Diversity of rhizobia associated with chickpea germplasm in relation to nodulation and nitrogen fixation

SUMMARY OF

Thesis

SUBMITTED TO

BABASAHEB BHIMRAO AMBEDKAR UNIVERSITY

LUCKNOW

FOR THE DEGREE OF

Doctor of Philosophy

IN

ENVIRONMENTAL MICROBIOLOGY

Submitted By

Renu Verma

(Enrolment No. 1233/15)

Under the Supervision of

Co-Supervisor

Dr. Senthil Kumar Murugesan

Principal Scientist
Basic Science Division,
Microbiology Department
Indian Institute of Pulses Research, Kanpur

Supervisor

Prof. Naveen Kumar Arora

Dean
School for Environmental Sciences
Babasaheb Bhimrao Ambedkar
University, Lucknow-226025

DEPARTMENT OF ENVIRONMENTAL MICROBIOLOGY

SCHOOL FOR ENVIRONMENTAL SCIENCES

BABASAHEB BHIMRAO AMBEDKAR UNIVERSITY

(A CENTRAL UNIVERSITY)

VIDYA VIHAR, RAEBARELI ROAD, LUCKNOW-226025 (UP) INDIA

2022

In this study 214 Chickpea minicore were screened under field conditions for variations in BNF related traits such as nodule number per plant (NNPP) nodule dry weight (NDW), nodule fresh weight (NFW), delayed nodule senescence, plant dry weight (PDW) at four crop-growth stages like 15 DAS (days after sowing) 30 DAS, 60DAS, and 90 DAS. Nitrogen content of plant tissues was analyzed at 60 DAS. Data of all parameter were categorized in four subgroups i.e. poor, average, good and excellent. Chickpea minicore presented large variations for nodule number. The data analysis 15 days after sowing revealed that 11 genotypes did not produce any root nodules and they were considered as late nodulating genotypes however majority of chickpea genotypes were early nodulating genotypes at stage 1. Further, 9 genotypes produced less than 1 root nodule per plant as mean of three replications. Whereas only two genotypes i.e. ICC 1356, ICC 7819 were found as excellent nodulating genotypes, forming more than 20 nodules per plant and remaining 178 genotypes produced root nodules between 1-20 per plant. All 214 chickpea genotypes were found to produce root nodules in the range of 0-29 NNPP at stage 2. Among them three genotypes viz ICC506, ICC867, and ICC15406 produced more than 20 nodules per plant and considered as excellent nodulation category. Highest number of root nodule (29 NNPP) observed in ICC506; though it did not produce any root nodule at stage-1. Seven genotypes viz. ICC12968, ICC7867, ICC13816, ICC867, ICC15264, ICC15510, and ICC283 are common under high nodulation groups (>10NPP) of stage-1 as well as stage-2. Stage-3 (60DAS), 17 genotypes were found as excellent nodulating genotypes that produce more than 20 nodules per plant whereas seven genotypes were observed with no nodulation. The maximum number of root nodules per plant (41) was recorded in ICC14815 while ICC3325 and ICC6874 produce 35 and 36 NNPP respectively. 36 genotypes were poor nodulating genotypes. Moreover ten genotypes viz. ICC16374, ICC13816, ICC9002,

ICC12968, ICC6816, ICC11944, ICC4567, ICC506, ICC4533, and ICC14815 are common to the high nodulation category of stage-2 (>10NNPP) and stage-3 (>20NNPP). Two genotypes viz. ICC13816 and ICC12968 are consistently categorized as high nodulating genotypes at all three stages i.e. >10NPP for S1, S2, and >20NNPP for S3. At stage 4th, root nodules of 140 genotypes were completely degenerated and 17 genotypes were observed with delayed nodule senescence while 43 genotypes were under the degeneration. The maximum 38 NNPP was observed in ICC11121. Eight genotypes viz. ICC12307, ICC14402, ICC6874, ICC12155, ICC4463, ICC9002, ICC12968, and ICC3325 are common for the high nodulation category (>20NNPP) of stage-3 and stage-4. ICC12968 is ranked under high nodulating group of all 4 crop growth stages in comparison to the rest of the genotypes in the same category. ICC3325 was observed with high number of root nodules per plant constantly with 33 and 35 NNPP during stage-3 and stage-4 respectively. Chickpea minicore lines belong to desi-type were considered as a good source of variations for nodulation trait that ranges from 0-21 nodules per plant, while Kabuli and pea-shaped types produced maximum of 13 and 12 root nodules per plant, respectively at 15 DAS. In general the nodulation behavior at all 4 stages of crop growth is poor in pea-shaped chickpea lines and followed by Kabuli-chickpea. Highest number of NNPP (36) at stage-3 was observed in ICC6874 originated from Iran, while Indian chickpea lines produced higher NNPP at remaining stages of plant growth. Chickpea genotypes viz. ICC7668 (Russian Federation), ICC12155 (Bangladesh), ICC3325 (Cyprus) were produced more than 25 NNPP at Stage-3 as well as stage-4. However, ICC12155 (Bangladesh), ICC3325 (Cyprus) were considered as poor nodulators at early stages with more than 10NNPP. Among chickpea germplasm lines the nodule fresh weight and dry weight varied widely, ranging from 60 to 810 mg/plant and 3mg/plant to 600 mg/plant at crop-growth

stage-3 respectively. Eight genotypes viz. ICC13764, ICC762, ICC13892, ICC13599, ICC1431, ICC13863, ICC9755 and ICC14077 were found to produce more than 500mg NFW whereas 192 genotypes were produce less than 500 mg NFW per plant. Nodule dry weight in 195 genotypes were recorded as more than 500 mg while five genotypes i.e. ICC 1431, ICC 1915, ICC 9755, ICC13863, and ICC13892 produced in the range between 500mg to 1000 mg dry weight of root-nodules per plant. The nodule fresh weight of chickpea germplasm lines at the 4th stage varied greatly, ranging from 140 to 2170 mg/plant. The genotypes such as ICC12028, ICC1431, ICC13599, ICC3946, ICC13764, and ICC13863 had more than1.0g/plant nodule fresh biomass whereas ICC13863 had more than 2.0 g/plant NFW. At both stage-3 and stage-4, four genotypes were prevalent for the high nodule biomass category: ICC1431, ICC13599, ICC13764, ICC13863 and except ICC1431 all were not considered as high nodulation group at all four stages of crop growth. Since these genotypes were having pink root nodules, they are reflected to have the trait of late nodule senescence. The nodule dry weight of chickpea varied greatly amongst germplasm lines, ranging from 1.5 to 1745 mg/plant. In 189 genotypes, nodule dry biomass was observed as less than 500 mg/plant, while nodule dry weight was between 500 mg/plant and 1000 mg/plant were recorded in nine genotypes. High nodule dry weight per plant was obtained by two genotypes, ICC13764 and ICC13863, with 1254 mg/plant and 1746 mg/plant, respectively. In both stages 3 and 4, the genotype ICC13863 is associated with high nodule dry weight.

Plant dry weight of chickpea genotypes at first stage (15 DAS) of growth showed that 10 genotypes viz. ICC1915, ICC4495, ICC12328, ICC13219, ICC13357, ICC14199, ICC15333, ICC15406, ICC15435, and ICC 7867 were having high PDW i.e. more than 250 mg/plant. The PDW of 38 genotype were recorded in the range of 151-250 mg/plant and poor PDW (101-150 mg/plant) were recorded in 89 genotypes. Although PDW was

found to be very low in 62 genotypes, ranging from 50 to 100 mg/plant. The highest PDW (>500 mg/plant) were recorded in the minicore lines i.e. ICC6306, ICC8261, and ICC9137. In the second stage, 45 chickpea lines were identified, with PDW ranging from 310 to 500 mg/plant. 10 genotypes had high PDW ranging from 410 to 500 mg/plant, whereas 35 genotypes had average PDW ranging from 310 to 400 mg/plant. The remaining 84 genotypes had low PDW, ranging from 210 to 300 mg/plant. However, in 68 minicore lines PDW was found to be very low that ranging from 60 to 200 mg/plant. At 60 DAS, the plant dry weight of minicore lines was measured, and the findings revealed that three genotypes, ICC6306, ICC8261, and ICC9137, had the highest PDW (>4000 mg/plant). Among 214 gentypes, 20 genotypes had PDW in the range of 2100-4000 mg/plant, 105 genotypes had PDW in the range of 1100-2000 mg/plant, besides 68 genotypes had poor PDW in the range of 380-1000 mg/plant. In the fourth stage of crop growth the maximum PDW (>12000 mg/plant) was found in ICC15697, which was 15150 mg/plant. Only 6 genotypes i.e. ICC13764, ICC14199, ICC14402, ICC5612, ICC15435, ICC15610 showed high PDW in the range of 10180-12350 mg/plant and PDW in 65 genotypes were recorded in the range 5100-10000 mg/plant whereas in 80 genotypes PDW were found in the range of 3100- 5000 mg/plant. Though, 48 genotypes had low PDW, ranging from 600 to 3500 mg/plant.

The 395 root nodule associated bacteria were isolated from healthy surface of sterilized root nodules collected from 214 chickpea minicore lines. Some of the colonies of bacterial isolates from YEMA showed the characteristic elevation and EPS production. The morphological characters of colony such as shape and size were differed on YEMA or CRYEMA. The appearance of bacterial colonies on YEMA were off-white to creamish, with a few colonies yellow, light pink, and dark pink. Based on morphological characters 95 bacterial isolates were selected from 395 bacterial isolates.

Among all 95 bacterial isolates many were belonged to Gram negative and remaining were Gram positive. Under different culture conditions all bacterial isolates were showed different physiological behaviour. The different salt concentrations used to test the salinity tolerance of root nodule-associated bacteria. Among all tested isolates two isolates i.e. CGNE-281 and CGNE-365 failed to grow at 2% salt and maximum number of bacterial isolates were grown at salinity range 2% to 8% whereas at 10% salinity more than 30% bacterial isolates were grown. Many of the selected isolates were grew at pH range 5-9 and however none of them could grow at pH 4.

The plant growth promotion activity of all isolates was also determined through qualitative analysis. Most of the selected bacterial isolates showed PGP activities a total of 53.68 percent of the isolates showed phosphate solubilisation. Bacterial isolate CGNE-48 exhibited maximum phosphate solubilisation ability by 2.4 psi with the formation of maximum clear zone (2.4 cm diameter) while isolates CGNE-69 and CGNE-75 showed the minimum solubilisation index i.e. 0.6 psi. The 33.68 percent of bacterial isolates were capable to produce HCN and 47.36 % percent isolates showed siderophore production. The bacterial isolates CGNE-9, CGNE-17, CGNE-18, CGNE-19, CGNE-20, CGNE-30, CGNE-34, CGNE-39, CGNE-48, CGNE-51, CGNE-52, and CGNE-88 were found to have a high ability for siderophore production on the CAS plate, with a larger orange zone. In the presence of tryptophan, 34.73 percent of bacterial isolates produced IAA. Among all positive isolates 14 isolates i.e. CGNE-9, CGNE-17, CGNE-18, CGNE-19, CGNE-20, CGNE-27, CGNE-30, CGNE-34, CGNE-39, CGNE-49, CGNE-55, CGNE-57, CGNE-88, and CGNE-97 were having high potential for IAA production.

Based on morphological characteristics 95 root nodule associated bacteria were selected for further genotypic identification using 16S-rRNA sequencing. The obtained

sequences of 16S rDNA gene of the bacterial isolates was further subjected to NCBI BLAST analysis and the fasta sequences were submitted to Gen Bank (NCBI) and consigned with corresponding accession numbers. These isolates were identified as strains from 15 different genera based on phylogenetic analysis. Some bacterial isolates belonged to the Gram positive genera viz. *Bacillus, Staphylococcus*, and *Bravibacillus,* whereas the majority of bacteria isolated belonged to the Gram negative genera *Bacillus, Staphylococcus,* and *Bravibacillu*s. Therefore, Gram negative group of bacteria are considered as most diverse group of root nodules that colonizing chickpea minicore lines. These genera comprise *Mesorhizobium, Rhizobium, Ochrobacterium, Agrobacterium, Klebsiella, Enterobacter, Pantoea, Brevundimonas, Pseudomonas, Stenotrophomonas, Burkholderia, Acinetobacter*. Whereas maximum numbers of isolates belong to genera *Bacillus* and minimum number of isolates identified from genera *Brevundimonas*, *Bravibacillus*, and *Klebsiella*. Although 76 isolates belonged to non-rhizobia group, 19 isolates were belonging to rhizobia group. Among 19 rhizobial isolates 3 (CGNE-9, CGNE-17, and CGNE-19) belonged to genera *Mesorhizobium* and 16 isolates (CGNE-18, CGNE-20, CGNE-39, CGNE-41, CGNE-44, CGNE-48, CGNE-49, CGNE-51, CGNE-52, CGNE-55, CGNE-57, CGNE-79, CGNE-88, CGNE-97, CGNE-98, and CGNE-275) belonged to genera *Rhizobium*.

There are 11 clades in the phylogenetic tree of 16S rDNA. It was observed that CGNE -18, CGNE -20, CGNE -49, CGNE -17, CGNE-9, CGNE-19 showed little similarity with USDA110AB9094301 (*Bradyrhizobium diazoefficiens*) though they arised from same node they showed divergence and remaining isolates does not showed similarity with reference strain. All identified rhizobia isolates further used for diversity analysis based on phylogenetic analysis of functional (*nod*AB and *nif*H) housekeeping (*atp*D and *dna*K) genes. The rhizobial isolates were subjected to *nod*AB gene amplification

using degenerate primers and all isolates were positive for *nod*AB gene and produce the amplicon with expected size of 700 bp. *Nod*AB positive isolates were subjected to *nif*H gene amplification using degenerate primers. Out of 19 rhizobial isolates 13 isolates were positive for *nif*H with amplicon size 399bp and sequencing of *nif*H PCR product was done by using Sanger sequencing. Obtained fasta sequences were further subjected to blast analysis using NCBI and sequences submitted to Gene bank. The sequence similarity of the *nif*H gene sequences of chickpea-root nodule associated bacteria with different *Rhizobium* species ranged from 95% to 99 %. The phylogenetic analysis showed that CGNE-79 has evolved much earlier than MT39588 (*Rhizobium sp. strain VFEP81 nif*H) this means more divergence has occurred in CGNE-79 and *nif*H gene sequences of bacterial isolates did not show sequence similarity with that of reference strains. All *nif*H positive isolates were selected for PCR amplification of *atp*D gene using 294F and 771R primers resulted in a single band at 450 bp**.** Among 13 isolates 12 were positive for *atp*D gene and PCR product subjected for sequence analysis. Blast analysis was done by using NCBI. The *atp*D gene sequences of bacterial isolates were shown sequence similarity with the *atp*D gene of two different genera i.e. *Rhizobium,* and *Brucella* in the range of 92%–99%. *atp*D gene sequences of CGNE-19, CGNE- 44, CGNE- 49 and CGNE-275 were showed similarity with the *atp*D gene sequence of *Rhizobium* in the range 98.95%, 94.91%, 98.73% and 99.57% respectively, whereas gene sequence of remaining isolates i.e. CGNE-9, CGNE-17, CGNE-18, CGNE-20, CGNE-39, CGNE-48, CGNE-57 and CGNE- 79 were similar to the sequences of *atp*D gene of genera *Brucella* with the similarity 95.65%, 98.03%, 97.38%, 98.08%, 89.03%, 95.22%, 92.04%, and 96.89% respectively. The Phylogenetic analysis of *atpD* gene revealed that CGNE-44, CGNE-39B, CGNE-9 have evolved earlier than WP_012067133.1 *(Sinorhizobium sp.)* while LC460924.1 (*Rhizobium pusense AV5 atp*D gene) has evolved earlier to CGNE-49 and 19, CGNE-275 and LC417131.1 (*Bradyrhizobium sp.*) were found to be evolved from single

common ancestor. Further *dna*K gene amplification all *atp*D positive bacterial isolates was done by using TSdnaK3 and TSdnaK2 primers and the amplicon size is 330bp. Bacterial isolates resulted in a single band at 330 bp. Amplicons of *dna*K positive isolates was selected for sequencing and fasta sequences were further subjected to blast using NCBI. *dna*K gene sequences of root nodules associated bacteria were shown sequence similarity with the *dna*K gene of three different genera i.e. *Rhizobium, Bradyrhizobium,* and *Agrobacterium*. Gene sequence similarity of all isolates was showed in the range of 76% –99%. Although among them *dna*K gene sequence of 3 isolates i.e. CGNE-17, CGNE-20 and CGNE-275 were similar to *dna*K gene of *Bradyrhizobium* with the similarity of 76.64%, 99% and 96.19% respectively. The *dna*K gene sequence of isolates CGNE-9, CGNE-18, CGNE-19, CGNE-39, CGNE-44, CGNE-49, CGNE-57, and CGNE-79 were similar to *dna*K gene of *Rhizobium* with sequence similarity of 99.79%, 79.64%, 99.32%, 99.31%, 99.28%, 99.64%, 81.85% and 97.33%, respectively. *dna*K gene sequence of remaining one isolate i.e. CGNE-48 is similar to that of *Agrobacterium* with 95.59% sequence similarity. The phylogenetic study of *dna*K shown that *Bradyrhizobium species* (KR071074.1) and CGNE-20 has most recent common ancestor and the branch lengths appear to be similar indicating they have evolved at the same time.

Nodulation potential of selected root-nodules associated bacteria of chickpea minicore lines was assessed through plant infection test using Leonard jar experimental, plastic cups with a diameter of 12 cm and pots with a diameter of 20 cm set-up during *Rabi* season of 2017-18, 2018-19 and 2019-20 respectively. Chickpea seedlings of negative control did not produce any root-nodules whereas only a few root-nodules were found in the seedlings of the positive control, which were inoculated with *Mesorhizobium ciceri* strain HRU and RVSGRS-119 under described both experimental condition such as Leonard jar and plastic cups. However, putative endophytic bacterial isolates does

not able to produced root-nodules in chickpea *cv*. *Shubhra*. During Rabi 2019-2020, Chickpea cv. Shubhra (Kabuli type), RSG888 (Desi) and the concerned plant genotypes were used for plant infection test for nodulation potential of chickpea endophytes. Sixteen endophytic bacteria fail to nodulate chickpea *cv*. RSG888 whereas seven of them such as CGNE-9, CGNE-17, CGNE-19, CGNE-20, CGNE-39, CGNE-41, and CGNE-44 found to nodulate concerned chickpea genotypes from which they were isolated and remaining isolates did not produced any root nodules. The seven positive isolates enhanced the plant dry weight in the range of 3.81 to 60.07% compared to un-inoculated seedlings of concerned chickpea lines but isolates which are not able to produced nodules also enhanced the plant dry biomass. The majority of them, 11 of the 16 strains, had a negative interaction with chickpea cv. RSG888, resulting in reduced plant dry weight in compared to the un-inoculated control. Though, CGNE-19 and CGNE-57, showed a positive interaction with chickpea cv. RSG888 and the genotype from which they were isolated. This research is beneficial for diversity analysis of chickpea root nodule associated endophytes as well as host genotypes.

Present investigation resulted in the identification of early nodulating chickpea genotypes, host genotypes with delayed nodule senescence, and chickpea genotypes with high number of root nodules. These genotypes can be used to understand the molecular basis for desirable BNF traits and also be used in breeding program for developing elite chickpea cultivars with superior symbiotic efficiency. In this study, root nodulating bacteria other than *Mesorhizobium* such as *Rhizobium pusence,* and *R. lucaenae* were isolated from surface sterilized root nodules of chickpea minicore lines. The diverse nature of rhizobia and rhizobacteria associated with chickpea minicore can be used effectively for improving plant establishment, growth and symbiotic efficiency.