoot dry weight was calculated and expressed as mg per plant. The best isolate in terms of its influence on plant parameters was selected for further study.

### **Characterization of most promising bacterial isolate for plant growth promoting traits**

Broth culture of the selected bacterial isolate was grown in nutrient broth with titer value of 10<sup>6</sup> cfu/mL. The isolate was screened for its functional attributes such as nitrogenase activity as Acetylene Reduction Assay (ARA), P solubilization, IAA production, HCN production and siderophore production.

### **Nitrogenase activity**

The promising bacterial isolate was characterized for nitrogenase activity by the method of Hardy *et al.*, (1973) using N-free Jensen's medium. Bacterial culture aliquots containing approximately 10<sup>5</sup>-10<sup>6</sup> cells were streaked on the slants of Jensen's medium and incubated at 28°C for 5-7 days. Following the incubation, the tubes were sealed with sterile suba seals. Ten percent of air space (v/v) was replaced with acetylene gas and tubes were then incubated for 24 hrs. Appropriate control was also maintained under similar conditions. One mL of air sample was injected into

preheated gas chromatograph (Nucon 5765 model) housing Porapak N column with Flame Ionization Detector (FID). The column temperature was maintained at 100°C whereas injector and detector temperature were maintained at 110°C. The peak area of standard ethylene was used for calculation and activity was expressed in terms of  $\mu\text{moles of C}_2\text{H}_4 / \text{mg protein} / \text{hr}$  as per equation.

$$\frac{C \times P_s \times A_s \times V}{P_{\text{STD}} \times A_{\text{STD}} \times T \times P}$$

Where, C = Concentration ( $\mu\text{moles}$ ) of standard ethylene,  $P_s$  = Peak area of the sample,  $A_s$  = Attenuation used for sample, V = Volume of air space in the test tube,  $P_{\text{STD}}$  = Peak area of standard ethylene,  $A_{\text{STD}}$  = Attenuation used for standard ethylene, T = Incubation time (hours), P = Protein content (mg/mL)

### **Phosphate solubilization ability**

Selected endophyte was characterized for its ability to solubilize phosphate which was detected by using NBRIP medium developed by Nautiyal (1999). The medium was autoclaved, poured in plates and grids were prepared on Petri plates. To each grid, 8 $\mu\text{l}$  of bacterial culture ( $10^4$  CFU) was spotted. After spot inoculation, plates were incubated at 28 $\pm$ 2°C for 6 days and observed for the development of P-solubilization zone around the colony.

### **Siderophore production**

Siderophore production by selected bacterial isolate was detected by using Chrome azurol-S assay (CAS) developed by Schwyn and Neilands (1987). The plates were prepared by mixing 100 mL CAS mixture with 300 mL nutrient agar medium. Selected bacterial culture (10  $\mu\text{L}$ ) containing at least  $10^4$  bacterial cells was spotted on CAS plate and

incubated at 28 $\pm$ 2°C for 7 days. The colony surrounded with deep yellow to orange colour was a positive indication of siderophore production.

### **Interactive effect of most promising bacterial isolate with *Mesorhizobium ciceri***

The interactive effect of most promising nodule associated bacteria and *Mesorhizobium ciceri* was studied on nodulation potential, growth attributes and nutrient uptake in chickpea variety PUSA 372 under pot culture conditions. Physio-chemical properties were analyzed in the soil used in the pots as per standard protocols. Surface sterilized seeds of chickpea were inoculated with exponentially grown bacterial culture for 30 min to have approximately  $10^6$  cells per seed. Pots of 4" size were filled with 3 kg of soil and three seeds were sown in each pot containing field soils. The treatment details of the pot experiment are as follow- T<sub>1</sub> - Absolute control (No fertilizer, No Inoculation), T<sub>2</sub> - *Mesorhizobium ciceri* alone, T<sub>3</sub> - NAB alone, T<sub>4</sub> - *Mesorhizobium ciceri*+NAB, T<sub>5</sub> - 50% Recommended dose of fertilizer (RDF), T<sub>6</sub> - *Mesorhizobium* + 50% RDF, T<sub>7</sub> - NAB + 50% RDF, T<sub>8</sub> - *Mesorhizobium ciceri*+NAB+50% RDF, T<sub>9</sub> - 100% RDF (positive control).

*Mesorhizobium ciceri* available in the Division of Microbiology was used in this study and Di-ammonium phosphate (DAP) @100 kg/ha was used in RDF treatment. The plants were carefully uprooted after 45 DAS and observations were recorded on dry matter accumulation, nodulation, nitrogenase activity and N and P uptake.

### **Nitrogenase activity as Acetylene Reducing Activity (ARA) in chickpea root nodules**

Plants were uprooted carefully and adhering soil was removed from roots carefully. Root nodules after recording fresh weight were

transferred into assay vials and sealed with Suba seal. In each vial, 10% of air was replaced with equal volume of acetylene gas and incubated at  $28 \pm 2^\circ\text{C}$  for one hr. ARA activity was determined as described in the earlier section. Nitrogenase activity was determined by Acetylene Reducing Assay and expressed as  $\mu\text{moles of C}_2\text{H}_4/\text{g}$  nodule fresh weight/hr.

### **Dry matter accumulation**

Root and shoot samples were dried in an oven at  $60^\circ\text{C}$  till constant weight and biomass was calculated as mg dry weight per plant.

### **Nodulation potential**

Nodules were detached from the roots and nodule number per plant as well as their fresh weight (mg) was recorded. These parameters were expressed on per plant basis.

### **Identification of promising nodule associated bacteria using 16S rRNA partial gene sequencing**

#### **Isolation of genomic DNA and 16S rRNA gene amplification**

Molecular identification of promising bacterial isolate was undertaken by 16S rRNA gene amplification followed by sequencing. The selected bacterial isolate was grown in LB broth and incubated under optimal conditions. The genomic DNA was extracted using Sigma GenElute Bacterial genomic DNA kits, USA. The PCR reaction was performed by selective amplification of 16S rRNA gene using equimolar concentrations of both forward primer - fD11 and reverse primer - rP2 (Weisburg *et al.*, 1991). PCR master mix containing Taq DNA polymerase, dNTPs, Tris-HCl,  $\text{MgCl}_2$  stabilizer and tracking dye was used according to the manufacturer's instructions (GE healthcare

Life Sciences). The PCR reaction conditions used were 1 min at  $94^\circ\text{C}$ , 1 min at  $55^\circ\text{C}$  followed by 2 min at  $72^\circ\text{C}$  for 30 cycles. The amplicon size of 1.4 kb was recovered from agarose gel using a gel extraction kit (Sigma Life Science GenElute gel extraction kit, USA).

### **Sequence analysis**

The purified PCR product was subjected to sequence analysis by Central Instrumentation Facility (CIF), Biotech Centre, Delhi University (South Campus), New Delhi and the nucleotide sequence of the amplified product was determined using same set of primers used for PCR amplification. The analysis of sequence was undertaken using Cap 3 software available online. Bacterial isolate was identified by comparative matching of the 16S rRNA gene sequence with homologous sequence using NCBI BLAST search tool (<http://blast.ncbi.nlm.nih.gov/>).

### **Statistical analysis**

SPSS 16.0 statistical software was used for all quantitative data analysis including standard errors, critical difference, and analysis of variance (ANOVA).

## **Results and Discussion**

### **Isolation and purification of nodule associated bacteria from Chickpea cultivars**

Different cultivars (BGD 72, PUSA 372, GNG 1581, PUSA 547, K 850, PUSA 256, ICC 5335, BGD 1005, SUBHRA) of chickpea grown at ICAR-IARI, New Delhi research field were used for isolation of Nodule Associated Bacteria (NAB) or endophytes. The plants were uprooted at vegetative stages (45 DAS) and nodules were used for isolation

of bacteria. A total of 73 endophytes showing different colony morphology were isolated using different media as discussed in material and method and bacterial isolates were selected on the basis of morphological parameters *viz.* size, shape, margin, colour, appearance and texture. Amongst the various media employed, Jensen's Medium showed highest number (19 morphotypes) of bacterial isolates followed by Yeast Extract Mannitol Agar (17 morphotypes), followed by Pikovskaya Medium and R2A Agar Medium (10 morphotypes each). Nine different morphotypes were also isolated on Trypticase Soy Agar. Minimum endophytic diversity was depicted by King's B Medium and Nutrient Agar Medium showing five and three different morphotypes respectively.

Out of the eleven different chickpea cultivars used, highest number (20 types) of morphotypes for nodule associated bacteria were obtained from cultivar BGD 72 followed by GNG 1581 which gave 13 types. In other cultivars, nine different morphotypes were isolated from PUSA 547 and PUSA 372, eight from PUSA 256 and five were obtained from K 850. The chickpea cultivars BGD 1005 and SUBHRA yielded four different types of nodule associate bacteria, and only one morphotype was obtained from cultivar ICC 5335. It is indicating that genotype mediated variation in endophytic colonization and the benefits conferred by endophytes can be cultivar specific (Pillay and Nowak 1997; Conn *et al.*, 1997; Bensalim *et al.*, 1998).

In addition to microsymbiont, chickpea nodules harbour non-rhizobial endophytic microorganisms, and different cultivars of the same host are normally associated with a diverse range of endophytic microorganisms (Strobel and Daisy, 2003). Endophytic bacteria have been isolated from flowers, fruits, leaves, stems, roots and seeds of

various plant species (Kobayashi and Palumbo, 2000) and their population have been reported in the range of  $10^2$  -  $10^4$  cfu g<sup>-1</sup> tissue (Kobayashi and Palumbo, 2000).

### **Preliminary screening of nodule associated bacteria by seed bioassay under *in-vitro* conditions**

Preliminary screening of nodule associated bacteria was undertaken for improved seedling growth of chickpea (cultivar PUSA 372) using water agar plates. A total of 73 bacterial isolates were used for seed bioassay. These bacterial isolates improved the seed germination and seedling growth significantly. In all the treatments, 100% seed germination was observed.

A total of 91.78% bacterial isolates showed increase in radical length as compared to uninoculated control treatment and six isolates showed negative effect on radical length promotion when compared to control. Seven isolates *viz* NAB 15, NAB 20, NAB 60, NAB 62, NAB 63, NAB 64, and NAB 69 improved chickpea radical length in the range of 60-75% increase over control. This range was followed by six isolates namely NAB 19, NAB 65, NAB 66, NAB 71, NAB 72 and NAB 73 which showed an increase of 45-60% over control whereas twenty two isolates showed 15-45% increase and the remaining showed less than 15% increase over control (Fig. 1). Endophytes isolated from diverse crops are known to produce different growth promoters as well as improve plant growth (Khan and Doty, 2009; Sgroy *et al.*, 2009; Camerini *et al.*, 2008; Panchal and Ingle, 2011; Zhao *et al.*, 2011).

Bioassay screening also showed improved plumule length of germinated seeds with inoculation of nodule associated bacteria. Out of the total, selected isolates of 83.56% showed a positive effect on increased plumule

length and twelve isolates showed reduced effect in comparison to control. Highest increase (63.04% over control) of plumule length with NAB 64 was observed followed by NAB 62 where as seven isolates showed a percent enhancement in the range of 45-60%; on the other hand, six exhibited an enhancement in the range of 30-45% and remaining isolates showed an enhancement which was less than 30% over control. On the basis of this, 24 NAB were selected for further screening using plant bioassay (Fig. 2).

### **Secondary screening of nodule associated bacteria for growth of chickpea under pot culture conditions**

The plant growth promoting efficiency of selected (total 24 isolates) bacterial isolates from *in-vitro* screening was assessed in chickpea, cultivar PUSA 372 under pot culture conditions.

Highest shoot dry weight increase (40.63% over control) was observed when seeds were inoculated with NAB 69 followed by an enhancement of 39.58% with NAB 37. Out of the total, 41.66% isolates showed a significant increase in the range of 30-50% in shoot dry weight and only one strain, NAB 55 exhibited the lowest increase of 5.21% in shoot dry weight (Fig 3). Endophytic and rhizospheric bacteria are known to play an important role in plant yield and growth promotion, plant health, and protection (Hallmann and Berg, 2006; Ryan *et al.*, 2008; Saini *et al.*, 2015).

There was also a significant improvement in root dry weight with inoculation of nodule associated bacteria and all isolates depicted positive effect. About 29.16% of selected endophytes improved root weight in the range of 40-60% over control where as NAB 14 and NAB 53 showed only 13.39% increase. Out of all the isolates tested, NAB 69 showed fivefold increase in shoot dry weight which

was 40.63% over control as well as threefold increase (45.09% increase over control) in root dry weight as compared to control. About 37.50% of isolates were observed to increase total plant weight in the range of 30-50% (Fig 3). Therefore, on the basis of secondary screening, this particular isolate, i.e, NAB 69 was selected and tested for its functional plant growth promoting (PGP) traits as well as its interactive effect with *Mesorhizobium ciceri*.

### **Characterization of most promising bacterial isolate for plant growth promoting traits**

The studies related to plant growth promoting traits in the selected bacterial isolate NAB 69 showed no nitrogenase activity and P solubilizing potential was moderate. The selected bacterial isolate did not show siderophore production. Endophytic and rhizospheric bacteria are known to play an important role in plant yield and growth promotion, plant health, and protection (Hallmann and Berg, 2006; Ryan *et al.*, 2008; Saini *et al.*, 2015). The selected endophytic bacteria produced IAA at a low level (1 ppm) and showed a positive result for P solubilization which was in congruence with the report of Li *et al.*, (2008).

### **Interactive effect of most promising bacterial isolate with *Mesorhizobium ciceri***

The interaction effect of NAB and *Mesorhizobium ciceri* was evaluated on dry matter accumulation, nodulation potential, nitrogenase activity and N and P uptake. Physico-chemical properties of soil used in the experiment were organic carbon 0.42%; available nitrogen 66.8 kg/ ha; pH of 7.8 and EC of 0.38 mS/ cm.

### **Dry matter accumulation**

The percent increase in root dry weight varied from 24.33 to 63.45% over uninoculated

control. All the treatments showed positive effect on root biomass and the treatment T<sub>2</sub> with *Mesorhizobium* alone improved root growth (63.45% over control) followed by the treatments T<sub>7</sub>, T<sub>9</sub> and T<sub>6</sub>. The treatment with 50% RDF alone showed minimum increase (24.33%). Similarly, all the treatments showed enhanced shoot growth when compared to absolute control (T<sub>1</sub>). Highest increase in shoot dry weight (76.87%) was observed in treatment T<sub>8</sub> (*Mesorhizobium*+NAB+75% RDF) followed by treatments T<sub>9</sub> (100% RDF) and T<sub>7</sub> (NAB+50% RDF). The lowest percent increase over control in the shoot biomass was observed in T<sub>2</sub> (14.02%) and T<sub>3</sub> (19.21%). The treatment with *Mesorhizobium ciceri* along with NAB (T<sub>4</sub>) showed 65.71% increase over uninoculated control. All the treatments increases total plant dry weight in the range of 20-70% over absolute control. The highest increase in total plant dry weight was shown by treatment T<sub>9</sub> (67.21%) followed by treatment T<sub>8</sub> (65.21%), T<sub>7</sub> (64.61%) and T<sub>4</sub> (61.01%) while the lowest increase was found in treatment T<sub>3</sub> (22.80%) NAB alone (Fig. 4).

### **Nodulation potential and nitrogenase activity**

Inoculation with NAB 69 increased nodule number/plant in comparison to control treatment (T<sub>1</sub>). The percent increase over control was in the range of 30-75%. Highest value was observed in treatment T<sub>4</sub> (72.53% increase over control) followed by treatment T<sub>2</sub> and T<sub>8</sub>. The treatment (T<sub>9</sub> -100% RDF) showed only 6.67 nodule number per plant which was less when compared to the treatments involving inoculation. Corresponding to nodule number, the nodule fresh weight also enhanced with inoculation in compare to control (T<sub>1</sub>). All the treatments

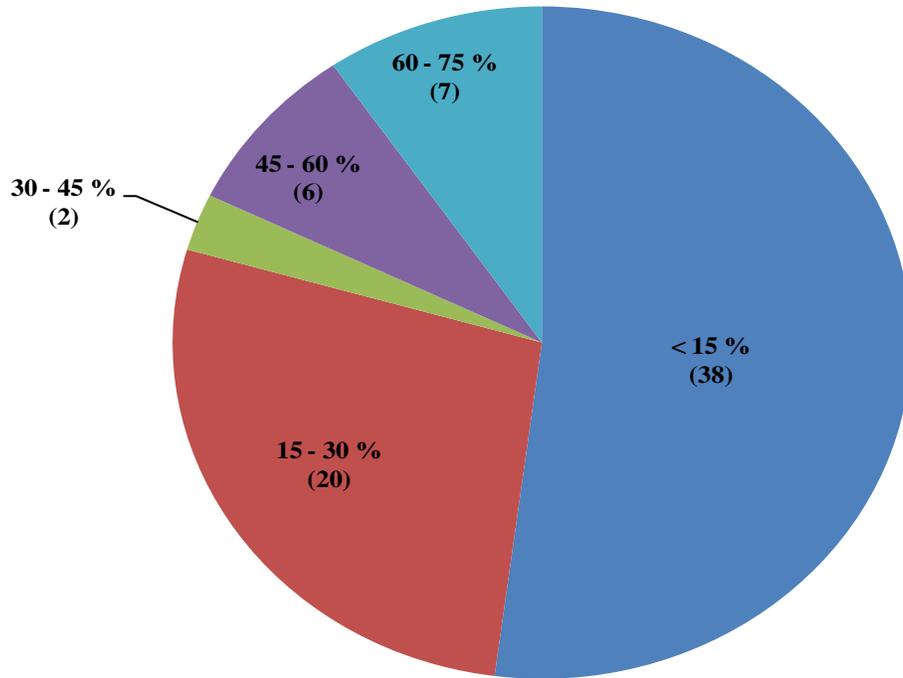
depicted improved nodule fresh weight in the range of 11-18 mg per plant. Treatment T<sub>4</sub> (*Mesorhizobium ciceri* + NAB) showed 63.64% increase over control followed by the treatment with *Mesorhizobium* alone. The Lowest percent increase over control (21.21%) in terms of nodule fresh weight was observed in treatment with 100% RDF (Fig.5). These are in consistent with the reports on alfalfa in which co inoculation of non-rhizobial strain with *Sinorhizobium meliloti* also influenced nodulation, however, no significant effect of *Sinorhizobium* sp. alone was reported (Stajkovoic *et al.*, 2009).

The nitrogenase activity was studied as Acetylene Reduction Assay (ARA) and was expressed in terms of  $\mu\text{moles of C}_2\text{H}_4/\text{g nodule fresh weight/hr}$ . The nodule ARA activity varied in the range of 11.64-74.12  $\mu\text{moles of C}_2\text{H}_4/\text{g nodule fresh weight/hr}$  under different treatments. The Highest ARA activity (74.12  $\mu\text{moles of C}_2\text{H}_4/\text{g nodule fresh weight/hr}$ ) was shown in treatment, T<sub>4</sub> (NAB with *Mesorhizobium ciceri*) followed by the treatment T<sub>2</sub> (*Mesorhizobium* alone) with the activity of 52.48  $\mu\text{moles of C}_2\text{H}_4/\text{g nodule fresh weight/hr}$ . The treatment, T<sub>8</sub> (*Mesorhizobium ciceri*+NAB +50% RDF) showed ARA activity of 49.55  $\mu\text{moles of C}_2\text{H}_4/\text{g nodule fresh weight/hr}$ . The Lowest ARA activity of 11.64  $\mu\text{moles of C}_2\text{H}_4/\text{g nodule fresh weight/hr}$  was observed in T<sub>9</sub> treatment (100% RDF), positive control (Fig.5).

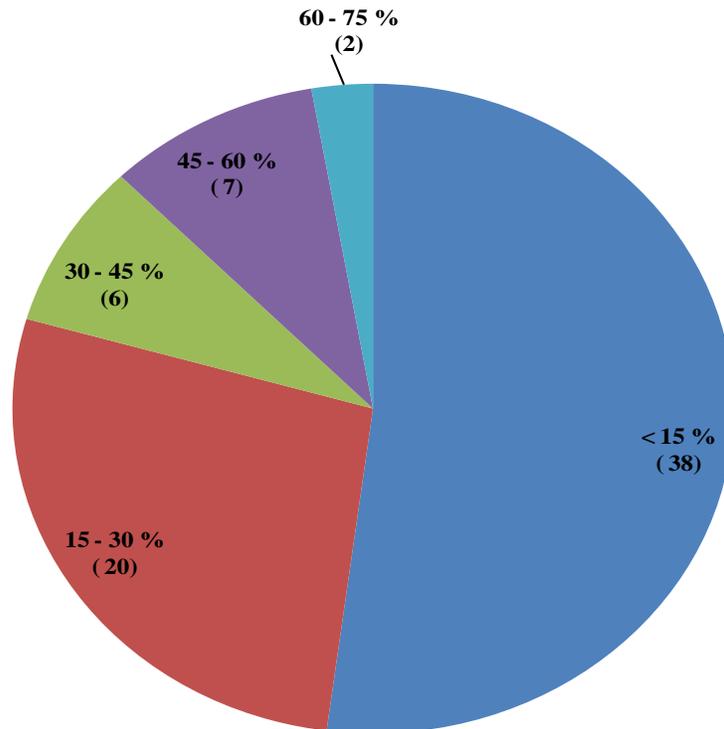
### **Identification of most promising bacteria based on 16S rRNA gene sequencing**

The blast search results using 16S rRNA sequence results using NCBI data base for NAB 69 showed a maximum identity of 99% with *Enterobacter* sp.

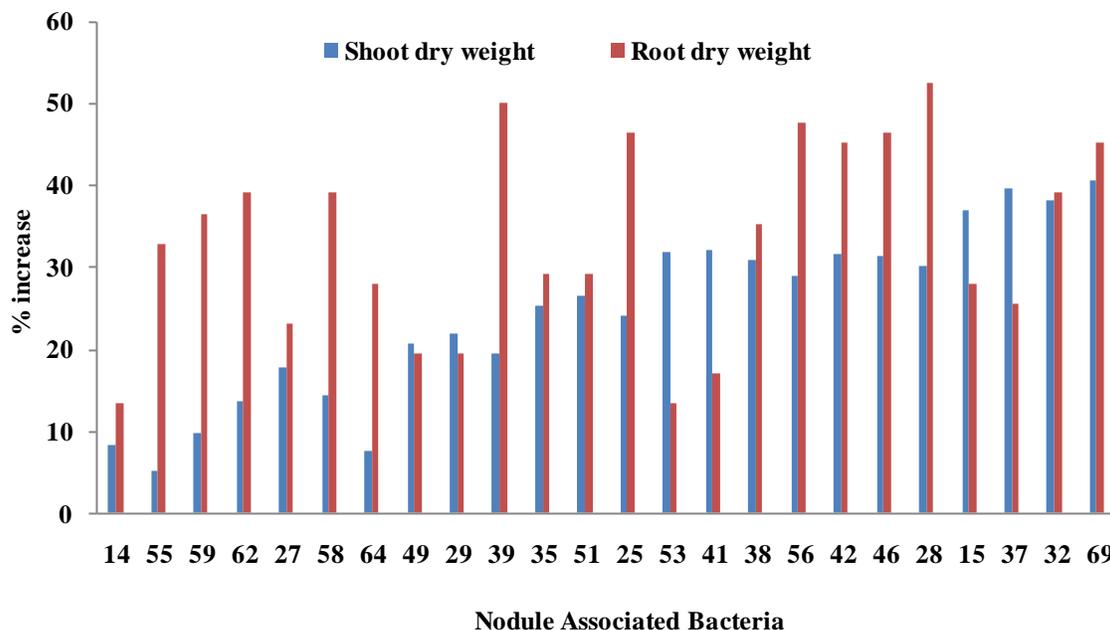
**Fig.1** Influence of nodule associated bacteria on the chickpea growth promotion in terms of radical length (% increase over control) using seed bioassay. Numbers in parentheses denote number of isolates



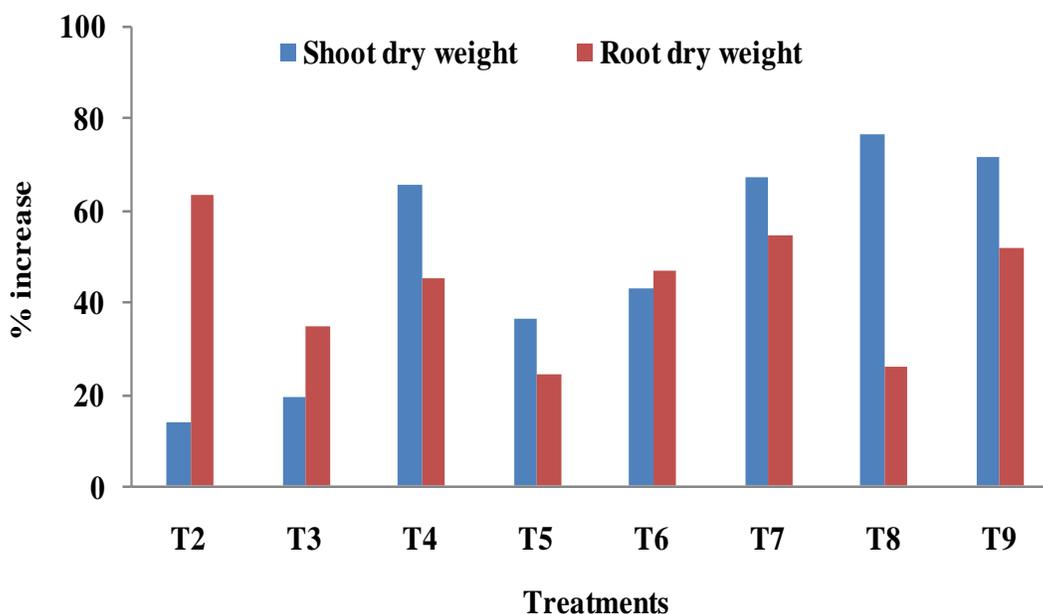
**Fig.2** Changes in the plumule lengths of chickpea due to the inoculation with nodule associated bacteria (percent increase over control). Numbers in parentheses denote the number of isolates



**Fig.3** Influence of nodule associated bacteria (isolates numbered differently) on chickpea plant growth in pot experiment (Percent changes in shoot and root dry biomass relative to the control treatment are provided)

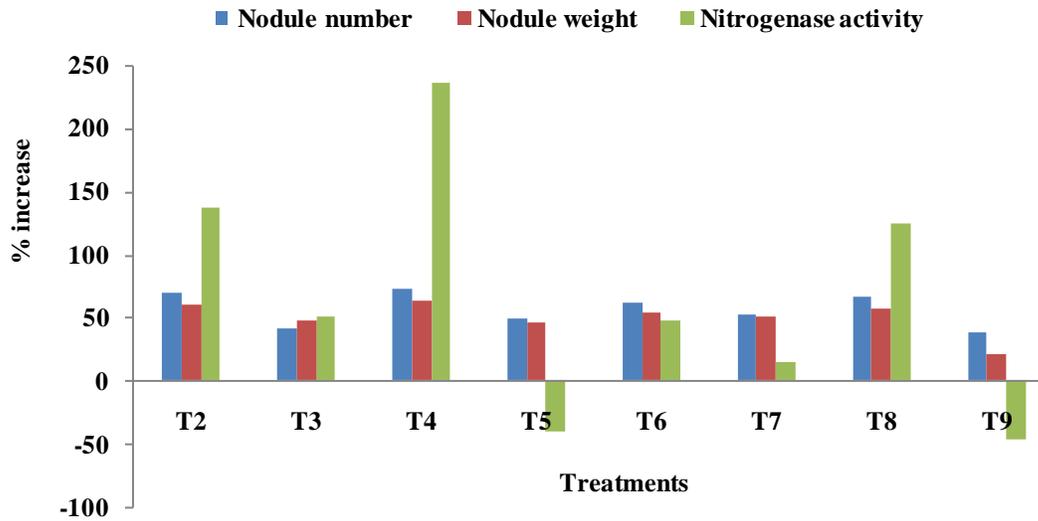


**Fig.4** Interactive effect of the promising nodule associated bacterium (NAB) and *Mesorhizobium ciceri* on chickpea growth



Percent increases or decreases over the control (T<sub>1</sub>) are provided. Treatment details are: Treatment details are: T<sub>2</sub>-*Mesorhizobium ciceri* alone; T<sub>3</sub>-NAB alone; T<sub>4</sub>- *Mesorhizobium ciceri*+NAB; T<sub>5</sub>-50% RDF (positive control); T<sub>6</sub>-*Mesorhizobium* + 50% RDF; T<sub>7</sub>-NAB + 50% RDF; T<sub>8</sub>-*Mesorhizobium ciceri*+NAB+50% RDF & T<sub>9</sub>-100% RDF (positive control).

**Fig.5** Nodulation potential and nitrogenase activity in chickpea as influenced by the interactive effect of the promising nodule associated bacterium (NAB) and *M. ciceri*



Percent increases or decreases over the control (T<sub>1</sub>) are provided. Treatment details are: Treatment details are: T<sub>2</sub>-*Mesorhizobium ciceri* alone; T<sub>3</sub>-NAB alone; T<sub>4</sub>- *Mesorhizobium ciceri*+NAB; T<sub>5</sub>-50% RDF (positive control); T<sub>6</sub>-*Mesorhizobium* + 50% RDF; T<sub>7</sub>-NAB + 50% RDF; T<sub>8</sub>-*Mesorhizobium ciceri*+NAB+50% RDF & T<sub>9</sub>-100% RDF (positive control)

The plant growth promoting functions of *Enterobacter* sp. isolated from poplar host has been reported (Taghavi *et al.*, 2010). However, systemic approach is required to understand its endophytic colonization, establishment as well as interaction with the plant (Taghavi *et al.*, 2009).

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