Journal of Crop and Weed, 12(3):14-19(2016)

Isolation of lentil-specific salt tolerant nitrogen fixing bacteria from Murshidabad district of West Bengal

A. HALDER, J. BANERJEE, P. BHATTACHARYYA, [1]K. PRAMANIK, AND [2]A. DEBNATH

Genetics and Plant Breeding, [1]Agricultural Biotechnology, [2]Agricultural Chemistry and Soil Science
Bidhan Chandra Krishi Viswavidyalaya, Mohanpur-741252, Nadia, West Bengal

Received :15-12-2016 ; Revised : 24-12-2016 ; Accepted : 28-12-2016

ABSTRACT

Lentil is a very important leguminous pulse crop in Indian agriculture. The unstable trend of lentil production is a major concern for food and nutritional security. Although different districts of West Bengal plays pivotal role in lentil production, very insignificant amount of production comes from the salinity stress affected area. Additionally the suitable Rhizobium strain and their performance in different environmental stress conditions in relation to lentil production is not yet evaluated. In the present study, we are interested to isolate different lentil-specific nitrogen fixing bacterial isolates from Murshidabad district of West Bengal. Sequence analysis revealed that some of the isolated strains are Rhizobium sp. either closely or distantly related to R. leguminoserum. Different isolated strains have been analyzed for salt tolerance. These salt tolerant strains might be useful to make bio-consortium which will be further used to popularize lentil production in the salt affected regions of West Bengal.

Keywords: Lentil, plant growth promoting rhizobacteria; Rhizobium sp. ; salt stress

Lentil *(Lens culinaris*) is one of the major important edible *rabi* pulses belonging to legumiosae family grown in India. The cultivated seeds are mainly taken as dal. As lentil seeds are relatively rich in protein, carbohydrate and calorie content compared to most of the pulse crop, it is highly preferred. In lentil about 30 per cent of calories come from protein and therefore after thsoybeans and hemp, lentil is the third placed legume to have highest level of protein, by weight (Singh and Singh, 2014).

In an experiment conducted with five different species of lentil, it was found that two amino acids namely methionine and cysteine were absent or deficient in all of them (Rozan *et al*., 2001). However, sprouted lentil contains sufficient levels of all essential amino acids, including methionine and cysteine (Singh and Singh, 2014). Lentil proteins are rich source of the essential amino acids isoleucine and lysine.

Lentil is a very important leguminous pulse crop in Indian agriculture. Eastern half of the Indo-Gangetic plain (IGP) covers the major share in lentil production (~ 32%) around the world and India occupies the second place in lentil production across the world just after Canada. (Talukdar, 2013). A number of lentil varieties like Moitree, Ranjan and KLS 218 are popularly cultivated as well as used in research purpose in this region (Sen et al. 2016). About 70 per cent of lentil production in India is contributed by the IGP and West Bengal has a big share in production after Uttar Pradesh, Bihar and Madhya Pradesh (Singh and Singh, 2014). Although the area and production of lentil in West Bengal is very less compared to other leading states namely, Uttar Pradesh, Bihar and Madhya Pradesh, the productivity is 833 kg ha^{-1} in West Bengal and it is significantly higher than the national average productivity which is 591 kg/ha (Singh and Singh, 2014). Among the Gangetic plains of West Bengal, it is predominantly grown in Murshidabad, Nadia and North 24 Paraganas.

Among the various abiotic stresses, salt stress drastically hamper the yield of salt sensitive legume crops by hampering biological nitrogen fixation and due to this lacuna lentil production is very scanty in the Southern part and salt affected districts of West Bengal. Several reports documented the involvement of *Rhizobium* sp. and other plant growth promoting rhizobacteria (PGPR) in salinity amelioration and subsequent improvement in crop growth. In the present study we are interested to isolate some salt tolerant lentil-specific bacteria and which will be utilized to generate biofertilizer for popularizing lentil production in salt affected regions of West Bengal.

MATERIALS AND METHODS

Survey of samples

A survey was made by B.C.K.V Survey Selection and Mass Production Unit during *rabi* season of 2015-2016 in different regions of Murshidabad district of West Bengal. Lentil root nodules were isolated from different block of Murshidabad for isolation of lentil-specific nitrogen fixing bacteria.

Surface sterilization

At first nodules from sampled lentil plants were collected separately in glass vials and washed thoroughly by plain water 8 to 10 times followed by distilled water

Email: jbanerjeebiotech@gmail.com

5 to 6 times. Then the nodules were allowed to soak in 0.1 % $HgCl_2$ for 5 minutes. Thereafter the nodules were taken out from $HgCl_2$ solution and treated with 0.1 % $AgNO_3$ solution for 1 minute for surface sterilization. Thereafter the sterilized nodules were washed repeatedly with distilled water.

Preparation of single cell suspension

The nodules were then separately crashed into test tubes each containing 10 ml of distilled water. Then the bacterial suspension was 7 fold diluted (10^{-7}) by serial dilution. From the finally diluted bacterial suspension 1 ml of solution was mixed with 25 ml of Yeast Extract Manitol Agar medium and poured in each petri plate. Then the plates were incubated at 28 ^{o}C temperature for two days for isolating bacteria.

DNA isolation

Bacterial colony of different lentil-specific bacteria was grown in YEM broth for 48 hrs at 28 ^{o}C with constant shaking and subsequently used for genomic DNA isolation. DNA was isolated from liquid culture of six different isolates using Chromous Biotech bacterial genomic DNA (spin) kit using manufacturer's protocol.

PCR reaction

For successfully identifying different lentil-specific bacterial isolates, forward primer R16-1 (52 -CTTGTACACACCGCCCGTCA-32) and reverse primer R23-3R (52 -GGTACTTAGATGTTTCAGTTC-32) was used for amplifying internally transcribed spacer (ITS) region. The PCR reaction was carried out using the following thermal profile: initial denaturation at 94 °C for 3 min followed by 35 cycles of 94 °C for 30 sec, 50 °C for 45 sec and 72 °C for 1 min and 30 sec along with a final extension at 72 °C for 7 min.

Gel electrophoresis

Genomic DNA isolated from six different isolates was subjected to 0.8% agarose gel electrophoresis along with 100 bp DNA molecular weight marker. The PCR product of six isolates carried out using R16-1 and R23-3R were subjected to 1% agarose gel electrophoresis along with 1 kb DNA ladder.

Salt stress treatment

For analyzing the salt tolerance nature, six different lentil-specific bacterial strains were grown in YEMA media without or with different concentrations of NaCl (100 mM, 200 mM, 300 mM and 400 mM) at 28 ^{0}C for 2 days.

Bioinformatics analyses

For analyzing the sequence homology among the two identified strain (BCKV MU2 and BCKV MU5) and the *Rhizobium sp*. O312 (Accession number AB529847) and *R. leguminoserum bv. phaseoli* (Accession number AB740456), CLUSTALW alignment was carried out using http://www.genome.jp/tools/clustalw/ online software. An evolutionary tree for the sequences was constructed by the UPGMA method.

RESULTS AND DISCUSSION

Isolation of lentil-specific bacterial isolates

Six different lentil-specific bacterial isolates were successfully isolated as pure culture from different parts of Murshidabad district namely #2, #3, #4, #5, #6 and #11.. . For successfully identifying different lentil-specific isolates, genomic DNA was isolated from them and checked on 0.8% agarose gel (Fig. 1). Agarose gel electrophoresis of the genomic DNA along with the DNA molecular weight marker depicted good quality genomic DNA from different bacterial isolates. These genomic DNA samples were used further for amplifying the internally transcribed spacer (ITS) region from six different isolates.

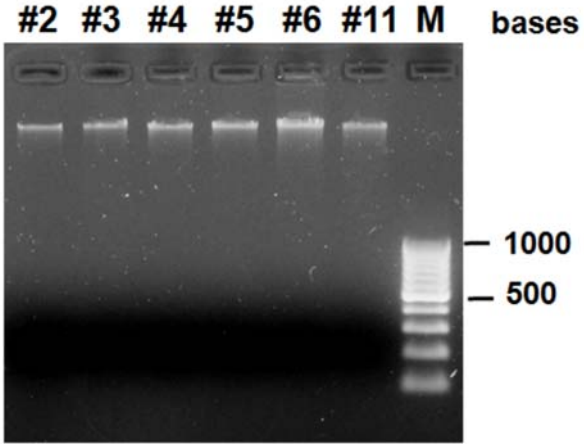

Fig. 1: 0.8% Agarose gel showing the genomic DNA samples isolated from six different bacterial isolates.

Lane 1: Genomic DNA from #2; Lane 2: Genomic DNA from #3; Lane 3: Genomic DNA from #4; Lane 4: Genomic DNA from #5; Lane 5: Genomic DNA from #6; Lane 6: Genomic DNA from #11; Lane 7: 100 bp DNA molecular weight marker (M).

Identification of bacterial strains

As the survey was carried out by BCKV at Murshidabad district of West Bengal, six different

isolates were designated as #2, #3, #4, #5, #6 and #11. *i.e.* BCKV MU2, BCKV MU3, BCKV MU4, BCKV MU5, BCKV MU6 and BCKV MU11, respectively throughout the text. Genomic DNA isolated from six different lentil-specific bacterial isolates was subjected to polymerase chain reaction for amplifying ITS region. PCR amplification showed single amplified band of ~1400 bp in most of the isolates except two namely, BCKV MU3 and BCKV MU6 (Figure 2). In BCKV MU3 DNA sample two amplified bands were detected at ~1300 and ~500 bp region whereas; a single PCR amplified band of ~500 bp was found in the BCKV MU6 strain. Earlier study documented that when similar set of primers were used in the phylogenetic study of *Bradyrhizobium*, *Mesorhizobium*, *Rhizobium* and *Sinorhizobium sp.*, the PCR amplified ITS region varied from 751 to 1236 nt (Kwon *et al.* 2005). Another report demonstrated that the PCR reaction for ITS region of *R. leguminosarum* strains belonging to the biovars *viciae*, *trifolii*, and *phaseoli* amplified bands between 1160 to 1400 bp (Andrade *et al*. 2002). As high sequential variation of the ITS region is very helpful to distinguish different closely related species (Chun *et al.* 1999), in the present study bacterial isolates having single band PCR amplified products were subjected to sequencing reaction for subsequent analysis.

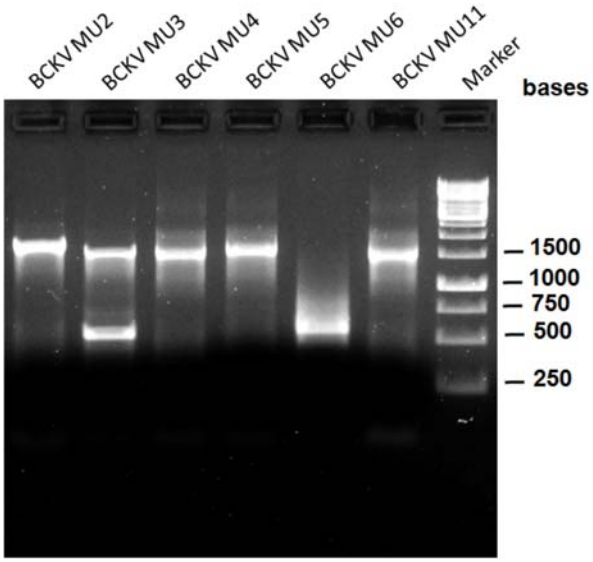

Fig. 2: 1% agarose gel showing the PCR amplified DNA of ITS region.

Lane 1 to 6: PCR product of ITS region from indicated bacterial isolates; Lane 7: 1 kb DNA marker

Sequence analysis

DNA sequences from different lentil-specific isolates were subjected to nucleotide BLAST analysis (https://blast.ncbi.nlm.nih.gov/Blast.cgi) against the public database at National Center for Biotechnology Information (NCBI). Out of six isolates two were belonging to *Rhizobium sp*. Sequences of those two isolates namely BCKV MU2 and BCKV MU5 were submitted at NCBI public database having accession number KY174316 and KY172969, respectively. These two sequences were aligned with the ITS region from *Rhizobium sp.* O312 and *Rhizobium leguminoserum bv. phaseoli* DNA by CLUSTALW software and a significant amount of homology was detected among themselves (Fig. 3).

The selected sequences are: part of ITS region from *Rhizobium sp.* O312 (Accession number AB529847); *R. leguminoserum bv. phaseoli* (Accession number AB740456); BCKV MU2 (Accession number KY174316) and BCKV MU5 (Accession number KY172969). Asterisks indicate the strictly conserved residues.

Phylogenetic study

The phylogenetic tree generated by UPGMA method using the ITS sequences of two newly identified strains (BCKV MU2 and BCKV MU5) and two known sequences from public database demonstrated that BCKV MU2 is closely related to *R. leguminoserum bv. phaseoli* compared to the distance between BCKV MU2 and *Rhizobium sp*. O312 (Fig. 4). The strain BCKV MU5 is distantly related to all other three strains used in the phylogenetic study (Fig. 4). Likewise using 23S rRNA sequences the phylogenetic analysis of 13 different *Rhizobium sp*. was performed by UPGMA method very recently (Abdel-Lateif *et al*., 2016).

Effect of salt stress on different lentil-specific bacterial isolates

Six different isolates were initially screened for salt tolerance nature in YEMA media along with differential NaCl concentrations (Fig. 5). Under control condition all isolates were grown properly (Fig. 5A) whereas their growth variations were detected under differential NaCl stress. From 200 mM NaCl stress onwards, #2 did not grow properly after 48 hrs of growth whereas #5 showed severe growth reduction from 300 mM NaCl stress onwards (Fig. 5C and Fig. 5D). Although at 400 mM NaCl stress, most of the isolates were killed after 48 hrs of growth, #11 and #4 showed very limited growth (Fig. 5E).

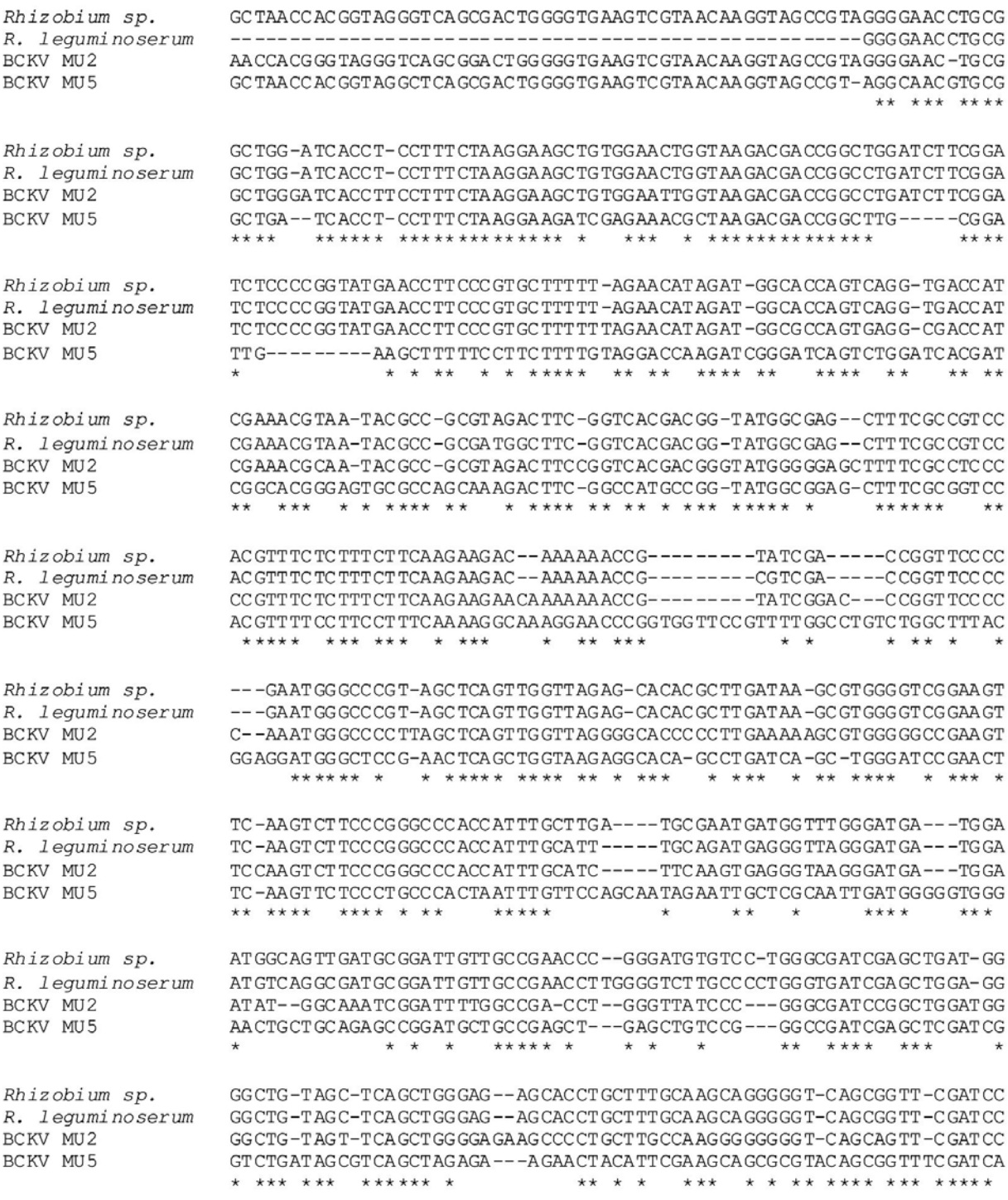

Fig. 3: Multiple alignment of nucleotide sequences of four different strains using ClustalW program

In the world near about 40% of land surface are affected by salinity and soil salinity is a serious problem in agriculture due to low salt tolerance capability of majority of the crop plants (Bouhmouch *et al*. 2005). Plants' reaction to salt stress is a versatile event comprising of signal transduction, gene expressional changes, biochemical activities, and physiological consequences, along with the changes in phenotype and developmental programmes (Greenway and Munns, 1980; Jiang *et al*., 2007; Banerjee *et al*. 2013). Lentil is an important pulse crop in Indian economy and it is very much sensitive to salt stress (Bandeoðlu *et al*. 2004). Numerous studies documented that soil salinity decreases number of nodule formation and typically

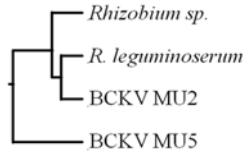

Fig. 4: UPGMA phylogenetic tree based on the aligned ITS region from *Rhizobium sp.* O312 (Accession number AB529847);
R. leguminoserum bv. phaseoli (Accession number AB740456); BCKV MU2 (Accession number KY174316) and BCKV MU5 (Accession number KY172969).

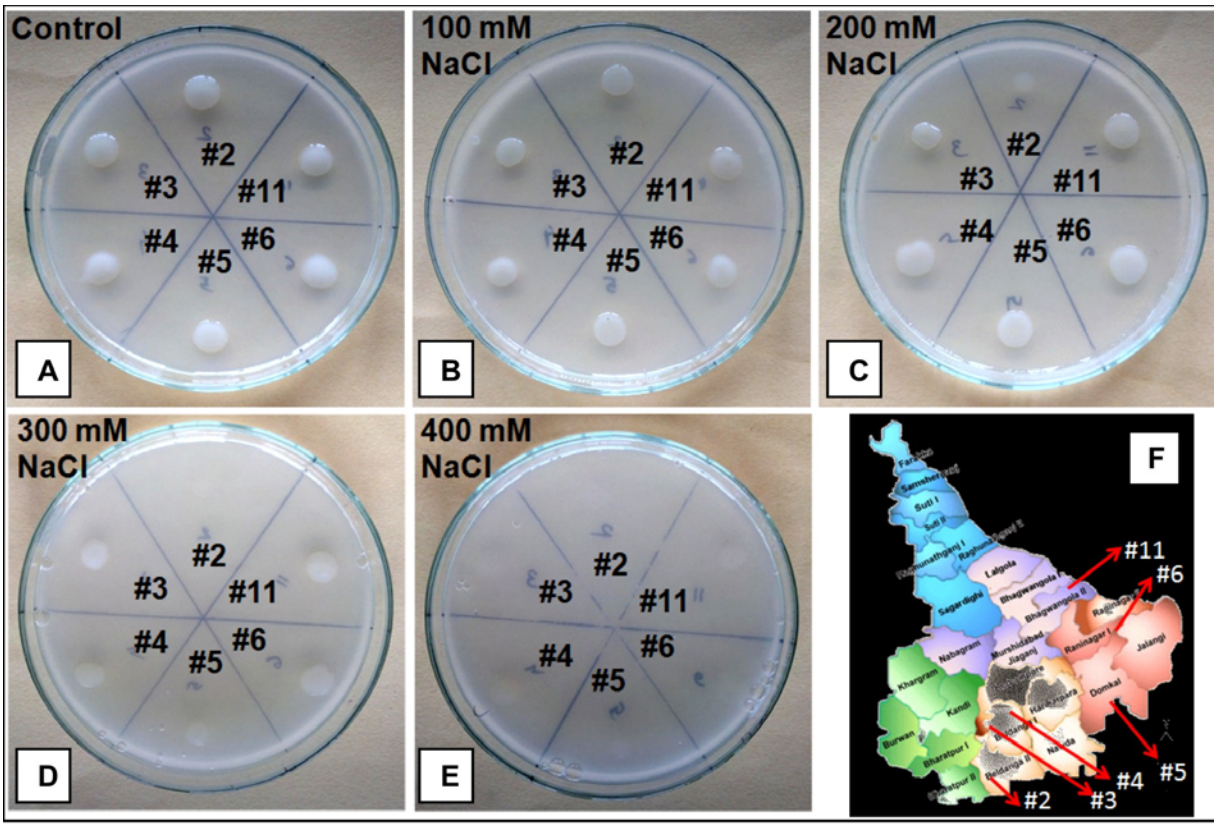

Fig. 5: Survivality of different lentil-specific isolates for NaCl tolerance and the origin of those strains. *(A-E): Six different isolates were grown on YEMA plates without (control) or with indicated concentrations of NaCl stress. (F) The map shows different blocks of Murshidabad district of West Bengal. Arrow shows the collection places of the indicated isolates.*

reduce N_2 fixation and nitrogenase activity of nodulated legumes. So, development of salt-tolerant symbioses is necessary to enable cultivation of legume crops in saline soils.

As salinity is globally growing problem, several researches are going on for improving the salt tolerance of crop plants including legumes. Although most of the legumes are salt sensitive, different types of *Rhizobium sp*. vary in salt tolerance (Bouhmouch *et al*. 2005). During salt stress plants also suffer from osmotic stress (Zahran, 1999). Osmotic stress tolerant *Rhizobium* strains can support significant modifications in the osmolarity without hampering the number of viable cells (Singleton *et al*. 1982). A number of studies documented that *Rhizobium sp*. alone or in combination with *Pseudmonas sp*. or other PGPR can improve the yield as well as nodulation efficiency of different legume crops under salt stress and drought condition (Zahran, 1999; Dardanelli *et al*. 2008; Egamberdieva *et al*. 2013; Biswas *et al*. 2015). During any stress reactive oxygen species (ROS) are generated in plants and many superoxide dismutase, peroxidase, glutathione reductase and catalase genes are available

in plants for combating those elevated ROS (Abogadallah, 2010; Barman and Banerjee, 2015); but how *Rhizobium* or other PGPR play role in stress alleviation of plant is not clear yet. In the present study two *Rhizobium sp*. were identified from Murshidabad district of West Bengal and BCKV MU5 isolate is more salt tolerant than BCKV MU2 isolate under 200 mM and 300 mM NaCl stress (Figure 5). It is worthy to mention here that the isolate BCKV MU5 is phylogenetically different from other *Rhizobium* sp. tested in the study (Fig. 4). Herefrom it can be concluded that BCKV MU5 isolate is a unique salt tolerant *Rhizobium sp*. which might be helpful to improve the nodulation efficiency of lentil under salt stress condition. This strain has a potential to be used for production of biofertilizer consortium and it will be very beneficial to popularize lentil production in the vast salt affected area of South 24 parganas and Medinipur in future.

REFERENCES

Abdel-Lateif, K.S., Hewedy, O.A. and El-Zanaty, A.F.M. 2016. Phylogenetic analysis of 23S rRNA gene sequences of some *Rhizobium leguminosarum* isolates and their tolerance to drought. *Afri. J. Biotech.*, **15**: 1871–76.

Abogadallah, G.M. 2010. Antioxidative defense under salt stress. *Pl. Signal. Behav.*, **5**: 369–74.

Andrade, D.S., Murphy, P.J. and Giller, K.E. 2002. The diversity of *Phaseolus*-nodulating rhizobial populations is altered by liming of acid soils planted with *Phaseolus vulgaris L.* in Brazil. *Appl. Env.. Microbiol*., **68**: 4025–34.

Bandeoðlu, E., Eyidoðan, F., Yücel, M. and Öktem, H.A. 2004. Antioxidant responses of shoots and roots of lentil to NaCl-salinity stress. *Pl. Growth Regul.*, 69–77.

Banerjee, J., Magnani, R., Nair, M., Dirk, L.M., DeBolt, S., Maiti, I.B. and Houtz, R.L., 2013. Calmodulin-mediated signal transduction pathways in Arabidopsis are fine-tuned by methylation. *Pl. Cell*, **25**: 4493–11.

Barman, A.R. and Banerjee, J., 2015. Versatility of germin-like proteins in their sequences, expressions, and functions. *Funct. Integr. Genomics*, **15:** 533–48.

Biswas, P.K., Bhowmick, M.K., Kundu, M.C., Mondal, S. and Ghosh, G.K. 2015. Cojoint application of biofertilizer and phosphorous levels on growth, nodulation, nutrient uptake and productivity of lentil [*Lens culinaris* Medikus] in red and lateritic soils of West Bengal. *J. Crop Weed*, **11**: 29–32.

Bouhmouch, I., Souad-Mouhsine, B., Brhada, F. and Aurag, J. 2005. Influence of host cultivars and *Rhizobium* species on the growth and symbiotic performance of *Phaseolus vulgaris* under salt stress. *J. Pl. Physiol.*, **162**:1103–13.

Chun, J., Huq, A. and Colwell, R. R. 1999. Analysis of 16S–23S rRNA intergenic spacer regions of Vibrio cholerae and Vibrio mimicus. *Appl. Env.. Microbiol.*, **65:** 2202–08.

Dardanelli, M.S., Ferna´ndez de Co´ rdoba, F.J., Espuny, M.R., Carvajal, M.A.R., Dý´az, M.E.S., Serrano, A.M.G., Okon, Y. and Megý´as, M. 2008. Effect of *Azospirillum brasilense* coinoculated with *Rhizobium* on *Phaseolus vulgaris* flavonoids and Nod factor production under salt stress. *Soil Biol. Biochem.*, **40** : 2713-21.

Egamberdieva, D., Berg G., Lindström, K. and Räsänen, L.A. 2013. Alleviation of salt stress of symbiotic Galega officinalis L. (goat's rue) by co-inoculation of *Rhizobium* with root-colonizing *Pseudomonas*. *Pl. Soil*, **369:** 453–65.

Greenway, H. and Munns, R., 1980. Mechanisms of salt tolerance in non-halophytes. *Ann. Rev. Pl. Physiol*., **31**: 149–90.

Jiang, Y., Yang, B., Harris, N. S. and Deyholos, M. K., 2007. Comparative proteomic analysis of NaCl stress-responsive proteins in *Arabidopsis* roots. *J. Exp. Bot*., **58**: 3591–3607.

Kwon, S-W., Park, J-Y., Kim, J-S., Kang, J-W., Cho, Y-H., Lim, C-K., Parker, M.A. and Lee, G-B. 2005. Phylogenetic analysis of the genera *Bradyrhizobium*, *Mesorhizobium*, *Rhizobium* and *Sinorhizobium* on the basis of 16S rRNA gene and internally transcribed spacer region sequences. *Int. J. Syst. Evol. Micr.*, **55**:263–70.

Rozan, P., Kuo, Y-H., and Lambein, F. 2001. Amino acids in seeds and seedlings of the genus Lens. *Phytochem.*, **58**: 281–89.

Sen, S., Ghosh, M., Mazumdar, D., Saha, B. and Dolui, S. 2016. Effect of sowing date and variety on phenology and yield of lentil during *rabi* season. *J. Crop Weed*, **12**: 135–38.

Singh, K.M. and Singh, A.K. 2014. Lentil in India: An Overview. *SSRN Electronic J*., *DOI*: **10**.13140/ 2.1.2191.1049.

Singleton, P.W., El-swaifi, S.A. and Bohlool, B.B. 1982. Effect of salinity on *Rhizobium* growth and survival. *Appl. Env. Microbiol.*, **44:** 884–90.

Talukdar, D. 2013. Bioaccumulation and transport of arsenic in different genotypes of lentil (*Lens culinaris* medik.) *Int. J. Pharm. Bio. Sci.* **4** : 694 – 701.

Zahran, H.H. 1999. *Rhizobium*-legume symbiosis and nitrogen fixation under severe conditions and in an arid climate. *Microbiol. Mol. Biol. Rev*., 968-89.