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Synergistic Effect of Arbuscular Mycorrhizal Fungi and Mycorrhizal Helper Bacteria on Physiological Mechanism to Tolerate Drought in *Eclipta prostrata* (L.) L

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A study was undertaken to determine the inoculation effects of arbuscular mycorrhizal fungus, *Funneliformis mosseae* and two plant growth promoting microorganisms, *Bacillus megaterium* and *Trichoderma harzianum* on growth and metabolites content of *Eclipta prostrata* (L.) L plant grown under irrigated and drought conditions. The mycorrhizal inoculation increased plant growth parameters like root length, shoot length, plant height, fresh weight, dry weight and chlorophyll content independent of the water regime, particularly when associated with *B. megaterium*. Physiological changes after drought stress as observed through relative water content (RWC) of plant leaf was evident, microbial treatments supported higher RWC. Exposure of plant to water stress led to cell damage which gets lesser in seedlings inoculated with consortia of *F. mosseae* and *B. megaterium*, as compared to other treatments including control. Dual consortia of *F. mosseae* and *B. megaterium* enhanced antioxidant enzyme activity like proline, catalase, peroxidase, phenols and flavonoids in plant maximally. This particular consortium was able to enhance plant defence system and ameliorate oxidative damages to membrane lipids. The results of the experiment indicated compatibility and synergy between *F. mosseae* and *B. megaterium* and was found to be the best for improving growth, biomass and antioxidant enzyme content of *E. prostrata* under drought stress.

Keywords: *Bacillus megaterium*, *Eclipta prostrata*, *Funnelliformis mosseae*, *Trichoderma harzianum*, drought, flavonoids, phenols, proline, catalase, peroxidase.

India has a rich wealth of medicinal plants and these may not only be considered as chemical factories for biosynthesis of a huge array of secondary metabolites utilized on commercial scale as medicine, dyes, scent and pesticides but are also a source of novel molecules looked at for human welfare. Demand for medicinal plants is increasing due to growing recognition of natural products and as a result many of them have now being listed as endangered. Changing climatic condition are further adding new challenges to the plant biologist and as predicted, by 2050 the average annual mean warming may rise by 2.2 to 2.9°C, which may expose plants to drought stress. Drought is recognized as one of the most important abiotic stress affecting plant vigour and its life cycle[1]. Drought stress induces morpho-physiological responses in plant such as reduction in leaf area and shoot growth, enhancement of root growth, stomata closure, reduction in growth rate, antioxidants and soluble compounds accumulation, and activation of some enzymes[2]. Hence, increasing plant growth and metabolites under limited land resources and abiotic

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stress may come up as a major issue. The issue can be partly addressed by the soil microorganisms, which are the key elements in ecosystem functioning and show wide adaptability under diverse environmental conditions. Microorganisms are now widely applied in field soils for its plant growth promoting characters[3], as bio-control agents against plant pathogens and in soil health improvement[4]. Plant growth promoting rhizobacteria (PGPR) helps plant growth promotion by improving nutrient uptake[5], enhancing phytohormone production[6] and showing synergistic association with bacteria-plant interactions[7]. The mechanism by which PGPR in presence of AM fungi contributes to plant establishment, growth and drought tolerance is the sum measure of cellular, physiological and nutritional effects[8,9]. PGPR have been reported to enhance the activity of mycorrhizal fungi and consequently plant growth[10, 11] by providing phosphorus availability to the host plant. The free living bacteria stimulate fungus contacts and root colonization[12]. The mycorrhizal colonization in the plant contributes in enhancing drought tolerance[13]. Role of *Trichoderma* spp. as biopesticides and biofertilizers to protect plants from pathogens[14] and promote plant growth under different abiotic stress has been widely studied[15]. It is versatile plant symbiont which ameliorates plant growth under abiotic stress conditions by lowering ethylene levels and enhancing antioxidative capacity[16]. There exist a lot of prospects in inoculating medicinal plants with these plant growth promoting microorganisms as an effective strategy to overcome drought.

Eclipta prostrata (L.) L commonly known as Bhringraj, in India belongs to family Asteraceae. The plant is a small and erect annual herb and is widely distributed in China, Thailand, Brazil and eastern countries like Indonesia, Srilanka, Phillipines, Nepal and Malaysia. The whole plant and seeds have great medicinal value. *E. prostrata* has been used in various pharmacopeia for a variety of purpose like to prevent aging, rejuvenate hair, teeth, bones, sight and hearing, kidney, enhance sleep and memory, improve complexion, treat hepatitis, skin disorders and remove worms. Wedelolactone the active ingredient present in *E. prostrata* has also been reported as anti-HIV-1[17] and anticancerous[18]. Studies on medicinal property of *E. prostrata* plant have gained potential in current scenario due to its potent antioxidant activities, no side effects and economic viability. Till date studies on the inoculation of plant growth promoting microorganisms (PGPM) have been focused on economically important agricultural crops. Their role in growth promoting effects on wild medicinal flora has been less worked out. Improving plant growth and content of secondary metabolites by PGPM could be achieved under abiotic stress condition as an agronomical approach. Use of microbes from arid areas as a sustainable method to induce drought tolerance is limited by under-performance of microbes under altered native conditions. Screening indigenous microbes for drought tolerance under *in vitro* conditions has given encouraging results but is area specific and studies lag at field level. Commercially available biofertilizers performing well under irrigated condition have not been tested under drought which is speculated as a future problem. Keeping in view the changing climatic conditions and sustainable approaches to meet the challenges, the present study was under taken with an aim to study field performance of commercially available bio-fertilizers under drought stress and methods to improve their performance by support of indigenous growth promoting microbes.

MATERIALS AND METHODS

Plant authentication and growth conditions

The study was conducted in the campus of Banaras Hindu University (B.H.U.) located at 25°182 N latitude, 83°12 E longitude and 76.19m above the mean sea level in the Eastern Gangetic plains of India. Plants of *E. prostrata* growing widely in B.H.U. campus was first authenticated at Botanical Survey of India, Allahabad, India with deposited specimen voucher number-91926.The field experiment was conducted from March to August 2013 at BHU. The mean monthly maximum and minimum temperature varied from 34.89-24.15°C, the relative humidity varied from 72.12% - 50.15%, mean sunshine was 7.22 h, with pan evaporation value 4.96 mm d^{-1}, rainfall 3.629 mm during the study period. Physicochemical analysis of the field soil showed pH 7.35$\pm$ 0.246, organic carbon (%) 0.95 $\pm$ 0.31, available P (ppm)19.09 $\pm$ 2.1

and available NO_3^{2-}(ppm) 8.7± 3.3. The soil texture was slity loamy (gravel– 4%, sand– 11%, slit–71% and clay– 14%). Seeds of *E. prostrata* collected widely from the campus and grown in plastic plots showed 85% seed viability. The experiment was performed in a randomized complete block design (RCBD) with three replications. On the basis of drought stress treatments the main plot (20 × 20 m^2) was subdivided into two subplot (10×10 m^2) and was prepared by ploughing and levelling soil properly for the experiment. After maintaining, proper field capacity condition, the plots were well prepared for sowing the seeds. In one subplot, daily irrigation was provided and in the second sub-plot, drought stress was given after 90 days of sowing seed. Seeds of *E. prostrata* was sown by hand drilling each subplot consisting of 7 rows having inter-row distance of 60cm. Plants were thinned 15 days after germination to maintain plant-to-plant distance of 30 cm. Weeding was done from time to time when required. The indigenous AM spores present in the field soil comprised mainly of *Glomus* sp. and *Acaulospora* sp. with total spore density of 100 per 10g of soil.

Inoculum preparation of Plant growth promoting microorganisms (PGPM's)

AM fungal inoculum preparation

Spores of *Funnelliformis mosseae* (syn.*Glomus mosseae* T.H. Nicolson & Gerd.)[19] were obtained from Tata Energy Research Institute, New Delhi, India. Seeds of *Pennisteum typhoides* used for AM inoculum production were procured from Indian Fodder Research Institute, Jhansi, India. Inoculum density of 50 spores per 10 g soil was maintained for selected treatment. Dried inocula containing AM infected *Pennisteum typhoides* roots and soil were spread 10 cm below the soil surface at the time of inoculation. Seeds of *E. prostrata* were sown in the field 2cm below the soil surface. The AM inoculum treatment given in selected plots were over the indigenous AM species already present in the field soil.

Bacillus megaterium (BHU1) (Accession no.- KC432646) an indigenous plant growth promoting bacteria isolated from Eastern U.P., India was obtained from Institute of Agricultural Science, B.H.U. The strain was well studied for its high nitrogen fixing capacity, IAA production and plant growth promoting characters[20]. The strain was maintained on nutrient agar plates and subcultured at every two weeks. For inoculum preparation nutrient broth media autoclaved at 121°C for 20 min was inoculated with a single colony of *B. megaterium* strain and was maintained at 32±2°C and 200 rpm for 48h. The sticker solution was prepared by boiling 2g gum acacia and 5g sugar in 100ml water for 15 min. Seeds of *E. prostrata* was inoculated with 0.1ml of 48 h old nutrient broth culture along with 1ml of 1% (w/v) sticker solution of gum acacia to ensure bacterial population in range 10^7 -10^8 seed^{-1}[21] and dried in shade for inoculation in the field.

Trichoderma harzianum (Accession no.- NRRL 30598) a fungal biofertilizer was procured from Institute of Agricultural Science, B.H.U. The culture was grown on potato dextrose agar medium at 27± 2°C for 7 days. Spore suspension of *T. harzianum* supplemented with 2% of starch (w/v) as an adhesive was prepared for seed coating. *E. prostrata* seeds were dipped in this suspension (5×10^6spores/ml) of *Trichoderma* spp. for 1-2 min and subsequently inoculated in the field[22].

Plant treatment under different moisture regime

Two set of soil water status was maintained during the experiment where one set was normally irrigated daily and the other set under drought was irrigated at 3 days interval. Both sets were sown with the seeds of *E. prostrata* with different microbial treatment. The different microbial inoculations given were 1. *Bacillus megaterium* (B), 2. *Trichoderma harzianum* (T), 3. *Funneliformis mosseae* (F), 4. *Funnelliformis mosseae* and *Trichoderma harzianum* (FT), 5. *Bacillus megaterium* and *Trichoderma harzianum* (BT), 6. *Funnelliformis mosseae* and *Bacillus megaterium* (FM) and 7. Control (C) without any inoculation.

Growth parameters and AMF colonization assessment

Fifteen plants were randomly uprooted from each plot after 120 days of treatment to maintain a composite sample for each treatment. The shoot length, root length, no. of leaves, total plant height, fresh weight and dry weight were measured. For AMF assessment fresh roots were cleared by autoclaving in 4% potassium hydroxide for 20 min and then stained with 0.1% Chlorazol Black E for 40 min in an autoclave at 121 °C[23] and stored in lactoglycerol. The percent of AMF colonization was calculated by the gridline intersect method by studying 100 intersections from each 1cm root sample [24]. The counts for mycorrhizal colonization included the presence of hyphae, vesicles and arbuscules within the roots.

Chlorophyll content

Chlorophyll extraction from fresh leaves of uprooted plants was done following method of Lichtenthaler[25]. Chlorophyll a and b content was calculated by the given formula:

$$\frac{\text{mg chlorophyll a}}{\text{g tissue}} = \frac{(12.7 \times A_{663} - 2.69 \times A_{645})}{1000 \times W} \times V$$

$$\frac{\text{mg chlorophyll b}}{\text{g tissue}} = \frac{(22.9 \times A_{645} - 4.68 \times A_{663})}{1000 \times W} \times V$$

where mg= milligram, V= volume prepared, W= weight of the leaf

Relative water content (RWC)

$$RWC = \frac{(FW - DW)}{(TW - DW)} \times 100$$

RWC in leaf was calculated following method of Jeon et al., [26].

where FW= fresh weight, DW= dry weight and TW= turgid weight

Proline content

The proline content in the fresh leaves after various treatments in plant was quantified by the acid-ninhydrin procedure of Bates et al[27].

Estimation of lipid peroxidation

Lipid peroxidation was estimated by measuring MDA using the thiobarbituric method[28] with some modification. About 0.25 g of leaf was homogenized in 1.5 mL of 1 % trichloroacetic acid (TCA) and centrifuged at 10,000 rpm for 5 min. To 1 mL of their supernatant, 4mL of 0.5 % TBA was added. The mixture was heated at 95 ºC for 30 min and then quickly cooled in an ice-bath. The centrifugation at 2000 rpm for 10 min at 4 ºC, the absorbance was taken at 532 nm and correction for specific turbidity was done by subtracting the absorbance at 600 nm. The 0.5 % TBA in 20 % TCA served as blank. The MDA content was calculated according to its extinction coefficient of 155mM^{-1} cm^{-1} and was expressed as µmol g^{-1} FW.

Estimation of antioxidative enzyme activity

Catalase activity (CAT) was estimated following the method of Aebi,[29] with some modification. About 0.2 g of plant leaf was taken and homogenized with 2 mL of phosphate buffer (0.5 M, pH 7.2). The mixed homogenate was centrifuged at 10,000 rpm for 20 min and supernatant was separated for enzyme assay. To 200 µL of enzyme extract, 1.6 mL phosphate buffer (pH 7.3), 0.2 mL H_2O_2 (0.3 %), EDTA (0.5 mM) was added in a test tube. The absorbance was taken at 240 nm at interval of 10 s upto 30 s. Enzyme activities was calculated by using extinction coefficient 39.4 mM^{-1} cm^{-1} and was expressed as mM H_2O_2 utilized min^{-1} g^{-1} FW.

Peroxidase (POX) activity was determined following the method of Kumar and Khan[30] with slight modification. About 0.25 g of leaf was crushed and homogenized with 2 mL of cold phosphate buffer (0.1 M, pH 6.8) containing 0.005 M cysteine and was centrifuged at 10,000rpm for 20 min and supernatant was removed for enzyme assay. The assay mixture contained 1 mL of 0.125 M phosphate buffer (pH 6.8), 0.5 mL of 0.05 M pyrogallol, 0.5 mL of 0.05 M H_2O_2 and 0.5 mL of enzyme extract. The solution was incubated for 5 min at 25ºC, after that the reaction was terminated by adding 0.25 mL of 5 % H_2SO_4. The amount of purpurogallin formed was determined by measuring the absorbance at 420 nm against a blank and was measured as µM purpurogallin formed g^{-1} FW.

Total phenols and flavonoids: Total phenolics (TP) concentration was measured by Folin–Ciocalteu assay[31]. The standard graph was prepared with quecertin as reference compound ($y = 0.4398x + 0.1879$, $R^2 = 0.9907$). $AlCl_3$ colorimetric method was used for total flavonoid (TF)[32]. The standard graph was prepared with rutin as reference compound ($y = 0.186x - 0.0055$, $R^2 = 0.9968$).

Statistical analysis

For each experiment three replicates were used and repeated for three times independently. The mean values were represented as mean ± standard deviation (SD). Statistical analysis was performed using one-way analysis of variance (ANOVA) and separation between means were calculated by using Duncan's Multiple Range Test (DMRT) at $p \leq 0.05$ (SPSS software, version 16) under irrigated and water stress conditions.

RESULTS

Effects of microbial treatments on plant growth parameters

Microbial inoculations (B, T, F, FT, BT, and FB) augmented plant growth attributes i.e. root length, shoot length, plant height, fresh weight, dry weight under both water stress and irrigated conditions. Co-inoculation with FB showed maximum and significantly higher growth and biomass when compared to their respective control. The root length, shoot length and plant height showed (73.44 %, 35.10 % and 42.66 %) increase after co-inoculation with *F. mosseae* and *B. megaterium* (FB) respectively as compared to control under water stress while same treatment showed increased root length, shoot length and plant height (58.85 %, 58.71 % and 60.14 %) respectively under irrigated condition. Fresh weight and dry weight was reduced under drought stress compared to irrigated plants irrespective of treatment provided (Table 1). However, co-inoculation of *F. mosseae* and *B. megaterium* increased fresh weight and dry weight (69.72 % and 143.17 %) as compared to control under drought stress. Fresh weight and dry weight of plant was increased 74.16 % and 196.2 % respectively as compared to control in seedlings co-inoculated with *F. mosseae* and *B. megaterium* under irrigated condition (Table 1).

There was a significant increase in chlorophyll a (42.50 %) and chlorophyll b (41.64 %) content after *F. mosseae* and *B. megaterium* inoculations under water stress (Fig.1A). The same treatment showed significant increase in chlorophyll a (36.14 %) and chlorophyll b (27.63 %) content under irrigated condition (Fig.1B).

AMF colonization

Drought stress showed strong effect on AMF development. Mycorrhizal colonization upto 66 % and 73 % was observed in roots of *E. prostrata* after FB treatment under drought and irrigated conditions (Fig.1C). Single inoculation of *F. mosseae* showed mycorrhizal colonization of 46.7 % and 60 % under water stress and irrigated conditions. As the experiment was performed under field conditions, colonization by indigenous AM fungi *Glomus* sp. and *Acaulospora* sp. was also observed in plants in range of 10 - 16.67 %.

Table 1. Effect of microbial treatments on *E. prostrata* growth parameters under irrigated and water stress conditions

Root length (cm plant^{-1})	Irrigated	Water stress
B	5.80 ± 0.80b	4.15 ± 0.80b'
T	5.72 ± 0.56b	4.35 ± 0.86b'
F	6.17 ± 1.17b	4.35 ± 0.86b'
FT	6.58± 0.93b	4.66 ± 0.53b'
TB	6.59 ± 1.21b	4.65 ± 0.78b'
FB	8.48 ± 0.69a	6.22 ± 0.56a'
C	5.34 ± 1.28b	3.58 ± 0.74b'
Shoot length (cm plant^{-1})	Irrigated	Water stress
B	47.86 ± 5.57b	26.81 ± 4.85a'b'
T	47.61 ± 4.67b	27.34 ± 2.89a'b
F	47.33 ± 4.49b	29.58 ± 5.74a'b'
FT	50.66 ± 4.13b	32.09 ± 5.46a'b'
TB	47.49 ± 4.08b	32.10 ± 4.76a'b'
FB	60.82 ± 3.47a	32.86 ± 1.78a'
C	38.32 ± 3.92c	23.76 ± 4.99b'
Plant height (cm plant^{-1})	Irrigated	Water stress
B	53.66 ± 4.97b	30.96 ± 4.90a'b'
T	53.3 ± 4.16b	31.70 ± 1.02a'b'
F	53.5 ± 5.40b	33.91 ± 4.90a'b'
FT	57.12 ± 4.52b	36.71 ± 4.98a'
TB	54.10 ± 4.68b	36.78 ± 4.89a'
FB	69.13 ± 3.33a	39.00 ± 2.26a'
C	43.66 ± 4.50c	27.32 ± 4.60b'
Fresh weight (g plant^{-1})	Irrigated	Water stress
B	14.54 ± 4.54ab	5.34 ± 1.51a'b'
T	14.34 ± 4.39ab	5.77 ± 1.13a'b'
F	14.77 ± 3.98ab	6.31 ± 1.16a'
FT	15.72 ± 4.59ab	6.33 ± 1.52a'
BT	16.72 ± 3.95ab	7.08 ± 2.02a'
FB	19.35 ± 4.15a	7.38 ± 1.46a'
C	11.11 ± 4.20b	4.35 ± 1.81c'
Dry weight (g plant^{-1})	Irrigated	Water stress
B	1.71 ± 0.63cd	0.31 ± 0.10a'b'
T	1.91 ± 0.52cd	0.29 ± 0.08a'b'
F	1.89 ± 0.50cd	0.36 ± 0.08a'b'c'
FT	2.97 ± 0.50ab	0.37 ± 0.08a'b'c'
BT	2.56± 0.49bc	0.45 ± 0.17a'b'
FB	3.73 ± 0.45a	0.54 ± 0.11a'
C	1.26 ± 0.51d	0.22 ± 0.09b'

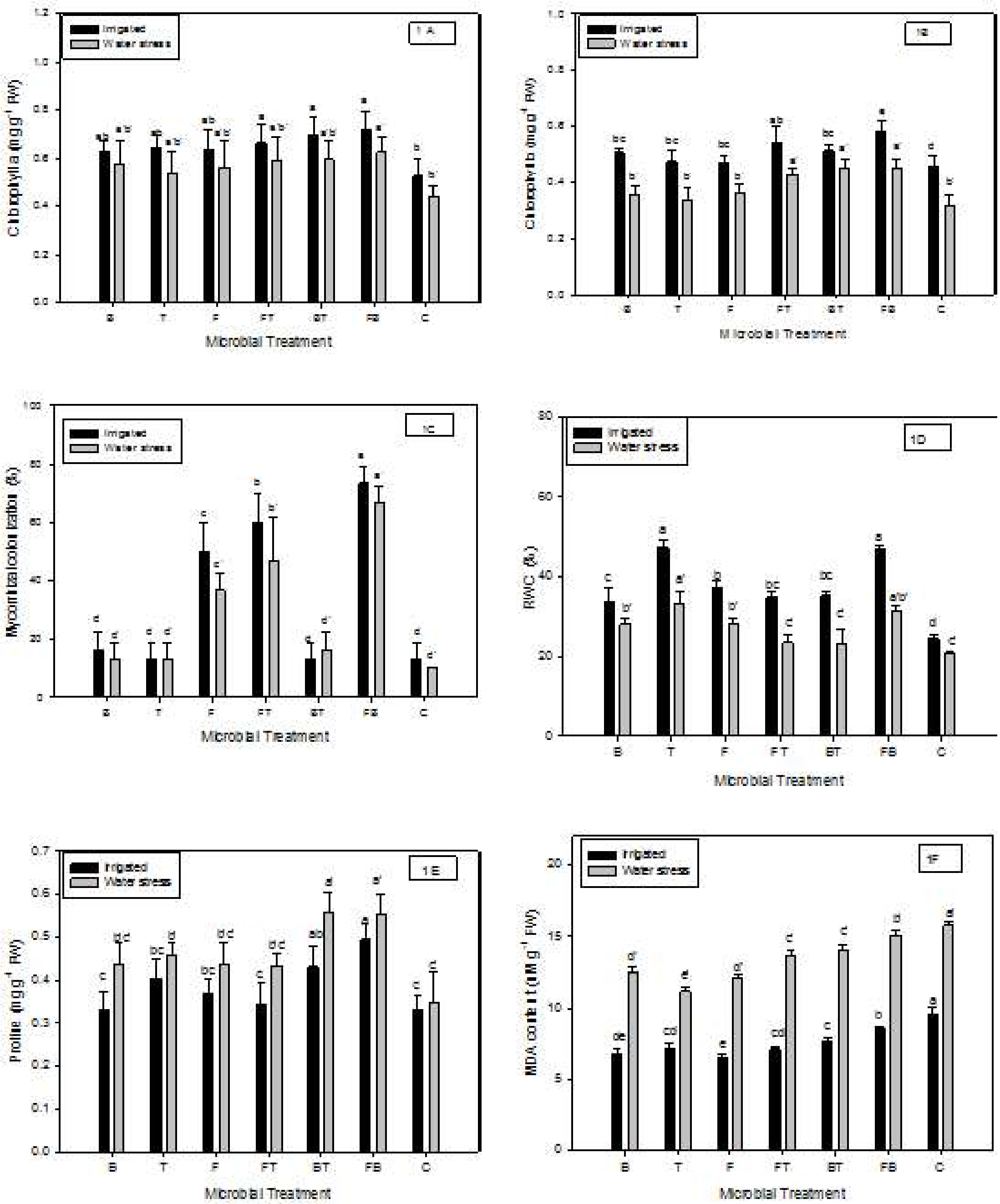
Mean ± standard deviation (n = 3). Values in columns sharing the same letter do not differ significantly ($p \leq 0.05$) as determined by the Duncan's test by one way ANOVA.

Relative Water Content (RWC)

Leaf RWC was insignificantly higher in the plants experiencing different microbial treatment under both the irrigated and water stress conditions than their respective controls, Table 1. Maximum increase in RWC was observed after microbial treatment of T (47.33%) and FB (46.72%) under irrigated condition and under water stress conditions too it was, T (33.21%) and FB (31.40%) showing maximum increment with respect to their control (Fig.1D).

Proline content

Under water stress conditions maximum increase in proline content was seen in FB (58.59 %) and BT (59.85 %) inoculated plants. Highest proline amount was observed in FB (48.58 %)



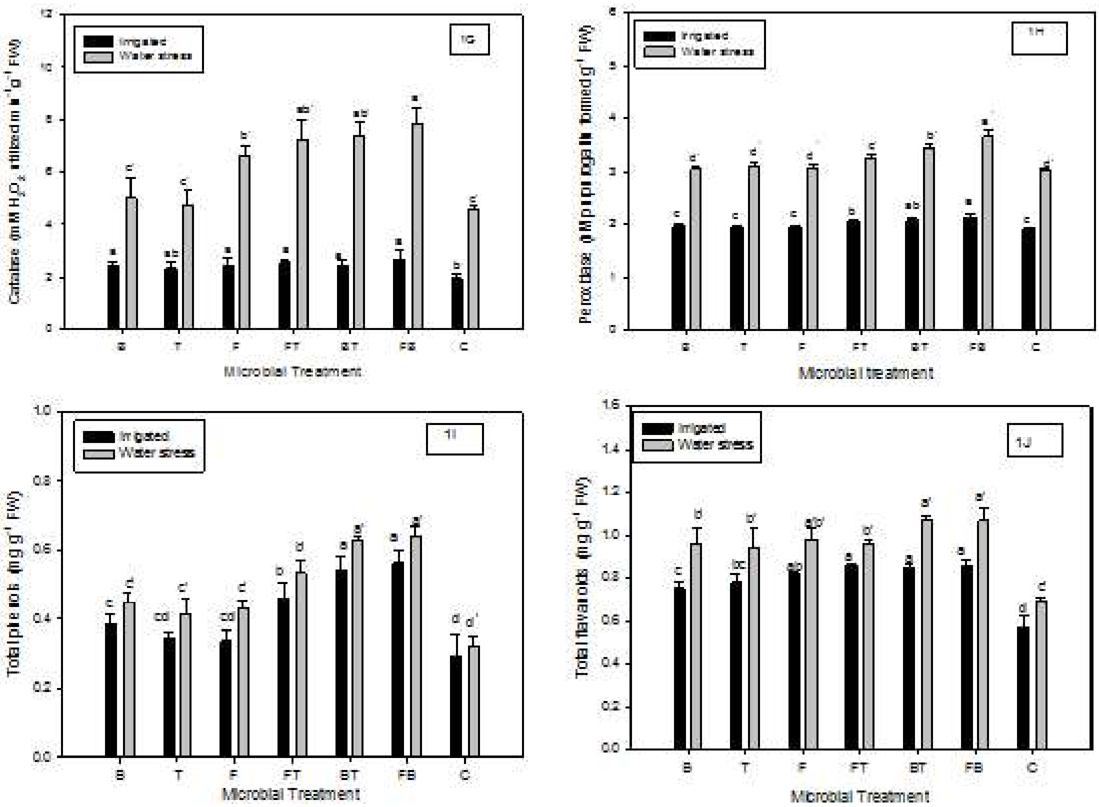


Fig. 1. Effect of microbial treatments on (A) Chlorophyll a, (B) Chlorophyll b, (C) Mycorrhizal colonization (D) Relative Water Content (E) Proline (F) Lipid peroxidation (G) Catalase (H) Peroxidase (I) Total phenols (J) Total flavonoids in *E. prostrata* under irrigated and drought conditions. Statistical analysis of irrigated and drought conditions are done separately by one way ANOVA. Results are expressed as means of three replicates, and vertical bars indicate standard deviations of the means. Same letters do not differ significantly according to DMRT at $p \leq 0.05$

inoculated plants under irrigated condition (Fig.1E).

Lipid peroxidation

Malondialdehyde content was increased under water stress. The MDA content in the plants was significantly improved after dual inoculation of *F. mosseae and B. megaterium* (FB) by 32.20 % and 28.84 % respectively under irrigated and water stress conditions (Fig.1F).

Enzymatic antioxidant

Drought stress showed a positive increase in activities of leaf enzymes of *E. prostrata*. The co-inoculation of *F. mosseae* and *B. megaterium* (FB) enhanced the activities of CAT (71.49 %) and POX (20.10 %) under drought stress while same treatment under irrigated condition showed maximum CAT (37.29 %) and POX (10.15 %) activity as compared to their respective control (Fig.1G-H).

Total phenols and flavonoids

Content of phenol and flavonoids got increased in *E. prostrata* plants exposed to drought. Highest phenolic content was observed after FB (0.641 mg/g) and BT (0.628 mg/g) treatments in drought stressed plants compared to control (0.32 mg/g) (Fig.1I). Under irrigated condition, treatment of FB (0.562 mg/g) and BT (0.543 mg/g) resulted in a maximum increase in phenol content compared to its control (0.293 mg/g). Content of flavonoid was found maximum after FB (1.068 mg/g) and BT (1.068 mg/g) treatments under water stress. The flavonoid content was obtained maximum after dual inoculation of FB (0.861 mg/g), BT (0.853 mg/g) and FT (0.86 mg/g) under irrigated condition compared to its control (0.569 mg/g) (Fig.1J).

DISCUSSION

Diazotrophic bacteria and PGPFs have been used as model organism in many crops for their beneficial influence on plant health. In this study we tried to develop compatible consortium taking into account the direct and indirect benefits they impart to plants. PGPR induced changes in plant under abiotic stress are looked under induced systemic tolerance[33]. The present study was based on the hypothesis that PGPRs induces metabolic adjustment in plants leading to augmented levels of antioxidants, organic solutes and secondary metabolites to alleviate the drought stress in plants. While microbial inoculations in the present study was found to promote plant growth maximum effect on plant in terms of root length, shoot length, plant height, biomass was observed after dual inoculation of FB under drought conditions. Beneficial effects of PGPM are usually enhanced when they are co-inoculated and this depends on the synergistic effect of the bacterium- fungus pair[34, 35] as observed in the present study. Synergistic effects are mostly seen when the partners are isolated from the same soil and tested in their native conditions although this restricts their wide applications as biofertilizer. In the present study *B. megaterium* was isolated from Eastern UP, India and was co-inoculated with *F. mosseae* and *T. harzianum* obtained from collection centres. A similar study done to compare effect of *Glomus intraradices* collected from commercial centre and *Glomus constrictum* autochthonous and *G. constrictum* from collection in combination with *Bacillus megaterium* isolated from Mediterranean calcareous soil of Spain[36]. Beneficial effects of mycorrhizal fungus have been positively co-related with percent colonization as also observed in our study although few studies differ in this respect[37]. The mycorrhizal colonization increase in roots of *E. prostrata* treated with *F. mosseae* and *B. megaterium* might be due to the production of metabolites like indole-3-acetic acid, amino acids etc by the bacteria[38,39] which might have enhanced fungal spores germination and AMF establishment in the soil[40]. Microbial inoculations in *E. prostrata* partially eliminated the deleterious water stress effects on chlorophyll content as also shown in previous studies done on *Hyoscyamus niger*[41], *Pisum sativum*[42] and *Catharanthus roseus*[43]. Dual inoculation of FB brought considerable improvement in chlorophyll a and chlorophyll b content of *E. prostrata*, not much difference was observed under irrigated and water stress conditions. Our findings suggest that PGPM could used as a biological tool to alleviate the detrimental effect of water stress on pigments, as pigments like chlorophyll and carotenoid are usually taken as suitable marker for leaf stress [44].

Decreased water potential in soil under drought condition reduces the RWC in leaves of plants. The physiological and biochemical processes in plants exposed to drought stress tend to accumulate more water for enhancing its tolerance against drought[45,46]. In the present study the RWC in *E. prostrata* was negatively affected by drought. Although all microbial inoculations improved the RWC, the effect was more significant in fungal inoculations as the physical presence of mycelial mass serves as appendages to the normal rhizosphere of plants[47, 48]. Among all the treatments *T. harzianum* showed maximum RWC as this could also be due to the increased lignification reported after *T. harzianum*[49]. The values of RWC in *Trichoderma* treated and dual consortia FB treated plants were comparable. Mycorrhizae and *Trichoderma* strains increases the deep root length which not only increases the root surface area but also access the deep scaled water for plants[50]. The effect can also be seen with respect to the growth hormones like cytokinin produced, by which these fungi alter root morphology. Treatment of *B. megaterium* along with *F. mosseae* also brought considerable improvement in the RWC of *E. prostrata*. Treatment of seeds with exopolysaccharide producing strains like *Pseudomonas putida*[51] and *Bacillus*[52] improve soil microaggregation which enhances the water content in soil thereby making it available to the plants as observed through RWC in leaves of *E. prostrata*. These bacteria form biofilm on the root surface. Improvement in RWC is an important aspect in drought as cellular process and temperature are conserved by tissue hydration[53]. Studies support the drought tolerance mechanism in plants by maintenance of RWC[54, 55].

Soluble sugars and proline are the two most important compatible solutes in plants for osmotic adjustment against drought[56]. Exposure to drought increased the proline content in *E.*

prostrata and response was increased two folds on inoculation with PGPMs indicating that response of plants to stress was improved. Similar studies have shown significantly low electrolyte leakage after inoculation of *Bacillus* sp. in maize[52]. Under drought stress conditions the plant invests more of its energy in increasing the osmolytes like sugar and proline[57,58] to alleviate stress effects as they enhance the stability of proteins and membranes[59] and prevent electrolyte leakage by acting as a potent antioxidant to scavenge the reactive oxygen species (ROS). Proline acts as a hydroxyl radical scavenger[60]. The proline level in *E. prostrata* was improved after *Bacillus* inoculation in drought stress conditions as it up regulates the proline biosynthesis pathway[61]. Upto 10 fold increase in proline level has been reported[62] under low water potential under drought in maize plant. In our study an increased level in proline was best observed after inoculations of consortia of *F. mosseae* and *B. megaterium* and its level was almost comparable in plant with consortia of *B. megaterium* and *T. harzianum*. *E. prostrata* showed higher capacity of osmotic adjustment in terms of accumulating proline which could protect the plant from damage of dehydration, although proline accumulation normally used as stress marker cannot be used as sole criteria for drought tolerance as it also accumulate under stresses such as high salt and starvation[63].

Two fold increases in phenolic content of *E. prostrata* was observed after FB microbial treatments under drought conditions compared to control. Phenols formed during phenyl-propanoid pathway is also as an important mediator of the PGPR's induced systemic response as it has major role in formation of various other biomolecules in plants helpful in stress defense[64]. Phenolic compounds are reported to indirectly promote lignification in plants [49,65] which act as barrier to water loss and pathogen attack and improve plant growth. Increased growth of *E. prostrata* under drought after dual inoculation could be related to increased phenolic content which in turn lignified the cell wall and prevented water loss, which is also evident by the high RWC in these plants. Similar reports show role of microbes in providing physical strength in form of lignin deposition in various vascular elements in chickpea[66] and *Capsicum*[49]. The study thus helps in understanding the role of compatible microbial consortia in management of drought response by plants.

In order to fight back the high level of ROS generated during stress, plants respond by generating antioxidants as an ISR. Though several studies have demonstrated induction of antioxidants by PGPR[67], the current study shows the effect of compatible microbial consortium (FB) on antioxidants catalase and peroxidase which got enhanced by 1-2 fold. ROS generated during abiotic and biotic stress trigger hypersensitive cell death of plants and therefore plants counter react by an array of antioxidant enzymes. Several *Bacillus* and *Trichoderma* strains have been reported to induce antioxidants like SOD, POX, PPO in plants which help in early defence much against stress[65]. Both abiotic and biotic stresses cause peroxidation of lipid membranes by overproduction of ROS. Increased ROS and altered pattern of antioxidant enzymes are reported to be involved in plant- AM interactions[68].

PGPMs trigger modifications of metabolic composition of whole plant. In present study too while slight enhancement in flavonoid content was observed in *E. prostrata* after microbial inoculations in drought, a significant 2 fold increase was observed after dual consortia treatment of FB and BT respectively. PGPM applied to roots can affect the composition of secondary metabolites in shoots, pointing towards systemic effects. Plant responds to drought stress by accumulating anthocyanin and other phenolic compunds. Elicitation of secondary metabolites like isoflavone[69], alkaloids, terpenoids [70-72] in medicinal plants is well reported. Flavonoid over accumulation having radical scavenging activity has been reported to mitigate against oxidative and drought stress in *Arabidiopsis thaliana*[73] and rice plants[74]. However, the signalling mechanism of flavonoid or the individual role of molecules in the strain mitigation mechanism is still unclear.

CONCLUSION

The present study clearly demonstrates an augmented acclimatization response of *E. prostrata* medicinal plant to drought stress under microbial inoculation of *F. mosseae* and *B. megaterium*. The consortia elicited the antioxidant activity and phenolic accumulation in plant thereby providing it with better capabilities to protect it from drought stress and maintain its integrity and growth are also enhanced by its primary and secondary metabolites level and augmented growth parameters. This further proves the synergistic behaviour of the microbes in double consortia which may be used to enhance drought tolerance in plants.

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