Journal of Pharmacognosy and Phytochemistry

Available online at www.phytojournal.com

J P P Journal of Pharmacognosy and Phytochemistry

E-ISSN: 2278-4136
P-ISSN: 2349-8234
JPP 2018; 7(2): 1305-1311
Received: 22-01-2018
Accepted: 23-02-2018

Ratul Moni Ram
Department of Mycology and Plant Pathology, Institute of Agricultural Sciences, Banaras Hindu University, Varanasi, Uttar Pradesh, India

HB Singh
Department of Mycology and Plant Pathology, Institute of Agricultural Sciences, Banaras Hindu University, Varanasi, Uttar Pradesh, India

Correspondence
Ratul Moni Ram
Department of Mycology and Plant Pathology, Institute of Agricultural Sciences, Banaras Hindu University, Varanasi, Uttar Pradesh, India

Bacillus sp. BHU 08 isolated from stressed regions of Eastern Uttar Pradesh and Bundelkhand for promotion of plant growth in chickpea (*Cicer arietinum* L.)

Ratul Moni Ram and HB Singh

Abstract
Drought is often considered as one of the major environmental stress the productivity of crop plants around the world. Role of various microorganisms in mitigating drought stress in several crops have been documented by various researchers in the past. The experiment was conducted for isolation of agriculturally important microorganisms (AIMs) having drought tolerant potential from six districts of eastern Uttar Pradesh and Bundelkhand Region. Screening for drought tolerance was performed for 120 bacterial strains isolated from different moisture stressed sites of the above mentioned region. Polyethylene glycol (PEG) solution was used for screening of drought tolerant bacteria. Selection of the bacteria was done on the basis of their long term survival potential under drought stress conditions. The colony forming unit (CFU) ml^{-1} counting of the isolates were done and 10 drought tolerant bacteria were selected on the basis of their high CFU counting. The isolates were also tested for their thermo tolerance ability and all of them found to survive for 5 days at 55 ˚C. Biocontrol potential of the isolates was checked against chickpea dry root rot *Rhizoctonia bataticola* pathogen and the isolate BHU 08 was found to show profound antagonism and demonstrated maximum similarity with *Bacillus* sp after 16S rRNA gene sequencing analysis. Isolation of bacteria having drought tolerance and against ability altogether will be a pioneer concept and if further explored, would help in alleviating the effect of drought on various crops in near future.

Keywords: Drought, *Rhizoctonia bataticola*, Polyethylene glycol (PEG), C.F.U

Introduction
Plants are subjected to various abiotic stresses having an adverse impact on their growth, metabolism, and yield. Drought, water logging, salinity, temperature extreme, radiation and pollutants are the major constraints in crop growth and yield (Lawlor, 2002) [14, 15]. Apart from these abiotic factors, biotic factors such as pests and pathogens also alter the growth and productivity in higher plants (Lichtenthaler, 1996, 1998) [16, 17]. Among the abiotic stresses, drought is regarded as a significant yield limiting factor in crop production. It affects plant growth and development adversely leading to reduction in crop yield and serious threat to food security. Drought is a recurrent climatic factor usually faced by plants in arid and semi-arid regions of the world (FAO, 2013) [9]. It causes nutrient deficiencies, hormonal imbalance and reduced photosynthesis and in crop plant which ultimately leads to low yield, reduced plant vigour and food insecurity. Drought stress becomes predominant when supply of water to roots is less compared to the demand or when the rate of transpiration becomes very high (Reddy *et al.,* 2004) [19]. These two factors frequently found to overlap under arid and semi-arid zones. However, plants have an intrinsic ability to tolerate water stress, but its degree varies both inter and intraspecies (Jaleel *et al.,* 2009) [12]. Such kind of abiotic stress affects the whole plant both at cellular and whole-plant level, resulting in specific and non-specific damage.
There are various reports of microorganisms *viz*. *Paenibacillus polymyxa*, *Rhizobium tropici, Pseudomonas* sp, *P. putida P45, P. polymyxa*, *Bacillus thuringiensis* AZP2, AM fungi, *Azospirillum* spp. etc. in ameliorating water stress in different plants (Timmusk *et al.,* 2014: Sandhya *et al.,* 2009 a,b; Figueiredo *et al.,* 2008; Arshad *et al.,* 2008; Cho *et al.,* 2006; Alami *et al.,* 2000) [26, 21, 22, 10, 3, 7, 1]. PGPR have survival ability under water stress conditions owing to the production of exopolysaccharides (EPS), which in turn defend them from water stress by facilitating water retention and maintaining the diffusion of carbon sources (Wilkinson 1958; Hepper 1975; Roberson and Firestone 1992; Chenu 1993; Chenu and Roberson 1996) [29, 20, 5, 6]. EPS also facilitate PGPR in attachment and root colonization through a network of fibrillar material that enduringly adhere the bacteria to the root surface (Bashan *et al*., 2004; Ali *et al.,* 2014) [4, 2].

The mechanism of water stress tolerance induced by PGPR may be briefly categorized as (1) production of phytohormones like indole-3-acetic acid (IAA), abscisic acid (ABA), gibberellic acid and cytokinins (2) induced systemic tolerance by bacterial compounds (3) production of exopolysaccharides (Yang *et al.,* 2009; Dimkpa *et al.,* 2009; Timmusk and Nevo, 2011; Kim *et al.,* 2013; Timmusk *et al.,* 2014) [30, 8, 27, 26] (4) reduction in ethylene level in the roots by releasing ACC deaminase. The first report on plant drought tolerance enhancement by PGPR was published in Uppsala, Sweden (Timmusk and Wagner, 1999) [28]. The reports revealed that *Arabidopsis thaliana* inoculated with *P. polymyxa* B2 have the ability to withstand prolonged drought stress compared to the control plants.

Therefore, an attempt was made to isolate and characterize drought tolerant bacteria from eastern Uttar Pradesh and Bundelkhand regions which could provide maximum benefits to the drought affected plants. For the reason, present study was planned on the hypothesis that inoculation with of drought tolerant bacteria in agriculturally important crop plants would further improve their growth under drought and in turn would facilitate in minimizing crop losses.

Materials and Methods

Isolation of drought tolerant bacteria

A roving survey was conducted during June-July, 2012 in Varanasi, Allahabad, Banda, Jhansi, Lalitpur and Jalaun districts of Uttar Pradesh in order to collect soil samples for isolating drought tolerant microorganisms. The regions of the districts mostly affected by moisture stress were selected for the study. Soil samples were collected from such stressed regions and the temperature of the region while collecting the samples was recorded (Table1).

Table 1: Isolation site of AIMs from extreme regions of Eastern Uttar Pradesh and Bundelkhand Region

S. No.	District	Latitude/ Longitude	Location	Temperature (°C)	Year of collection
1	Varanasi	25.2765° N/ 83.0335° E	Ramnagar	45.2	2012
2	Allahabad	25.4253° N/ 81.9126° E	Phulpur	43.6	2012
3	Jhansi	25.5739° N/ 79.2987° E	Garautha	46.4	2012
4	Lalitpur	24.5831° N/ 78.7278° E	Mahroni	44.5	2012
5	Banda	25.5503° N/ 80.7048° E	Baweru	47.4	2012
6	Jalaun	26.0296° N/ 79.6558° E	Pipraya	43.5	2012

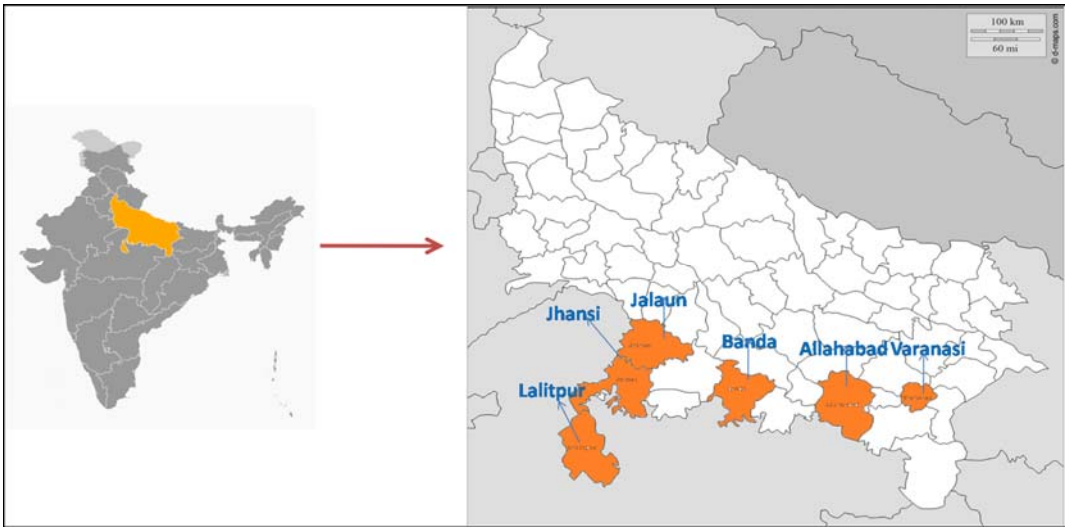


Fig 1: Different districts of Eastern Uttar Pradesh and Bundelkhand region surveyed for isolation of drought tolerant bacteria

The bacteria were isolated from the collected soil samples through serial dilution method. The bacteria having distinct colony characteristics were selected and grown in individual NA plates. 120 bacteria were selected having different morphology and colony characters and are designated serially from BHU 01 to BHU 120. Purification of the bacterial isolates was done in NA plates. The Pure cultures were maintained in NA slants at stored at 4 °C for future use.

Drought tolerance assay

The isolates were grown in glass vials containing 60% (NB+ PEG) media and were incubated for 96 hours in shaking incubator at 28 ± 1 °C. Firstly, the bacteria were inoculated in the NB tubes and kept in shaking incubator shaker for 24 hours. When growth of the bacteria is seen in the NB tubes then 50 µl of the suspension was transferred to 60% NB+ PEG media containing glass vials. The tubes were labelled properly. The tubes were placed in the shaking incubator and the bacterial growth was monitored continuously for 5 days. After 24 hours, 20 µl of the bacterial suspension was transferred in NA Petriplates to check the growth of the corresponding bacterial isolates. Those isolates which showed prominent growth up to 5 days were selected and their $CFUml^{-1}$ was calculated.

Thermo tolerance assay

The selected drought tolerant strains were tested separately for their thermotolerance ability. The bacteria were inoculated in glass vials containing NB and were kept for about 5 days in different B.O.D with temperature maintained at a range of 40-55 °C. The growth potential of each bacterial isolate was checked per day by transferring 20 µl of the bacterial suspension to fresh NA plates for up to 120 hours. Those isolates which showed prominent growth up 120 hours to were selected for antagonistic test against the pathogen *R. bataticola.*

Antagonistic test of the isolates

The Petriplates containing PDA media were inoculated with the corresponding bacterial isolates at one edge as a straight line and with 5 mm mycelia disc of the pathogen on the opposite edge at a distance of 5 cm. Inoculated plates were incubated at 25 °C in BOD incubator. Observations were recorded up to 7 days of inoculation on area covered by tested bacterial isolates and the pathogen (*R. bataticola).* Inhibition of mycelial growth of pathogen fungi by each strain was recorded.

Characterization of BHU 08 by 16 S rRNA gene sequencing

Pure cultures of the selected bacterial isolate BHU 08 was maintained on NA slants and revived on freshly poured NA plates for 1-2 days. A small loopfull of bacteria was inoculated in flask containing NB and incubated at 25 ± 2 °C for 2 days without shaking. The bacterial DNA was isolated using Hi PurA TM SP Bacterial DNA Mini Kit (Himedia Laboratories Pvt., Ltd., Mumbai, India). The DNA quality was assessed on 1.2% agarose gel in 1X TAE buffer. Sharp and discrete bands reveal a fine quality of isolated DNA (Saxena *et al.,* 2014).

Taxonomic identification of the selected isolate was performed by 16S rDNA amplification was done using universal bacterial primers forward (5' AGAGTTGATCYTCGCTC 3') and reverse (5' GYTACCTTGTTACGACT 3') obtained from Bangalore GeneI Pvt. Ltd., Bengaluru, Karnataka, India. 25 ml reaction mixture consisting of 50 ng of genomic DNA, 1 X PCR Buffer, 0.5 µL Taq DNA Polymerase and 1 pM primer was taken for PCR amplification in thermocycler (Technie, UK). The reaction mixture was denaturated at 94 °C for 4 min followed by 35 cycles of 30 sec each at 94 °C and 55 °C followed by 60 s at 72 °C and final extension at 72 °C for 15 min. The PCR products obtained was assessed on 2% agarose gel in 1 X TAE buffer containing Ethidium bromide (0.5 mg/ml). Finally, the phylogenetic tree was prepared using MEGA 6 software to authenticate the classification.

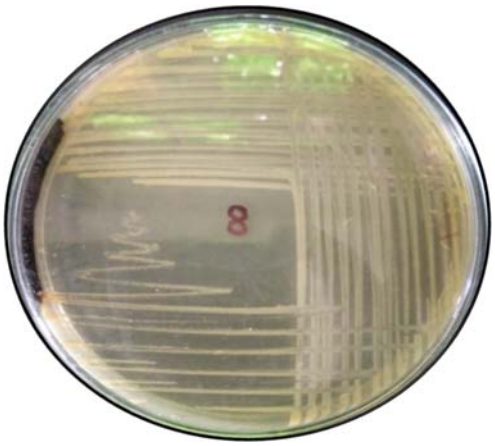


Fig 2: Bacterial isolate BHU 08

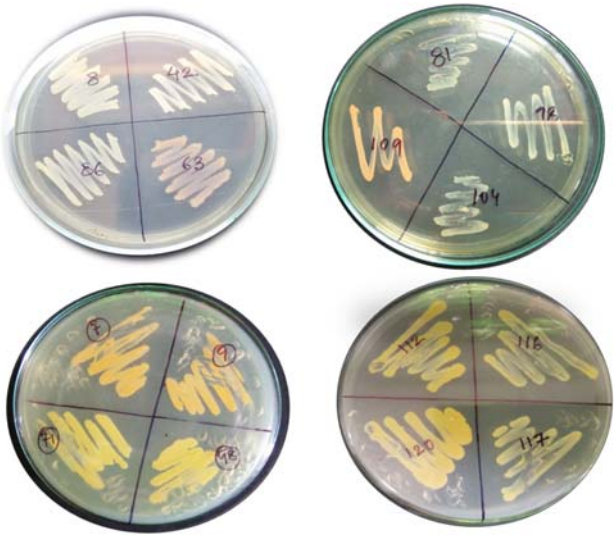


Fig 3: Different types of drought tolerant bacteria isolated from extreme soils

Plant growth promotion assay

The growth promotion ability of bacterial isolate BHU 08 was conducted on chickpea cultivar "Radhey". The drought condition was imparted on chickpea plants by applying 40 % PEG 6000. The chickpea seeds were inoculated with BHU 08 and are sown in pots. Fresh inoculum of BHU 08 was prepared having uniform cell density of 10^8 CFU ml^{-1}. For inoculation, surface sterilized seeds of chickpea cultivar "Radhey". The seeds were dipped in bacterial suspension at 20-25 °C and were maintained in the controlled room temperature. Sampling for plant growth promotion was done after 30, 60 and 90 days of sowing and growth parameters like plant height, shoot and root length, dry and fresh weight of plants, number of roots, pod number and number of grains per pod were measured (Singh *et al.,* 2016 a,b; Ray *et al.,* 2015). The experiment was conducted with three replications and the data were pooled for analysis.

Table 2: Order of treatments for studying growth promotion in chickpea

Treatment No.	Treatment
T_1	Control (C)
T_2	Chickpea+ drought stress (D)
T_3	Chickpea plant + drought stress + Biocontrol agent (D + B)
T_4	Chickpea Plant + Biocontrol agent (B)

Results

***In vitro* drought tolerance assay**

The 120 bacteria were subjected to drought tolerance test and their growth up to 5 days after inoculation (DAI) in NB+PEG media was checked. Ten isolates *i.e* BHU7, BHU 8, BHU 9, BHU 42, BHU 63, BHU 71, BHU 81, BHU 86 and BHU98 were selected on the basis of their high CFU after 5 DAI compared to the rest and were subjected to thermo tolerance test. The CFU ml^{-1} of the 10 isolates were calculated from 1 - 5 DAI and data was recorded (Figure 4).

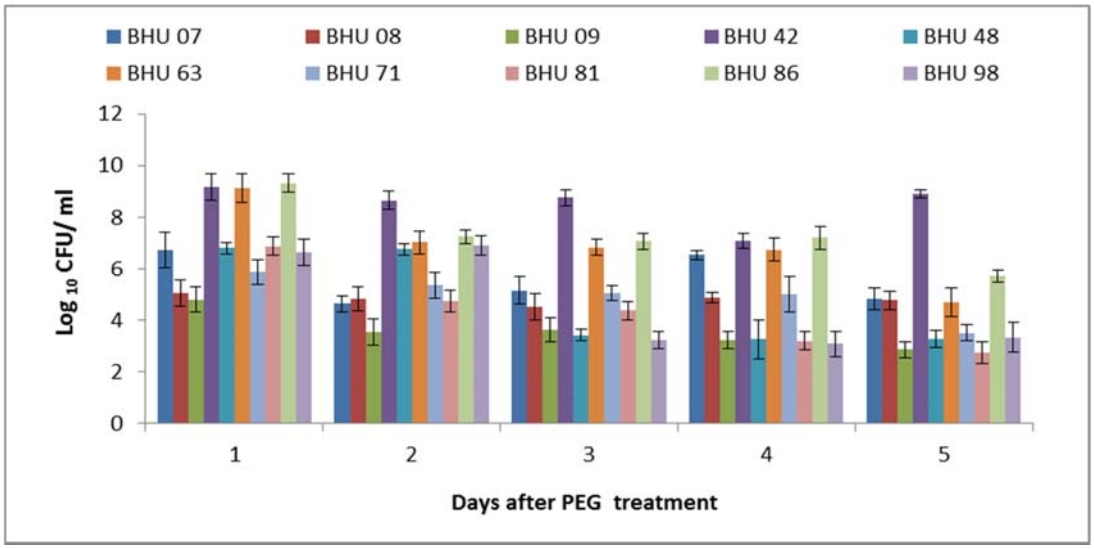


Fig 4: Survival of selected bacterial isolates at different time intervals. Results are expressed as mean of three replicates with vertical bars indicate standard deviation of the mean.

Thermotolerance assay

10 isolates were selected for checking their thermotolerance ability. Out of the 10 isolates, BHU 07 and BHU 81 showed growth up to 45 °C. However, 4 isolates namely BHU 08, BHU 42, BHU 63 and BHU 86 showed growth at temperature range of 55 °C (Table 2). The 4 isolates were selected and subjected to antagonism test against *R. bataticola*.

Antagonism test against *R. bataticola*

Out of the 4 isolates, only BHU 08 showed antagonistic ability against *R. bataticola* in dual culture, while the remaining 3 isolates BHU 42, BHU 63 and BHU 86. So for carrying out further experiments BHU 08 was selected as it is drought tolerant, thermotolerant and antagonistic to dry root rot pathogen *R. bataticola*.

Table 3: Thermotolerance test of the selected bacterial isolates

Isolates	40 ºC	45 ºC	50 ºC	55 ºC
BHU07	+	+	-	-
BHU08	+	+	+	+
BHU09	-	-	-	-
BHU42	+	+	+	+
BHU48	+	-	-	-
BHU63	+	+	+	+
BHU71	+	-	-	-
BHU81	+	+	-	-
BHU86	+	+	+	+
BHU98	+	-	-	-

Fig 5: Dual culture test of BHU 08 with *R. bataticola*

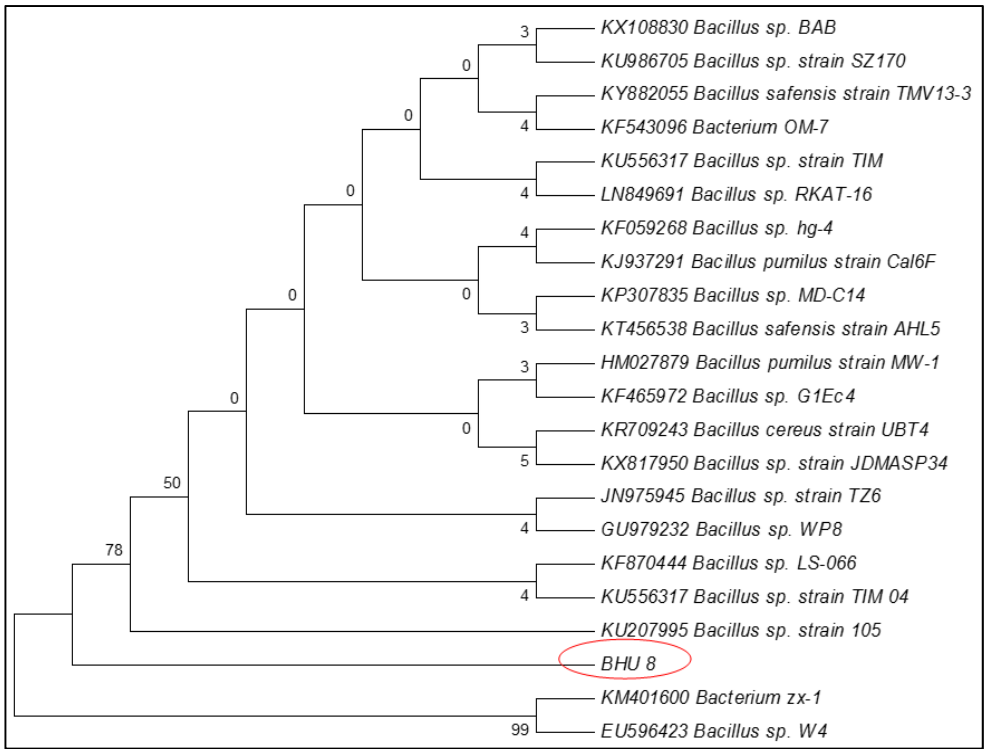
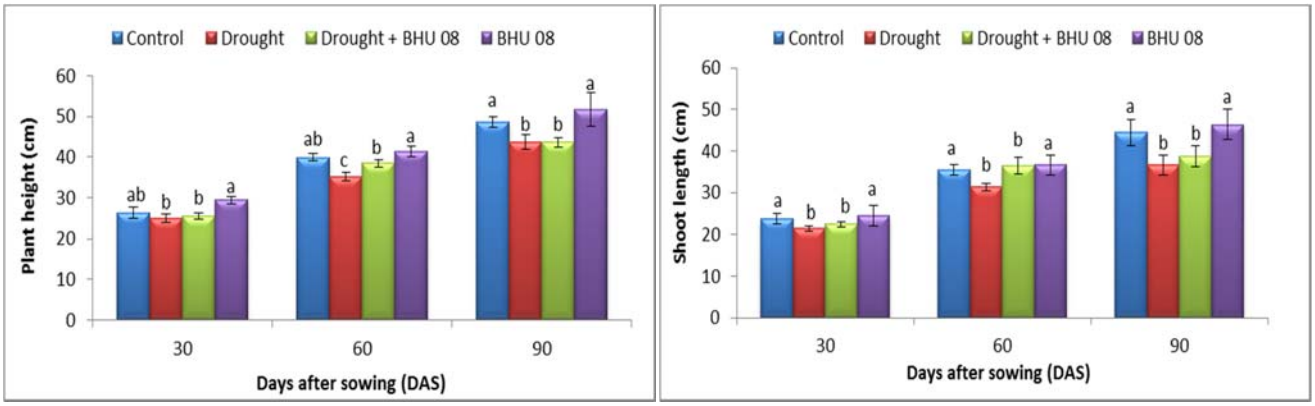


Fig 6: The phylogenetic tree of BHU 08 isolates constructed according to 16S rDNA sequence. The bootstrap values are based on 1,000 replications. The BHU 08 isolate is indicated through red circle. Bar 0.005 substitutions per site.

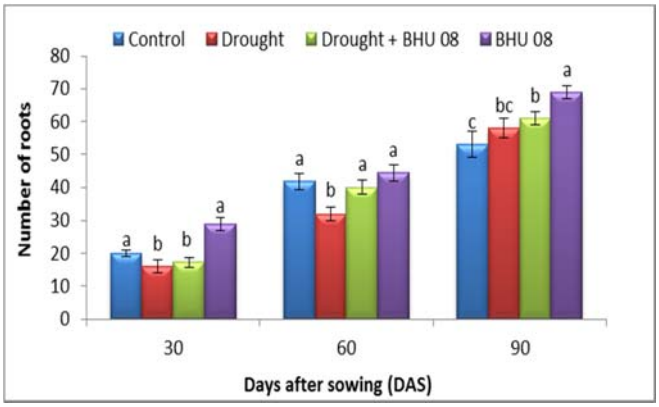
Growth promotion assay

The chickpea plants were checked for growth promotion by calculating the various morpho-physiological parameters. The results revealed that among all the treatments, T4 recorded the maximum values of all parameters which confirm the potential of BHU 08 in plant growth promotion abilities and mitigating drought stress to a certain extent.

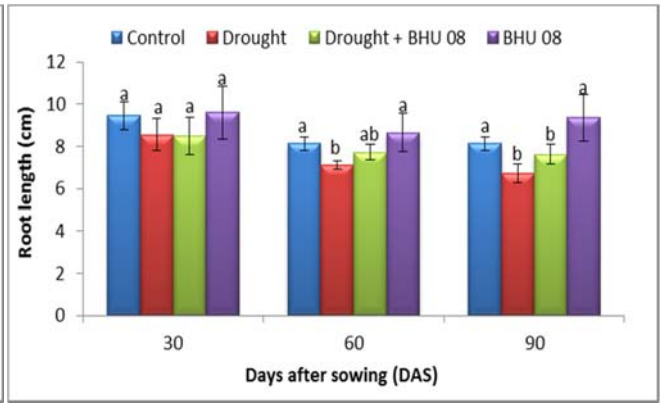


(a)

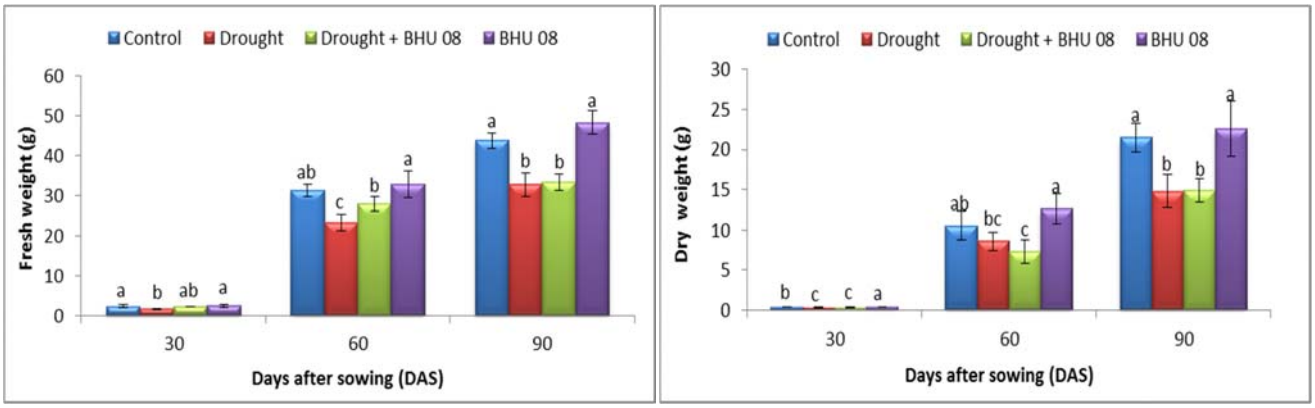
(b)



(c)



(d)



(e) **(f)**

Fig 7: Effect of *Bacillus* sp. BHU 08 on various morpho-physiological parameters in chickpea plant. (a) Plant height (b) Shoot length (c) Number of roots (d) Root length (e) Fresh weight (f) Dry weight. The results are expressed as means of three replicates and vertical bars indicate standard deviation of the mean. Different letters indicate significant difference among treatment results taken at same time interval according to Duncan's multiple range test at $p \leq 0.05$.

Fig 8: Comparison of Control (C) with *Bacillus* sp BHU 08 treated plants (T)

Conclusion

It is inferred from the present study that bacterial isolate BHU 08 has drought tolerant potential as well as antagonistic ability to chickpea dry root rot pathogen (*R. bataticola*). The data on growth promotion suggests that inoculation of chickpea seeds with this isolate led to increase in all growth parameters compared to the control. Thus further work is needed to check its potential in ameliorating drought stress in other agricultural crops and also to check its ability to control dry root rot of chickpea in field.

Acknowledgement

Ratul Moni Ram is highly grateful to UGC for providing Rajiv Gandhi National fellowship under SC/ST scheme.

References

1. Alami Y, Achouak W, Marol C, Heulin T. Rhizosphere soil aggregation and plant growth promotion of sunflowers by exopolysaccharide producing *Rhizobium sp.* strain isolated from sunflower roots. Applied Environmental Microbiology. 2000; 66:3393-3398.
2. Ali SZ, Sandhya V, Rao, LV. Isolation and characterization of drought-tolerant ACC deaminase and exopolysaccharide-producing fluorescent *Pseudomonas* sp. Annals of Microbiology. 2014; 64**:**493-502.
3. Arshad M, Sharoona B, Mahmood T. Inoculation with *Pseudomonas* spp. containing ACC deaminase partially eliminate the effects of drought stress on growth, yield and ripening of pea (*Pisum sativum* L.). Pedosphere. 2008; 18: 611-620.
4. Bashan Y, Holguin G, de-Bashan LE. 2004. *Azospirillum*-plant relationships: physiological, molecular, agricultural, and environmental advances. Canadian Journal of Microbiology. 2004; 50:521–577.
5. Chenu C. Clay or sand polysaccharide associations as models for the interface between microorganisms and soil: water-related properties and microstructure. Geoderma. 1993; 56:143-156.
6. Chenu C, Roberson EB. Diffusion of glucose in microbial extracellular polysaccharide as affected by water potential. Soil Biology and Biochemistry. 1996: 28:877-884.
7. Cho K, Toler H, Lee J, Ownley B, Stutz JC, Moore JL *et al.* Mycorrhizal symbiosis and response of sorghum plants to combined drought and salinity stresses. Journal of Plant Physiology. 2006; 163:517-528.
8. Dimkpa, C, Weinand T, Asch F. Plant-rhizobacteria interactions alleviate abiotic stress conditions. Plant Cell Environment. 2009; 32:1682-1694.
9. FAO. Drought facts-Report on Land and Water Day, 11th March, 2013. Available online at: http://www.fao.org/ docrep/017/aq191e/aq191e.pdf [Accessed on: 20/10/ 2014]
10. Figueiredo MV, Burity HA, Martínez CR, Chanway CP. Alleviation of drought stress in the common bean (*Phaseolus vulgaris* L.) by co-inoculation with *Paenibacillus polymyxa* and *Rhizobium tropici*. Applied Soil Ecology. 2008; 40:182-188.
11. Hepper CM. Extracellular polysaccharides of soil bacteria. In: Walker N (ed) Soil microbiology, a critical review. Wiley, New York, 1975, 93-111.
12. Jaleel CA, Manivannan P, Wahid A, Farooq M, Somasundaram R, Panneerselvam R. Drought stress in plants: a review on morphological characteristics and pigments composition. International Journal of Agriculture and Biology. 2009; 11:100-105.
13. Kim YC, Glick B, Bashan Y, Ryu CM. Enhancement of plant drought tolerance by microbes, In: Aroca, R. (Ed.), Plant Responses to Drought Stress, Springer Verlag, Berlin. 2013.

14. Lawlor DW. Limitation to Photosynthesis in Water-stressed Leaves: Stomata vs. Metabolism and the Role of ATP. Annals of Botany. 2002; 89(7):871-885.
15. Lawlor DW, Cornic G. Photosynthetic carbon assimilation and associated metabolism in relation to water deficits in higher plants. Plant, Cell & Environment. 2002; 25(2):275-294.
16. Lichtenthaler HK. Vegetation stress: an introduction to the stress concept in plants. Journal of Plant Physiology. 1996; 148(1):4-14.
17. Lichtenthaler HK. The stress concept in plants: an introduction. Annals of the New York Academy of Sciences. 1998; 851(1):187-198.
18. Ray S, Singh S, Sarma BK, Singh, HB. Endophytic *Alcaligenes* isolated from horticultural and medicinal crops promotes growth in Okra (*Abelmoschus esculentus*). Journal of Plant Growth Regulation. 2015; 35:401-412.
19. Reddy AR, Chaitanya KV, Vivekanandan M. Drought-induced responses of photosynthesis and antioxidant metabolism in higher plants. Journal of Plant Physiology. 2004; 161:1189-1202.
20. Roberson EB, Firestone MK. Relationship between desiccation and exopolysaccharide production in soil *Pseudomonas* sp. Applied Environmental Microbiology. 1992; 58:1284-1291.
21. Sandhya V, Ali Z, Grover M, Kishore N, Venkateswarlu B. *Pseudomonas* sp. strain P45 protects sunflowers seedlings from drought stress through improved soil structure. Journal of Oilseed Research. 2009a; 26:600-601.
22. Sandhya V, Ali SZ, Grover M, Kishore N, Venkateswarlu B. Alleviation of drought stress effects in sunflower seedlings by exopolysaccharides producing *Pseudomonas putida* strain P45. Biology and Fertility of Soil. 2009b; 46:17-26.
23. Saxena A, Raghuwanshi R, Singh HB. *Trichoderma* species mediated differential tolerance against biotic stress of phytopathogens in *Cicer arietinum* L. Journal of Basic Microbiology. 2014; 54:1-12.
24. Singh V, Upadhyay RS, Sarma BK, Singh, HB. Seed bio-priming with *Trichoderma asperellum* effectively modulate plant growth promotion in pea. International Journal of Agriculture, Environment and Biotechnology. 2016; 9(3):361-365.
25. Singh V, Upadhyay RS, Sarma BK, Singh HB. *Trichoderma asperellum* spore dose depended modulation of plant growth in vegetable crops. Microbiological Research. 2016.193:74-86.
26. Timmusk S, Islam A, Abd El D, Lucian C, Tanilas T, Ka Nnaste A. Drought tolerance of wheat improved by rhizosphere bacteri from harsh environments: Enhanced biomass production and reduced emissions of stress volatiles. PLOS One. 2014; 9:1-13.
27. Timmusk S, Nevo E. Plant root associated biofilms, In: Maheshwari, D.K. (Ed.), Bacteria in Agrobiology, Plant Nutrient Management, 3, Springer Verlag, Berlin, 2011, 285-300.
28. Timmusk S. Wagner EG. The plant-growth-promoting rhizobacterium *Paenibacillus polymyxa* induces change in *Arabidopsis thaliana* gene expression: A possible connection between biotic and abiotic stress responses. Molecular Plant Microbe Interaction 1999; 12: 951.
29. Wilkinson JF. The extracellular polysaccharides of bacteria. Bacteriological Review. 1958; 22:46-73.
30. Yang J, Kloepper JW, Ryu, CM. Rhizosphere bacteria help plants tolerate abiotic stress. Trends in Plant Science. 2009: 14:1-4.