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PLANT PATHOLOGY

Biocontrol Potential of Drought Tolerant Rhizospheric Bacteria Isolated from Stressed Ecosystems against Dry Root Rot of Chickpea (*Cicer arietinum* L.) Caused by *Rhizoctonia bataticola*

Ratul Moni Ram and H.B. Singh*

Department of Mycology and Plant Pathology, Institute of Agricultural Sciences, Banaras Hindu University, Varanasi, India

*Corresponding author: hbs1@rediffmail.com

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ABSTRACT

Drought is regarded as one of the major constraint in the productivity of crop plants around the world. Application of drought tolerant bacteria mitigating the effect of drought would be a novel approach in this regard. In the present study, the effect of *Bacillus* sp. BHU 08 was observed on growth promotion and disease suppression in chickpea. The treatment T8 (*Bacillus* sp. BHU 08) shows the highest value of morpho-physiological parameters that were observed at 30, 60 and 90 DAS. Application of *Bacillus* sp. BHU 08 induced accumulation of high level of phenol, proline, activities of PAL, SOD and catalase in chickpea plants when compared with its control. The treatment T7 recorded the highest value of all these enzymes at different hours after the pathogen challenge that varied from 48-72 h which then declined. Increased levels of these plant defense enzymes upon pathogen attack suggests that the ISR and SAR mechanism of the plant gets initiated and thus it will trigger a defense response in the host. These antioxidant enzymes quench the toxic reactive oxygen species and in turn protects the plant from oxidative damage. PAL activity precedes rapid recognition of a microbial invader which potentiates the accumulation of disease resistance factors including phenolics, phytoalexins and lignin. An increase in lignification is often observed in response to the attack by pathogen and is believed to represent one of the chief mechanisms adopted to block pathogen invasion due to its highly non-degradable and antimicrobial nature.

Highlights

- *Bacillus* sp. BHU 08 effectively enhanced plant growth promotion under stress condition.
- *Bacillus* sp. BHU 08 proved to be effective in managing dry root rot of chickpea.

Keywords: Drought, *Rhizoctonia bataticola*, *Bacillus* sp., Chickpea

Plants are subjected to several environmental stresses that have an adverse impact on their growth, metabolism, and yield. Drought, salinity, temperature extreme, water logging, pollutants, and radiation are few of the important stress factors limiting the productivity of crops (Lawlor, 2002). Apart from the above mentioned abiotic factors, several biotic factors such as insects, bacteria, fungi, and viruses also alter the growth and productivity in higher plants (Lichtenthaler, 1996, 1998). Among these, drought is regarded as a major abiotic factor that affects the agricultural crop production. Drought stress becomes predominant when the water supply to the roots is less compared to the demand or when the transpiration rate becomes very high. These two criteria are often found to coincide under arid and semi-arid climates. However, plants have an inherent ability to tolerate water stress, but its extent varies from species to species.

Although the general effects of drought on plant growth are well known, the primary effects of water deficit at the molecular and biochemical levels is still in dark (Zhu, 2002; Chaitanya *et al.,* 2003; Chaves *et al.,* 2003). In order to improve the agricultural productivity within the limited land resources, it is important to ensure higher crop yields against unfavorable environmental stresses. Understanding plant responses to the external environment is of greater importance and also a fundamental part to make the crops stress tolerant.

Chickpea (*Cicer arietinum* L.) is the third most important pulse crop in the world and is believed to be originated in South-eastern Turkey. It is a self-pollinated crop having a genome size of 740 Mbp. In India, chickpea ranks second in area and third in production. India is the largest producer of chickpea in the world covering 80 per cent area and 85% of total production with a productivity of 844kg/ha (www.iipr.res.in). However, the production of chickpea in the Indian continent and other countries in Asia is severely affected by many plant pathogenic fungi, bacteria, virus, and nematodes which cause the diseases such as *Fusarium* wilt, dry root rot, *Colletotrichum* blight, collar rot, bacterial blight, filiform virus and dirty root nematode (www.icrisat.org). Among the several constraints affecting the productivity of chickpea, 10-35% loss in yields are due to wilt and dry root rot diseases. Among them, dry root rot caused by *Rhizoctonia bataticola* is becoming severe in most of the chickpea growing regions of the country. *Rhizoctonia bataticola* is a polyphagous soil borne pathogen infecting more than 500 plant species worldwide causing huge loss. The pathogen becomes dominant during the flowering and the pod bearing stages of the chickpea crop. Biological method has proved to be an efficient tool for managing dry root rot disease. However, if any alternate method could be developed to minimize the combined loss *i.e.* abiotic and biotic; it will turn to be a great boon to the farmers.

MATERIALS AND METHODS

Green house experiment

Chickpea seeds were surface sterilized by using 1% sodium hypochlorite for 30 s and was rinsed twice with sterilized distilled water and then air-dried. The seeds were treated with the prepared spore suspensions of *Bacillus sp.* BHU 08 was isolated as mentioned above for 1 h. Soil mixture containing sandy loam soil, vermin compost, and farmyard manure (2:1:1) was autoclaved for 30 min at 15 lbs pressure for three consecutive days. Treated seeds were sown in 15 × 10 cm^2 pots under greenhouse conditions. The pots were timely irrigated with 50% polyethylene glycol (PEG) in order to create drought condition. Sampling for plant growth promotion was done after a gap of 30 days and various growth parameters like plant height, shoot and root length, total dry and fresh weight, number of roots, pod number and number of grains per pod were measured. The experiment was conducted with three replications and the data were pooled for analysis.

In vivo evaluation of biocontrol potential against *R. bataticola*

R. bataticola was grown in meal-sand medium at 25±2 °C for 15 days, was mixed with potting soil mixture and kept for two days for better colonization of pathogen in the soil. Ten treated chickpea seeds were sown in each pot under green house conditions. Untreated seeds sown in pathogen infected and pathogen uninfected soil served as positive and negative controls, respectively. When the plants was 40 days old, the soil was again infected with crushed mycelium of *R. bataticola* at the root zone. Three replications for each treatment were used and data were pooled for analysis.

Sample collection for biochemical analysis

From each treatment, randomly three plants were uprooted (one from each pot) carefully without causing any damage at 24 h intervals after the pathogens inoculation up to 96 h. Nodal leaves (3^{rd} to 5^{th} nodes) from the bottom were collected as samples. Collected leaves were washed in the running tap water, dried with blotting paper and stored in a deep freezer (-80°C) until used for the biochemical analysis.

Plant growth promotion test

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. The experiment was conducted with three replications and the data were pooled for analysis.

Phenylalanine ammonia-lyase (PAL) assay

Leaf sample of 0.1 g from each of the treatments was homogenized in 2 ml of 0.1 mol l^{-1} sodium borate buffer (pH 7.0; 4°C) containing 1.4 mmol l^{-1}ß-mercaptoethanol and centrifuged at 16,000 g at 4°C for 15 min. The supernatant was used as the enzyme source. To the reaction mixture containing 0.2 ml of enzyme extract, 0.5 ml of 0.2 mol l^{-1} borate buffer (pH 8.7) and 1.3 ml of water were added. The reaction was initiated by the addition of 1ml of 0.1 mol l^{-1} L-phenylalanine (pH 8.7) and incubated for 30 min at 32 °C. The reaction was terminated by addition of 0.5 ml of trichloroacetic acid (TCA, 1 mol l^{-1}. PAL (EC 4.1.3.5) activity was measured following the formation of trans-cinnamic acid at 290 nm as described by Brueske (1980) and was expressed in terms of µ mol l^{-1} TCA per g fresh weight (FW) (Jain *et al*. 2012).

Total phenolic content (TPC)

The TPC was determined following the method of Zheng and Shetty (2000). Leaf tissue (0.1 g) was placed in 5 ml of 95% ethanol and kept at 0°C for 48 h. The samples were homogenized individually and centrifuged at 13,000 g for 10 min. To 1 ml of the supernatant, 1 ml of 95% ethanol and 5 ml of sterile distilled water and 0.5 ml of 50% Folin–Ciocalteau regent were added, and the content was mixed thoroughly. After 5 min, 1 ml of 5% sodium carbonate was added, the reaction mixture was allowed to stand for 1 h and the absorbance of the colour developed was recorded at 725 nm. Standard curves were prepared for each assay using various concentrations of gallic acid (GA; Sigma-Aldrich-27645) in 95% ethanol. Absorbance values were converted to mg GA equivalents (GAE) per g FW.

Superoxide dismutase (SOD) assay

SOD (EC 1.15.1.1) activity was assayed following the method of Fridovich (1974) by measuring the ability of enzyme extract from samples, to inhibit the photochemical reduction of nitroblue tetrazolium (NBT) chloride. Fresh leaves (0.1 g) from each of the treatments were homogenized in 2.0 ml of extraction buffer (0.1 mol l^{-1} phosphate buffer containing 0.5 mmol l^{-1} EDTA at pH 7.5) in a pre-chilled mortar and pestle. The homogenate was centrifuged at 15000 g for 20 min at 4°C. The reaction mixture contained 200 mmol l^{-1} methionine, 2.25 mmol l^{-1} NBT, 3 mmol l^{-1} EDTA, 100 mmol l^{-1} phosphate buffer (pH 7.8), 1.5 mmol l^{-1} sodium carbonate and enzyme extract. The final volume was maintained to 3 ml. Reaction started by adding 2 µmmol l^{-1} riboflavin (0.4 ml), and the tubes were illuminated with two 15-W fluorescent lamps for 15 min. Reaction mixture without enzyme served as a control (Singh *et al*. 2013a). The reaction was terminated by putting the light off and keeping the tubes in dark until the absorbance was recorded at 560 nm. One unit of the SOD activity was defined as the amount of enzyme reducing the absorbance to 50% in comparison to control lacking enzyme.

Catalase assay

Catalase (CAT) (E.C. 1.11.1.6) activity was measured according to the method described by Aebi (1984). Leaf samples (0.1 g) were homogenized in 50 mM Tris–HCl buffer (pH 8.0) containing 0.5 mM EDTA, 2% w/v polyvinylpyrrolidone, and 0.5% (v/v) Triton X100 using a pre-chilled mortar and pestle. The homogenate was centrifuged at 15,000 g for 10 min at 4 °C, and the supernatant was used for enzyme assay. The reaction mixture consisted of 300 µM phosphate buffer (pH 7.2) and 100 µM H_2O_2 in 1 ml enzyme extract. Activity was determined by recording O_2 released from enzymatic dissociation of H_2O_2 in darkness for 1 min. O_2 produced by the enzymatic reaction was estimated by measuring the decrease in H_2O_2 absorption at 240 nm (extinction coefficient of H_2O_2 is 0.036 mM^{-1} cm^{-1}) and the enzyme activity was expressed as µM H_2O_2 oxidized min^{-1} g^{-1} FW (Singh *et al*. 2013b).

Estimation of Proline

Estimation of proline was followed according to Bates *et al.* (1973). 0.1 gram of plant sample was crushed in 10 ml of sulphosalicylic acid (3%). The sample was centrifuged at 10,000 rpm for 10 minutes and the volume was maintained to 10 ml by the addition of sulphosalicylic acid. In fresh test tube 2 ml of sample was pipetted out and 4 ml of ninhydrin solution was added, followed by boiling it in water bath at 100°C for 30 minutes.

After cooling, 4 ml of toluene was added and upon vortexing pink color was observed. With the help of a separating funnel, the upper layer was collected and its OD was measured at 520 nm against toluene blank.

Preparation of the samples

The extract was prepared by crushing 1.0 g of fresh leaf in mortar and pestle with 5 ml of methanol: H_2O: HCl (80:19:1) and incubated overnight at room temperature. The extracts obtained were filtered through sterilized Whatman No. 1 filter paper. The samples were then subjected to fractionation. The extracts were mixed with equal volume of ethyl acetate in a fractionating column and were shaken vigorously. After some time two different layers were formed in the column. The ethyl acetate fractions formed at the upper layer was collected separately, and the residue was extracted again using the ethyl acetate. The step was repeated thrice and the overall collected fractions were then allowed to evaporate. Dried samples were suspended in 1.0 ml of HPLC grade methanol, vortexed and used for analysis.

HPLC analysis

HPLC analysis of the samples was performed with an HPLC system (Shimadzu, Japan) equipped with two LC-10 pumps and a UV detector SPD-10A. A C-18 HPLC column (4 μm, 250 × 4.6 mm, Phenomenex, USA) was used, and the data were integrated by Shimadzu Class VP series software. Samples were filtered through membrane filter (pore size 0.2 μm, Merck) prior to injection. The sample (20 μl) was injected and separated with a two pump linear gradient program for pump A (water containing 1% acetic acid) and pump B (Acetonitrile), with detection at 254 nm. It initially was started with a gradient of 18% from pump B, changing to 32% in 15 min and finally to 50% in 30 min followed by washing for 25 min. Results (μg g−1 FW) were obtained by comparing the retention time (Rt) and the peak area of the samples with that of standard.

Statistical Analysis

The experiments were performed in a completely randomized design. The data are expressed as the mean of three independent replications ± standard deviation (SD). One-way ANOVA was carried out in order to test the significance of the observed differences using SPSS version 16. The differences between the various parameters were evaluated by means of Duncan's multiple range test and P values ≤ 0.05 were considered as statistically significant.

Table 1: Order of treatments used in the study

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 + Pathogen (**P**)
T_5	Chickpea Plant + Pathogen + Biocontrol agent (**P + B**)
T_6	Chickpea Plant + Drought stress + Pathogen (**D + P**)
T_7	Chickpea Plant + Drought stress + Pathogen + Biocontrol agent (**D + P + B**)
T_8	Chickpea Plant + Biocontrol agent (**B**)

RESULTS

Plant growth promotion test

The plants were recorded for their various morphological parameters on 30, 60 and 90 days after sowing. The data is presented in Table 2.

Plant height (cm)

Table 2 represents the data of plant height recorded at 30, 60 and 90 DAS. The plant height increased with the increasing age of the plants. The sole BHU 08 treated (T8) plant showed maximum height in comparison to control and stressed plants in each treatment level. It was clear from the data that the maximum height was recorded in T8 treatment level which was followed by control (T1) at 60 and 90 DAS. While comparing the stress regimes, T6 showed minimum plant height at all the time durations. While comparing to the individual stress provided to the crop, T4 treatment showed less plant height when compared to T2. The values of plant height did not significantly vary at 30 DAS as the pathogen *R. bataticola* shows symptoms only after 50 DAS. However the data recorded at 60 DAS and 90 DAS reflects a significant difference in the plant height of stress challenged (T2, T4, and T6) and BHU 08 treated plants (T3, T5, T7 and T8).

Table 2: Effect of various treatments on the growth parameters of Chickpea crop (Year 2013-14**).** *Results are expressed as the mean of triplicate ± SD. Different letters indicate significant difference among the treatment results taken at the same time interval according to Duncan's multiple range test at p ≤ 0.05.

Days after sowing (DAS)	Growth parameters	Treatments								
		T1	T2	T3	T4	T5	T6	T7	T8	CD at 5 %
30	Plant height (cm)	28.2 ± 1.10^{ab}	28.5 ± 0.3^{ab}	28.2 ± 1.4^{ab}	26.1 ± 2.31^{bc}	28.6 ± 1.1^{ab}	25.6 ± 1.74^{c}	28.3± 0.96^{ab}	29.1 ± 0.88^{a}	2.25
	Shoot length (cm)	23.7±1.81^{ab}	21.9±1.68^{ab}	24.2 ± 2.68^{ab}	22.1 ± 2.05^{bc}	23.3 ± 0.55^{ab}	21.4 ± 0.66^{c}	24.0 ± 0.97^{ab}	24.5 ± 0.30^{a}	2.40
	Root length (cm)	8.9 ± 3.23^{a}	7.1 ± 0.8^{a}	9.1 ± 2.31^{a}	7.6 ± 1.26^{a}	8 ± 1.45^{a}	6.1 ± 0.45^{a}	9.7 ± 3.41^{a}	9.3 ± 0.20^{a}	3.32
	Number of roots	21 ± 2.0^{a}	18 ± 2.0^{a}	21 ± 6.5^{a}	15.6 ± 2.5^{a}	18.3 ±1.15^{a}	15.6 ±1.52^{a}	18 ± 3.0^{a}	20.6 ± 3.0^{a}	5.09
	Fresh weight (g)	2.6 ± 0.2^{ab}	1.63 ± 0.09^{e}	2.2 ± 0.6^{cd}	1.48± 0.08^{e}	2.41 ± 0.7^{bc}	0.86 ± 0.3^{f}	2.16 ± 0.41^{d}	2.66 ± 0.36^{a}	0.15
	Dry weight (g)	0.57 ± 0.03^{a}	0.43±0.07^{ab}	0.41± 0.02^{ab}	0.38±0.03^{ab}	0.51 ±0.05^{a}	0.24 ± 0.01^{b}	0.47 ± 0.02^{a}	0.55 ± 0.13^{a}	0.78
	Relative water content (%)	67.20 ±0.28^{ab}	59.71±0.54^{a}	61.87±0.37^{ab}	56.21±0.46^{bc}	62.20±1.25^{ab}	54.36±0.86^{bc}	60.44±0.58^{ab}	72.45±1.10^{ab}	1.24
60	Plant height (cm)	41.8 ± 2.45^{a}	37.6 ± 3.75^{a}	39.8 ± 4.45^{a}	30.4 ± 2.45^{b}	41.6 ± 3.05^{a}	29.7 ± 3.45^{b}	40.1 ± 3.75^{a}	45.6 ± 3.15^{a}	5.57
	Shoot length (cm)	37.6 ±1.15^{a}	34.8 ± 3.35^{a}	36.4 ± 3.95^{a}	26.3 ± 1.25^{b}	38.4 ± 3.12^{a}	24.2 ± 2.55^{b}	35.8 ± 3.15^{a}	38.8 ± 2.45^{a}	5.15
	Root length (cm)	8.6 ±0.45^{abc}	7.3 ± 0.40^{e}	8.2 ±0.35^{bcd}	7.2 ± 0.70^{e}	9.2 ± 0.55^{a}	7.4 ± 0.55^{de}	7.9 ± 0.30^{cde}	9.00 ±0.45^{ab}	2.90
	Number of roots	38.6 ±6.02^{bc}	32.6 ±5.50^{cd}	35.0±3.60^{bcd}	27.6 ± 1.52^{d}	43.6 ±4.04^{ab}	28.0 ± 4.58^{d}	37.6 ±4.04^{bc}	48.0 ± 4.58^{a}	8.09
	Fresh weight (g)	32.8 ± 1.35^{a}	22.5 ± 1.05^{e}	27.6 ± 1.75^{c}	20.4 ± 0.85^{f}	30.5 ± 0.95^{b}	18.5 ± 0.75^{f}	25.4 ± 1.35^{d}	34.7 ± 0.65^{a}	1.61
	Dry weight (g)	11.6 ± 1.15^{b}	8.6 ± 0.85^{d}	6.9 ± 0.26^{e}	7.2 ± 0.05^{e}	10.3 ± 0.55^{c}	5.8 ± 0.15^{f}	7.6 ± 0.26^{de}	14.5 ± 0.40^{a}	0.76
	Relative water content (%)	76.24±0.74^{ab}	55.16±0.38^{bc}	61.12±0.46^{ab}	50.47± 0.75^{b}	73.18±1.16^{bc}	42.54±0.78^{ab}	58.15±1.65^{bc}	80.66±0.74^{ab}	1.16
90	Plant height (cm)	54.6 ±4.05^{ab}	47.1± 3.25^{bc}	50.2 ±6.45^{ab}	42.1 ± 1.32^{c}	52.1 ±2.45^{ab}	34.6 ± 4.85^{d}	49.4±4.25^{abc}	56.8 ± 3.85^{a}	4.01
	Shoot length (cm)	46.7±1.8^{a}	39.4 ± 3.85^{c}	44.3±1.55^{abc}	34.2 ± 3.35^{d}	44.6 ±3.15^{ab}	28.2 ± 2.45^{e}	41.2± 1.65^{bc}	49.4 ± 3.25^{a}	4.82
	Root length (cm)	9.76 ± 1.84^{a}	7.4 ± 0.7^{a}	7.7 ± 0.55^{a}	7.5 ± 0.65^{b}	8.76 ±0.85^{a}	6.8 ± 0.45^{b}	7.56± 0.65^{a}	8.43±0.85^{a}	13.19
	Number of roots	42.7 ± 8.08^{a}	36.4± 4.0^{bc}	38.7 ± 7.0^{bc}	22.6± 6.42^{bc}	39.4± 8.08^{ab}	16.25 ± 4.0^{c}	40.6 ± 7.0^{bc}	48.6± 6.42^{bc}	1.70
	Fresh weight (g)	42.31 ± 1.59^{a}	28.60± 0.75^{f}	32.36 ± 0.55^{d}	29.81± 1.55^{e}	41.43 ±1.45^{b}	21.60 ±2.15^{f}	28.2 5± 0.45^{e}	47.26 ±0.95^{c}	2.26
	Dry weight (g)	20.3 ± 0.39^{a}	10.5 ± 0.74^{f}	13.9 ± 0.58^{d}	11.9 ± 0.63^{e}	18.76 ±1.28^{b}	9.19 ± 1.12^{f}	13.7 ± 1.15^{d}	24.16 ±1.18^{c}	0.58
	Relative water content (%)	71.42 ±0.29^{bc}	58.16 ±0.35^{a}	64.85±0.95^{bc}	52.74±1.05^{bc}	69.48±0.63^{ac}	42.15±0.75^{bc}	60.37±1.25^{ac}	76.54±0.58^{bc}	1.58

Shoot length (cm)

Shoot length was recorded at 30, 60 and 90 DAS. Shoot length increased along with the increasing age of the plants. Maximum shoot length was attained in T8 at all time durations. At 30 DAS, T3 recorded the second highest value. While comparing the stress regimes, T6 showed minimum shoot length at all the time durations. On comparison of the individual stress provided to crop, T4 showed less plant height when compared to T2. The data recorded at 60 DAS and 90 DAS reflects a significant difference in shoot length of stress challenged (T2, T4, and T6) and BHU 08 treated plants (T3, T5, T7 and T8).

Root length (cm)

Root length was recorded at 30, 60 and 90 DAS. At 30 DAS, T7 recorded the highest value, while at 60 DAS, T5 was maximum. At 90 DAS, T1 recorded the highest root length. While comparing the stress regimes, T6 showed minimum root length in 30 DAS and 90 DAS, while in 60 DAS, T4 recorded the minimum root length. The data recorded at 60 DAS and 90 DAS reflects a significant difference in root length of stress challenged (T2, T4, and T6) and BHU 08 treated plants (T3, T5, T7 and T8).

Number of roots

Number of roots was recorded at 30, 60 and 90 DAS. Maximum number of roots was attained in T8 at 60 DAS and 90 DAS. At 30 DAS, T1 recorded the maximum number of roots value. While comparing the stress regimes, T6 showed minimum number of roots at all the time durations. On comparison of the individual stress provided to the crop, T4 showed less number of roots at 30 DAS along with T6. The data recorded at 60 DAS and 90 DAS reflects a significant difference in number of roots of stress challenged (T2, T4, and T6) and BHU 08 treated plants (T3, T5, T7 and T8).

Fresh weight (g)

Fresh weight was recorded at 30, 60 and 90 DAS. Maximum fresh weight was attained in T8 at all time regimes. While comparing the stress regimes T6 showed minimum number of roots at all the time durations. The data recorded at 60 DAS and 90 DAS reflects a significant difference in fresh weight of stress challenged (T2, T4, and T6) and BHU 08 treated plants (T3, T5, T7 and T8).

Dry weight (g)

Dry weight was recorded at 30, 60 and 90 DAS. Maximum dry weight was attained in T8 at 60 DAS and 90 DAS, while at 30 DAS T1 showed maximum dry weight. While comparing the stress regimes, T6 showed minimum number of roots at 30 DAS and 90 DAS, while at 60 DAS T1 exhibited minimum dry weight. The data recorded at 60 DAS and 90 DAS reflects a significant difference in dry weight of stress challenged (T2, T4, and T6) and BHU 08 treated plants (T3, T5, T7 and T8).

Relative water content (%)

Relative water content was recorded at 30, 60 and 90 DAS. Maximum relative water content was attained in T8 at all-time regimes. While comparing the stress regimes,T6 showed minimum number of roots at 30 DAS and 90 DAS, while at 60 DAS T4 exhibited minimum relative water content. The data recorded at 60 DAS and 90 DAS reflects a significant difference in the relative water content of stress challenged (T2, T4, and T6) and BHU 08 treated plants (T3, T5, T7 and T8).

Biochemical analysis

PAL activity was found to be significantly higher in all the treatments up till 48 hours and declined thereafter. T7 showed maximum accumulation of PAL at 48 h, followed by T3 at 24 h. At 48 h, T7 exhibited 1.95 folds increase compared to control (T1). The treatments in which only stress was applied i.e. T2, T4 and T6 recorded 1.32, 1.29 and 1.51 folds increase compared to control. T3 and T5 in which both stress and biocontrol agent i.e. BHU 08 was applied showed an increment of 1.69 and 1.48 folds as compared to control. Similarly, the plants treated with only biocontrol agent (T8) showed 1.52 folds increase in PAL values with reference to control (Fig. 1).

The TPC followed a similar trend that of PAL with maximum increment at 48 h in T7 followed by a sharp decline in its activity. The amount of TPC content shows significant variation among different treatments. The highest phenolic content was observed at 48 h in T7. The total phenolic content was higher by 0.89, 1.08, 0.86, 1.1, 1.02, 1.39 and 1.01 folds in T2, T3, T4, T5, T6, T7, and T8 respectively, when compared to control (T1) at 48 h (Fig. 2).

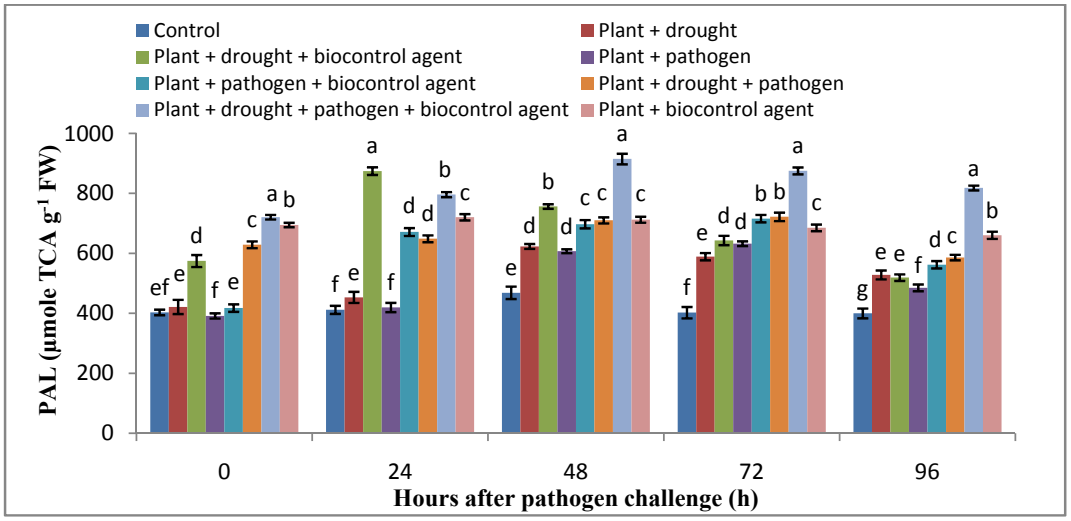

Fig. 1: PAL activity at different time intervals in chickpea raised from seeds treated with *Bacillus* sp. BHU 08, challenged with *R. bataticola* and drought individually and in combination. Results are expressed as means of the three replicates and the vertical bars indicate standard deviation of the mean. Different letters indicate significant difference among the treatment results taken at the same time interval according to Duncan's multiple range test at $p \leq 0.05$

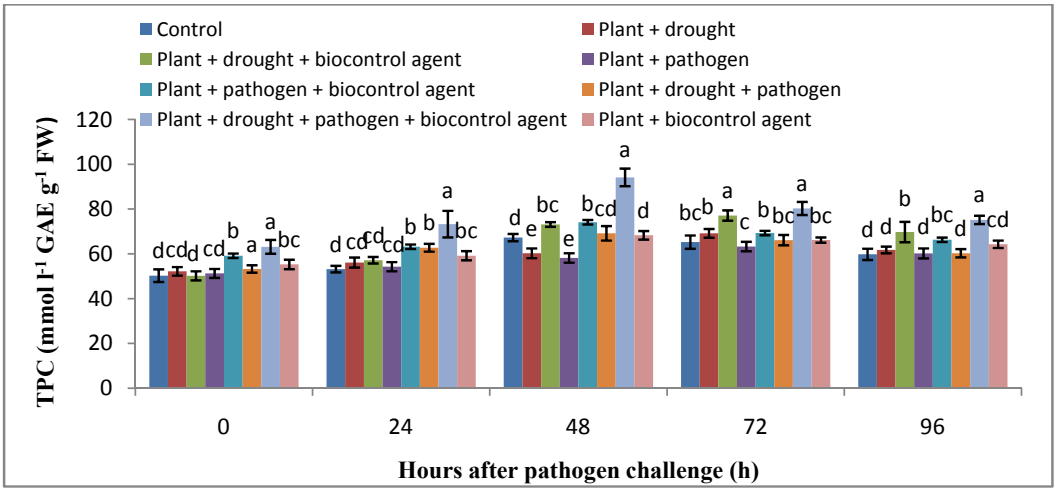

Fig. 2: TPC activity at different time intervals in chickpea raised from seeds treated with *Bacillus* sp. BHU 08, challenged with *R. bataticola* and drought individually and in combination. Results are expressed as means of the three replicates and the vertical bars indicate standard deviation of the mean. Different letters indicate significant difference among the treatment results taken at the same time interval according to Duncan's multiple range test at $p \leq 0.05$

SOD activities increased consistently and attained their maximum levels at 72 h in all treatments and thereafter its value declined. The highest SOD activities were observed at 72 h in T7 followed byT6. The SOD values were 1.11, 1.48, 1.00, 1.70, 1.89, 2.17 and 1.83 folds in T2, T3, T4, T5, T6, T7, and T8 respectively, when compared to control (T1) at 72 h (Fig. 3).

Proline accumulation followed a trend similar to that of SOD and increased significantly in all the treatments up to 72 h, thereby declining up to 96 h. Proline accumulation increased consistently and attained their maximum levels at 72 h in T7 followed by T8 and T3. Proline accumulation increased by 1.13, 1.55, 1.44, 1.54, 1.36, 1.74 and 1.55 folds in T2, T3, T4, T5, T6, T7, and T8 respectively, when compared to control at 72 h (Fig. 4).

Catalase activity has increased from 24 h and reached its maximum level at 48 h, thereafter it started declining. The Catalase activity was found to be highest at 48 h in T7. On comparing the other treatments it can be seen that the catalase activity

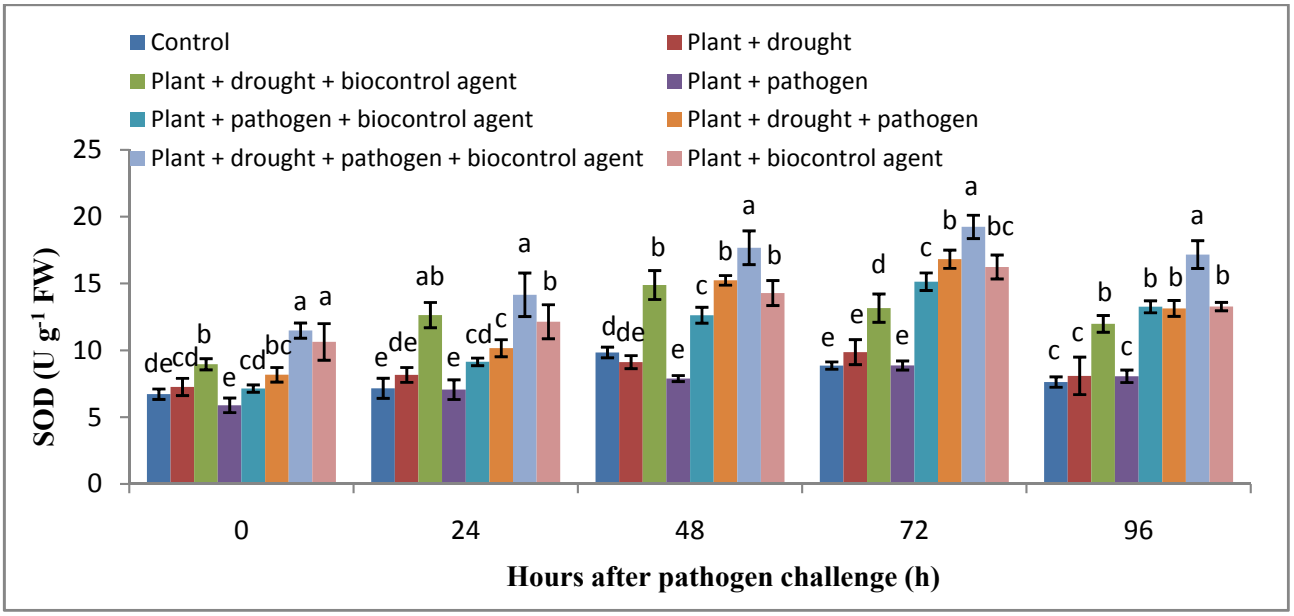

Fig. 3: SOD activity at different time intervals in chickpea raised from seeds treated with *Bacillus* sp. BHU 08, challenged with *R. bataticola* and drought individually and in combination. Results are expressed as means of the three replicates and the vertical bars indicate standard deviation of the mean. Different letters indicate significant difference among the treatment results taken at the same time interval according to Duncan's multiple range test at $p \leq 0.05$

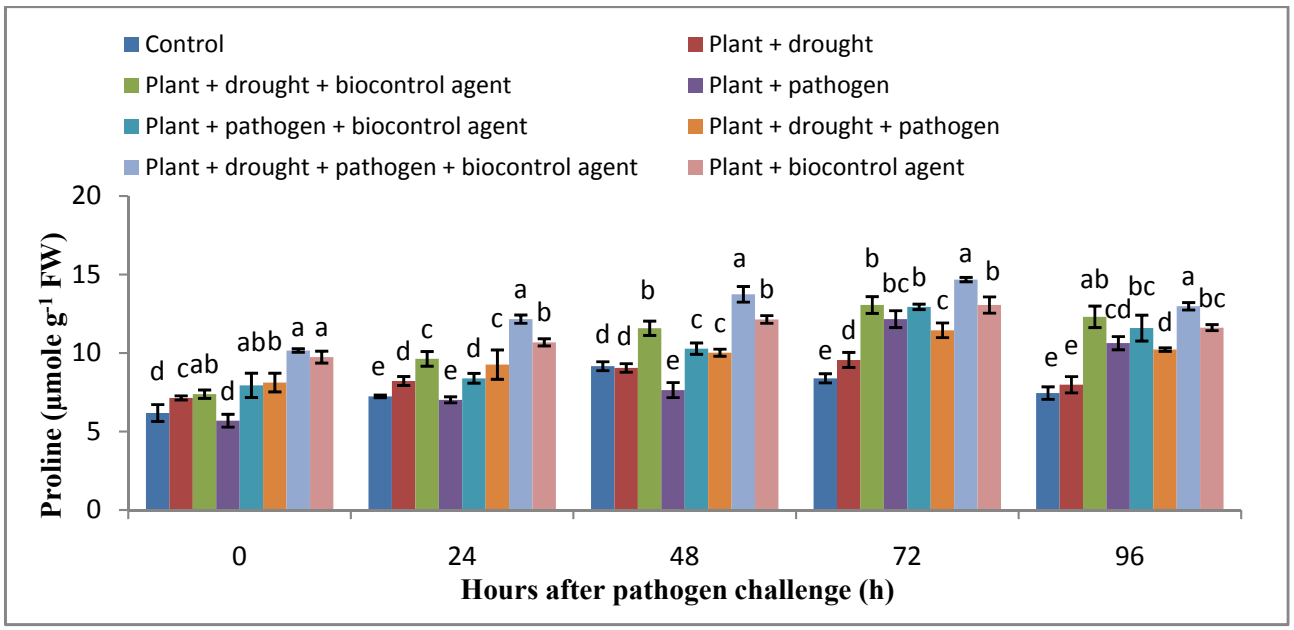

Fig. 4: Proline activity at different time intervals in chickpea raised from seeds treated with *Bacillus* sp. BHU 08, challenged with *R. bataticola* and drought individually and in combination. Results are expressed as means of the three replicates and the vertical bars indicate standard deviation of the mean. Different letters indicate significant difference among the treatment results taken at the same time interval according to Duncan's multiple range test at $p \leq 0.05$

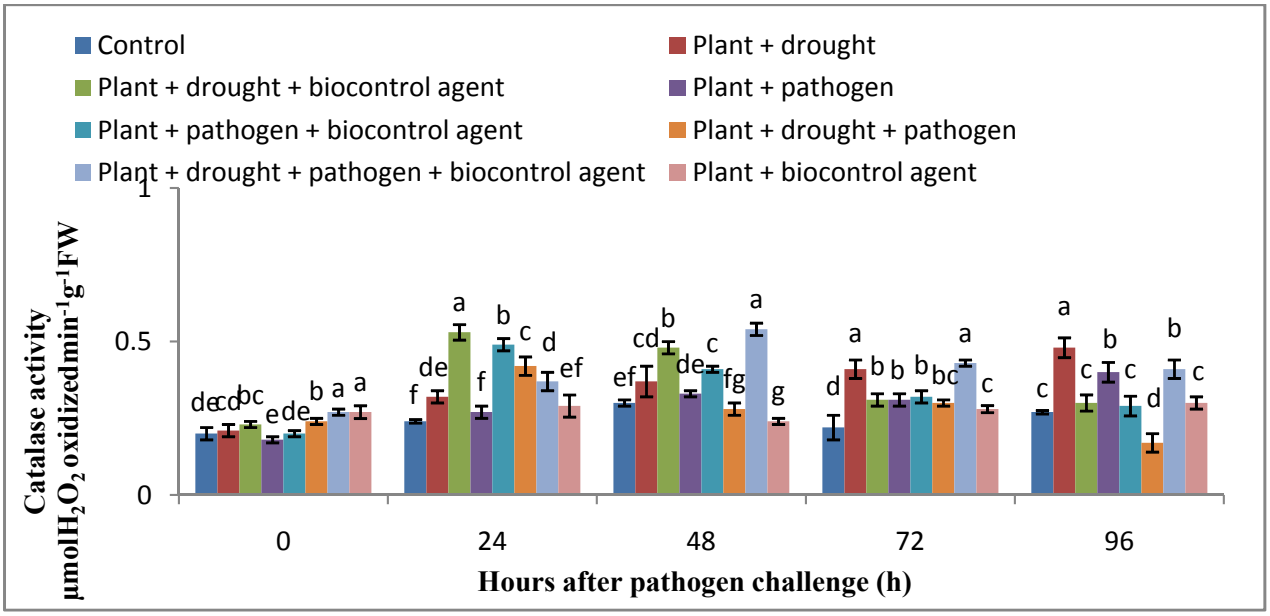

Fig. 5: Catalase activity at different time intervals in chickpea raised from seeds treated with *Bacillus* sp. BHU 08, challenged with *R. bataticola* and drought individually and in combination. Results are expressed as means of the three replicates and the vertical bars indicate standard deviation of the mean. Different letters indicate significant difference among the treatment results taken at the same time interval according to Duncan's multiple range test at $p \leq 0.05$

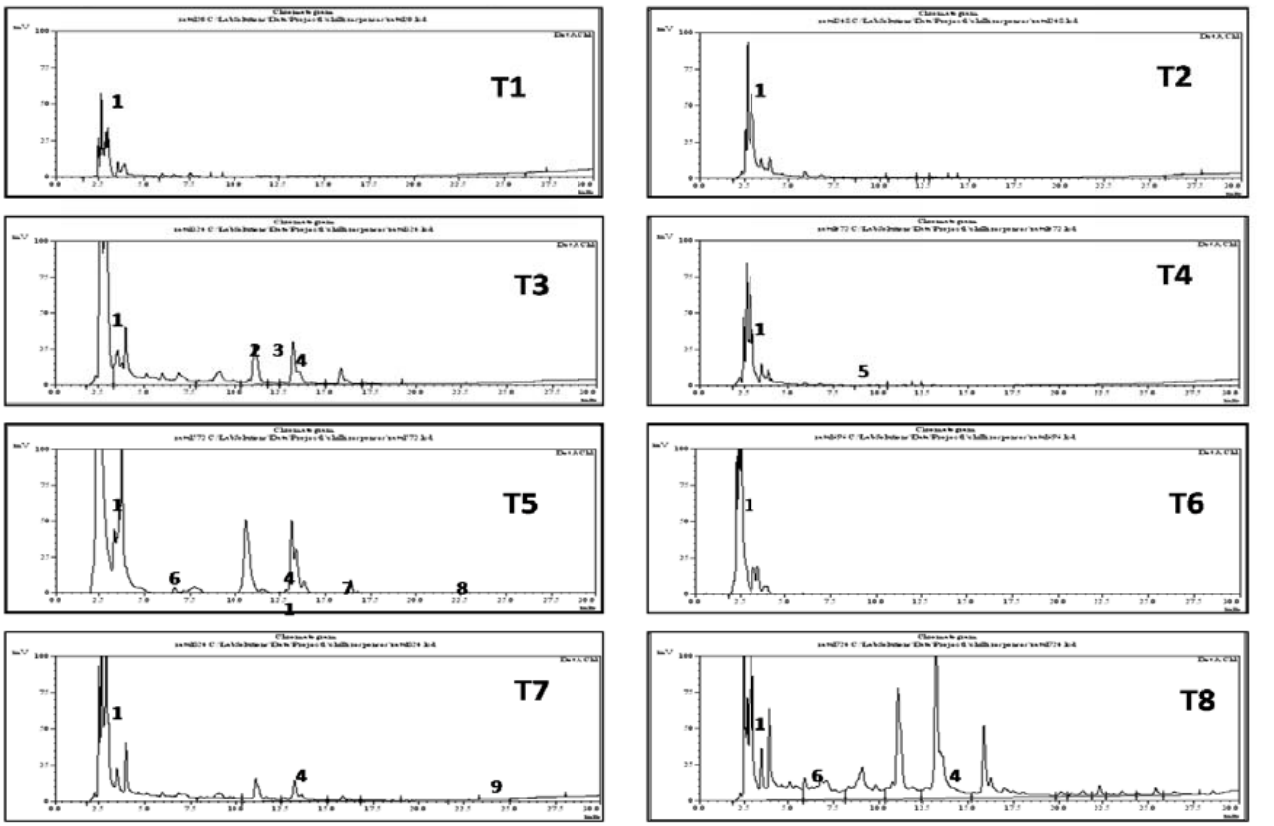

Fig. 6: HPLC Profiles Exhibiting Induction of Phenolics in the Leaves of Chickpea at 24 hours

increased by 1.23, 1.60, 1.10, 1.36, 0.93, 1.80 and 0.80 folds in T2, T3, T4, T5, T6, T7, and T8 respectively, when compared to control (T1) at 48 h (Fig. 5).

HPLC Analysis

Qualitative and quantitative analysis of phenolics

In order to gain an insight regarding the interaction of various stress parameters with the biocontrol agent (*Bacillus* sp. BHU 08), qualitative and quantitative enhancement of phenolic profiles were studied from the leaf samples of the combined treatments of *R. bataticola* and selected bioagents (*Bacillus* sp. BHU 08) through HPLC at 24 hrs after pathogen challenge and the results were presented in Fig. 6. Nine different phenolic compounds were detected in chickpea leaves, however the systemic accumulation of shikimic acid was observed in all the treatments.

The control plants (C), recorded the lowest production of shikimic acid i.e. 3920 µg/g FW whereas in other treatments there was a significant increase in the level of shikimic acid compared to C. The highest amount of shikimic acid was recorded in T5 (P + B) i.e. 30120 µg/g FW. The treatments in which drought and pathogen stress was applied i.e. T2 (D), T4 (P) and T6 (D + P) recorded shikimic acid content of 7620, 7140 and 10300 µg/g FW respectively. Similarly in T3 (D + B), T7 (D + P + B) and T8 (B), shikimic acid values were 14740, 14632 and 14286 µg/g FW which is more than the individual stress challenged plants.

p-caumaric acid and cinnamic acid was present in T3 (D + B) only with a value of 136 µg/g FW. The level of ferulic acid ranged in-between 44 – 204 µg/g FW. T8 (B) recorded highest amount of ferulic acid i.e. 204 µg/g FW followed by T5, T3 and T7 which recorded 116, 54 and 44 µg/g FW respectively. However, in treatments T1, T2, T4 and T6, it was found to be absent. Tannic acid was present in treatments T5 and T8 with values of 18 and 304 µg/g FW respectively. Rutin was present in only one treatment (T4) with value of 1.028 µg/g FW. Capsaicin, quercetin and kaempferol also followed the pattern of rutin and were present in single treatment only i.e. T5 and T7. Capsaicin and quercetin are present in T5 and recorded a value of 370 and 0.194 µg/g FW respectively. Kaempferol was present in treatment T7 and its concentration was 2.2 µg/g FW.

DISCUSSION

A large number of microbes possessing many useful attributes are known to harmoniously colonize the plants root without causing any disease. The association between plants and beneficial microbes not only stimulates plant growth but also checks the pathogen attack. Beneficial microbes usually enhance the plants defense signaling capacity in order to withstand pathogen attack (Van der Ent, 2009). *R. bataticola* is regarded as a dreadful pathogen of chickpea causing significant yield loss (Singh, 1989). The disease aggravates during moisture stress condition which usually predominates during the flowering stages of the crop. Many researchers have attempted towards controlling the incidence of dry root rot through different ways (Ved Ratan *et al.* 2010).

However, management of the disease through biological methods is still regarded as one of the best methods. In the experiment, the chickpea plants were inoculated with strain of *Bacillus* sp. BHU 08 and checked for their response individually and along with presence of drought stress. The plants were checked for various morphological features like plant height, shoot and root length, total dry and fresh weight, number of roots, pod number and number of grains per pod. The results revealed that when plants are inoculated with *Bacillus* sp. BHU 08, it exhibited better growth compared to the other treatments, whereas in the treatment where both pathogen and drought stress were combined exhibited least growth. This feature may be attributed to the synergistic association of moisture stress and pathogenicity of *R. bataticola*.

CONCLUSION

Inoculation of *Bacillus* sp. BHU 08 in chickpea leads to significant plant growth promotion and root rot tolerance in the treated plants. Also, an increase in the activities of defense related enzymes especially PAL, TPC, SOD, Proline and Catalase is recorded, suggesting that these enzymes are up regulated through the microbial signals. HPLC studies also revealed the enhancement in the level of various phenols in treatments compared to control. Study

of these responses at molecular level would be definitely an innovative work and prove to unravel new aspects in the management of both biotic and abiotic stress.

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