Biological Forum – An International Journal **15(7): 15-20(2023)**

ISSN No. (Print): 0975-1130
ISSN No. (Online): 2249-3239

Bacterial Endophytic Approach in drought stress Alleviation and Plant Growth Promotion

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(Received: 05 April 2023; Revised: 23 April 2023; Accepted: 18 May 2023; Published: 05 July 2023)
(Published by Research Trend)

ABSTRACT: Drought stresses have significant impact on plant growth, from seed germination to seed set in plant life cycle. This results in wide range of both biochemical and physiological changes in plant, ultimately leading to reduced economic output. The endophytic bacteria which survive within plant tissues are the most appropriate technologies that can influence positively on plant growth and yield under drought conditions. These bacterial endophytes in plant tissues are known to release various phytochemicals that assist plant to withstand in harsh environmental conditions, here in this context its drought stress. In the present study, five bacterial endophytes *viz*., P7L1, P2L2, P6R1, P3L2 and P7R1 were evaluated for the plant growth promoting and drought stress alleviating traits under *invitro* condition. Among plant growth promoting traits, all the bacterial endophytes were able to produced phytohormones IAA, GA, ABA and SA, while only one P7L1 bacterial endophyte showed positive results for phosphate solubilization, potash solubilization and siderophore production. Similarly, all the isolates were analyzed for production of drought alleviating compounds like accumulation of proline, production of antioxidants, phenols and flavonoids, both under drought stress (matric induced water stress using PEG 8000 molecule) and without stress condition. Among all the bacterial isolates under study P7L1 and P7R1 were observed to combat the drought stress significantly.

Keywords: Bacterial endophytes, drought, phytohormones, antioxidant activity.

INTRODUCTION

Among all abiotic stresses, drought puts severe restrictions on plant growth and development, which is due to lack of desired precipitation level. This in turn leads to loss of available internal plant water to a great extent due to high temperature resulting in climatic change, which is already causing water deficit issues in the global agricultural system (Pepe *et al.*, 2022). Similarly, variation in rainfall distribution pattern and intensity continue to aggravate drought stress specially, in areas where production mainly depends on availability of rainfall (Konapala *et al.*, 2020). However, plants have their own survival strategies to combat against drought stress which includes, reduced water conduction through stomata to avoid excess transpiration loss, decreased photosynthetic activity and enhanced accumulation of osmo-protectants like free amino acids, proline in cell for maintenance of osmotic pressure (Anjum *et al*., 2017) to protect plant.

Taken literally, the word endophyte means “in the plant” (endon = within, phyton = plant). The usage of this term is as broad as its literal definition and spectrum of potential hosts and inhabitants, e.g., bacteria in plant, fungi in plant, and insects in plants. Any organ of the host can be colonized. Equally variable is the usage of the term “endophyte” for variable life history strategies of the symbiosis, ranging from facultatively saprophytic to parasitic to exploitive to mutualistic. Anton de Bary in 1866 coined the term "endophyte" referring to the microorganism that live symbiotically inside the plant.

To provide food, shelter, and clothing to ever-growing population, crop production must be improved under drought stress. In this regard, various approaches have been used to improve drought tolerance in plants to overcome yield loss. The approaches must be cost effective, renewable and environment friendly in agriculture under drought conditions. In this regard, the use of beneficial microbes could be a stress-protecting agent for plants and lead to promising solutions for a sustainable agriculture.

Drought stress mainly results in reduced plant growth, nutrition, photosynthetic activity and increase oxidative stress in plants that in turn activates the expression of antioxidant enzymes. Schulz and Boyle (2006) reported that bacterial endophytes benefit by improving plant growth by microbial synthesis of phytohormones, access to minerals and other nutrients from the soil and

during environmental stress. Several reports have shown that microbial activity in the plant tissue plays an important role in drought-induced antioxidant responses since it can alleviate effects of drought stress by changing proline and antioxidants accumulation in plant tissues (Ruiz-Lozano *et al*., 2008).

Endophytic bacteria have been reported to increase compatible solutes, for instance, sugars and proline content under drought stress. For example, Asaf *et al.* (2017) showed that *Sphingomonas* sp. LK11 isolated from the leaves of *Tephrosia apollinea* enhanced drought tolerance *via* increased production of sugars and amino acids (glycine, glutamate, and proline) in inoculated Soya bean plants. Similarly, Shahzad *et al.* (2017) showed another endophytic bacteria *Bacillus amyloliquefaciens* increased amino acids content such as phenylalanine, aspartic acid, glutamic acid, cysteine, and proline content in inoculated rice plants under drought stress.

The plant growth regulators also known as plant growth substances, plant hormones, or phytohormones are naturally producing phytochemicals that regulate the growth and developmental processes in plants. Plant growth regulators were first introduced for their role in plant growth and normal operations within the plant, but later on their role were also studied as to regulate external factors (biotic and abiotic stresses) affecting plant growth, development, and yield (Atkinson and Urwin 2012).

Researchers are investigating on new bacterial endophytes from extreme plants growing in extreme climate for imparting drought tolerance in plants. In the previous study bacterial endophytes were isolated form drought adapted plants growing in arid and semi-arid regions of Karnataka and screened for imparting drought tolerance in maize seedling (Kale *et al.,* 2022). The present study is focused on evaluating the plant growth promoting and drought alleviating characteristics of bacterial endophytes.

MATERIALS AND METHOD

Bacterial cultures. Five bacterial endophytes *viz.* P7L1, P2L2, P6R1, P3L2 and P7R1were used in this study which were previously screened for imparting drought tolerance in maize (Kale *et al.,* 2022). Bacterial endophytes were cultured on nutrient agar (NA) media grown at 30ºC.

Determination of phytohormones in bacterial filtrate using High Performance Liquid Chromatogram (HPLC). Nutrient broth (20 mL) with PEG-8000 for stress and nutrient broth alone (20mL) for without stress was prepared in 100 ml conical flask. Tryptophan was amended in the broth for IAA production. The broth was inoculated with the bacteria and incubated at 30°C for 7 days. After incubation, the culture was centrifuged at 6000 rpm for 10 minutes and supernatant was collected. The pH 2.8 was adjusted using 1 N HCl. The acidified supernatant was taken in 100 mL conical flask and equal volume of diethyl ether was added and incubated for 4 h at 4 °C. The solvent phase (upper layer) formed was collected and allowed to evaporate. Then 2 mL of HPLC grade methanol was added and stored at -20 °C after membrane filtration to perform high performance liquid chromatography (Patten and Glick 2002).

HPLC analyses was carried out on a Shimadzu instrument (Prominence–I, LC-2030C) equipped with a UV detector (LC-2030 UV detector) and fitted with a C_{18} reverse phase HPLC column (Shim-pack GIST C_{18}, Dimension 250 × 4.6 mm, particle size 5 µm). The column temperature, 30ºC was maintained for all the samples with other specific conditions as described in the Table 1.

Table 1: Details of HPLC conditions.

Phytohormone	Solvent	Wavelength (nm)	Flow rate (mL/min)
SA	Acetonitrile: Acetic acid 0.5 % (90:10)	302	1.0

Total phenolic and flavonoid contents. The bacterial cultures grown for 24 h were inoculated in 20 mL nutrient broth containing PEG 8000 and only with nutrient broth for without stress. After 7 days bacterial growth was separated by filtration using sterile filter paper. The filtrate was extracted three times in ethyl acetate using separatory funnels and concentrated using a rotary evaporator until dried (Salini *et al.*, 2015). The dried extract was dissolved in ethanol and stored at -20°C for estimation of phenols and flavonoids.

Phenol content of bacterial extract was estimated using Folin–Ciocalteu reagent method described by Hameed *et al.* (2017). Bacterial extracts (250 µL) were mixed with 250 µL of Folin-Ciocalteu reagent and incubated for 2 minutes. Then, 500 µL saturated sodium carbonate (10%, w/v aqueous solution) was added and incubated in dark for 1h. The reaction mixture was measured at 765 nm using spectrophotometer (UV-VIS, Systronics Ltd., India). The concentration of total phenols was calculated based on a calibration curve using gallic acid. Total phenolic content was expressed as mg of gallic acid equivalent per gram of dried extract (mg GAE/ g dry weight).

Flavonoid content of the extract was estimated using the method described by Hameed *et al.* (2017). Bacterial extract (0.5 mL) was mixed with 1.0 mL of a 2% (v/v) $AlCl_3.6H_2O$ ethanolic solution and incubated for 10 minutes. The absorbance was measured at 430 nm using spectrophotometer (UV-VIS, Systronics Ltd., India). The concentration of total flavonoid was calculated based on a calibration curve using quercetin. Total flavonoid content was expressed as mg quercetin equivalent to per gram of dried extract.

$ABTS^+$ scavenging activity. The antioxidant activity assay was performed by free radical scavenging method using ABTS [2, 2'-azino-bis (3-ethyl-benzthiazolin-6-sulfonicacid)] as described by Hameed *et al.* (2017). A stock solution of ABTS (7 mM) was prepared. ABTS radical cations (ABTS+) was produced by reacting ABTS stock solution with 2.45 mM potassium

persulfate (1:1) and incubated in dark for 16 h. ABTS+ solution was diluted in 95% ethanol to an absorbance 0.75 at 734 nm. Endophytic bacterial extracts (20µL) were added to 180 µL of diluted $ABTS^+$ solution and incubated for 2 minutes at room temperature. The scavenging activity of the bacterial extracts was assessed from the percentage of decolorization at 734 nm using spectrophotometer (UV-VIS, Systronics Ltd., India). The $ABTS^+$ scavenging activity (%) was calculated using the equation as follows:

$ABTS^+$ scavenging activity = $[(OD_{734control}-OD_{734sample})/OD_{734control}] *100$

Proline production. Proline content was estimated in nutrient broth with stress (15% PEG-8000) and without stress. The bacteria inoculated broth was incubated for 48 h and quantified following the method described by Mishra *et al*. (2011). Two mL of culture was centrifuged at 10,000 rpm for 10 minutes. Cell pellets were kept in water bath with 80 % ethanol at 60 °C for 45 minutes. The suspension was centrifuged at 8000 rpm for 15 min and 1 mL of supernatant was collected. The supernatant was mixed with 1 mL of acid ninhydrin and 1 mL of glacial acetic acid. The reaction mixture tube was kept in boiling water for 1 h and transferred to ice bath for cooling. Extraction of proline from the reaction mixture was done by adding 2 mL of toluene. Then the extracted proline appears pinkish to red in colour, which was separated and transferred to new tubes and the absorbance was measured using the spectrophotometer, BioMate 3S, USA at 520 nm. The results were expressed as µg of proline per mL (µg mL-1) of bacterial culture (Ceylan *et al*., 2012).

Phosphate solubilization. The phosphate solubilizing potential of bacterial endophytes was evaluated *in- vitro* as described by Pikovskaya (1948). The ability of bacterial endophytes to solubilize phosphorus was tested by growing bacterial culture on Pikovskaya's agar medium. The clear zone formation around the colony was recorded for phosphate solubilization. The diameter of zone of TCP solubilized was measured in mm.

Potassium solubilization. The ability of potassium solubilization by bacterial endophytes was determined by growing the bacteria on Aleksandrow agar medium (Hu *et al.*, 2006**).** Each10µl of bacterial endophytes were spotted on plates containing Aleksandrow medium incubated at 30ºC for 2 days. After incubation, the clear zones formed around the colonies were recorded.

Siderophore production. Chomeazurol S indicator (CAS)Reagent was prepared by dissolving 60.5 mg dehydrated chomeazurol S in 50 ml double distilled water and further mixed with 10 ml of iron solution (1 mM FeCl3. 6H2O in 10 mM HCl).

For the assay of siderophore production, all the glass wares were first soaked in 2 N HCl solution for 24 h to avoid contamination of iron from the glassware. The CAS solution was slowly added to 40 ml aqueous solution containing 72.9 mg cetyl trimethyl ammonium bromide with continuous stirring and the final solution was autoclaved. The starch casein agar was prepared using PIPES buffer (30.2 g) and the pH was adjusted to 6.8 by addition of 0.1 N NaOH before autoclaving. After cooling, the CAS solution (100 ml) was added along the wall of flask with gentle agitation to avoid formation of foam. The CAS agar thus prepared was poured into the plates. After solidification, the plates were kept in the refrigerator (4 °C) for 24 h. The 10µl of freshly grown cultures of bacterial endophytes were spotted on CAS agar plates and incubated at 28°C for five days. Formation of orange coloured zone around the colony was considered as positive for the siderophore production. The diameter of orange coloured zone was recorded and expressed in mm **(**Schwyn and Neilands 1987).

RESULTS AND DISCUSSION

A. Evaluating plant growth promoting activity of bacterial endophytes

Phytohormones play a vital role on growth and development of plants. The production of Indole acetic acid (IAA), gibberellic acid (GA) and abscisic acid (ABA) for the five bacterial endophytes *viz.*, P7L1, P2L2, P6R1, P3L2 and P7R1 were previously confirmed by Kale *et al.* (2022). However, in this study results of salicylic acid (SA) production using high performance liquid chromatography (HPLC) is presented in the Fig. 1.

SA is as an important plant growth–regulating substance. The overall production of SA is high under stress condition compared to without stress condition (Fig. 1). Because genes responsible for SA are silent during normal condition, upon stress occurrence cell perceive the signal and transcribe to produce SA, which trigger drought-responsible signaling pathways upon exposure to drought stress and subsequently regulate morphophysiological and biochemical responses accordingly (Wilkinson e*t al.*, 2012). Under drought stress conditions, these regulators improve root system to enhance optimal water and nutrient acquisition by increasing root length and density to get deeper and contact with greater moisture content respectively (Vysotskaya *et al*., 2009). In this study the isolate P7L1 has produced highest amount of SA under both stress and without stress condition, however the production is higher under stress condition with 88.62 mg/L respectively and it is significantly higher compared to other isolates. This difference might be due to difference in signal perception among the isolates under stress condition. These observations are in agreement with, Shahzad *et al.* (2017) who isolated *Bacillus amyloliquefaciens* from rice seeds which has the potential to produce ABA and SA under normal and stressed conditions. Similarly, endophytic bacterial strains *Achromobacter xylosoxidans,* and *Bacillus pumilus* isolated from sunflower grown under drought, were both strains produced SA to improve plant health under water stress (Forchetti *et al.*, 2010).

The 5 bacterial isolates were qualitatively analyzed for the phosphate solubilization, siderophore production and potassium solubilization. In this regard isolate P7L1 showed zone of tricalcium phosphate solubilization in case of phosphorus nutrition in Pikovskaya's Agar medium, potassium nutrition in Aleksandrow agar medium and orange colored zone

around the colony in NA media supplemented with chrome azurol s indicating siderophore production, which is required for Fe scavenging activity. Similarly, P6R1 showed positive results only for P and K solubilization (Table 2). The remaining isolates *viz*P2L2, P7R1 and P3L2 had no response towards the above-mentioned activities, which might be due to lack off or slow release of organic acids to surrounding medium. The ability of P7L1 and P6R1 in nutrient solubilization may be due to production of organic acids and their release in to the surrounding medium, which drops pH to solubilize the unavailable form of nutrients to available form (Walia *et al.,* 2017). Similar observation was recorded by Baghel *et al.* (2020) in bacterial endophytes isolated from corn root, out of 24 endophytic isolates, 10 could solubilize phosphorus from tricalcium phosphate, and 8 isolates possessed potassium solubilizing ability from glauconite. The isolates also showed siderophore production. Recently Verma *et al.* (2022) revealed the ability of endophytes *Microbacterium, Pseudonocardia, Bacillus, Cellulosimicrobium, Staphylococcus, Luteimonas, Bordetella, Brevundimonas, Streptomyces, Cupriavidus, Sphingomonas, Ralstonia, Ochrobactrum, Conyzicola, Paenibacillus* and *Leifsonia* isolated from maize in solubilizing nutrients in media.

B. Evaluating stress alleviating traits of bacterial endophytes

(a) Proline production. Osmotic adjustment is one of the active phenomena of accumulating organic and inorganic solutes in plant cell under drought stress, so as to maintain the water potential in the cell. In the present study, the production of proline content in all bacterial endophytes increased subjected to drought stress compared to without stress condition (Fig. 2). This indicates that the proline is an important constituent imparting abiotic stress tolerance to maintain the osmotic potential in the cell. Among the isolates P7L1 (39.05 µg/mL) produced highest proline and it is on par with P7R1 (39.02 µg/mL) and significantly different from other isolates in the study. The similar results were observed in *Sphingomonas* sp. LK11 isolated from the leaves of *Tephrosia apollinea* enhanced drought tolerance via increased production of proline in inoculated Soya bean plants (Asaf *et al*., 2017). Similarly, the endophytic bacteria *Bacillus amyloliquefaciens* increased amino acids content such as phenylalanine, aspartic acid, glutamic acid, cysteine, and proline content in inoculated rice plants (Shahzad *et al*., 2017). And also, Danish *et al.* (2020) reported accelerated proline accumulation in *Pseudomonas* sp. was observed when grown under water deficit condition.

(b) Total phenols and flavonoids. Phenols and flavonoids are the antioxidant compounds produced by plants and bacteria. Which are non-enzymatic antioxidants induced under stress. These lower the oxidative stress under drought conditions and also observed to provide profit in terms of non-systematic oxidative stress (Barzana *et al.,* 2015). An increase in phenol and flavonoid content in culture filtrate of all the bacterial isolates was observed under stress condition compared to normal condition (Fig. 2). Highest phenol and flavonoid content were observed in bacterial culture filtrate of isolate P7R1, with 0.094 mg/ mL and 52.98 µg/ mL respectively when grown in matric modified media. The similar results were recorded by Joe *et al.* (2016), where higher phenolic content was produced by endophyte *Bacillus* sp. PVMX4 followed by *Acinetobacter* sp. ACMS25 treatments, compared to control under abiotic stress. Akter *et al.* (2022) reported that in medicinal plants *Bacillus*, *Pantoea*, and *Pseudomonas* were the most prevalent bacterial endophytic genera, accounting for 58.92%. *Bacillus*, *Pseudomonas*, and *Paenibacillus* influenced the growth under stress resistance by production of phenols and flavonoids.

(c) ABTS $^{+}$ scavenging activity. Antioxidant activity of the bacterial filtrate was evaluated based on the ability to scavenge synthetic radicals ABTS. An increase in antioxidant activity was observed in bacterial filtrate of all isolates when grown in matric modified media. Isolate P7L1(76.49%) showed maximum antioxidant activity under stress condition followed by P7R1 (72.77%). This might be because the aforementioned isolates might be more receptacle to ROS sensitivity as result to overcome reactive oxygen species that generated due partial reduction of oxygen during respiration the isolates might have showed higher scavenging activity. This assay represents the antioxidant activity of the compound that breaks the free radical chain through donation of a hydrogen atom (Ravindran *et al.,* 2012). According to Molyneux classification the antioxidant activity is categorized as the highly active compounds, the active category, medium category and weak categories. Thus, the results clearly show that the extract has promising ABTS scavenging activity and could be a potential source of natural antioxidant. The similar results were recorded by Joe *et al.* (2016), where higher ABTS activity was observed in endophyte *Bacillus* sp. PVMX4 compared to control under abiotic stress.

Table 2: Plant growth promoting traits of bacterial endophytes (Qualitative data).

Bacterial endophytes	Siderophore	Phosphate solubilization	K solubilization
P7L1	+	+	+
P2L2	-	-	-
P6R1	-	+	+
P3L2	-	-	-
P7R1	-	-	-

Note: +: Indicate zone of solubilization, **-** : Indicate no zone of solubilization

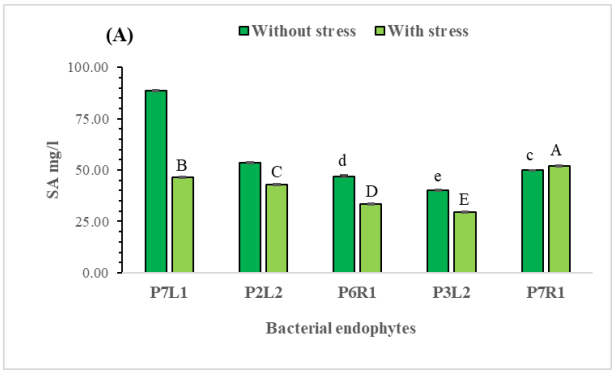

Fig. 1. Phytohormones secreted by bacterial endophytes **(A)** salicylic acid, SA. Means ± SEM are shown. Bars with same alphabets do not differ significantly at p≤0.05 as per Duncan Multiple Range Test (DMRT).

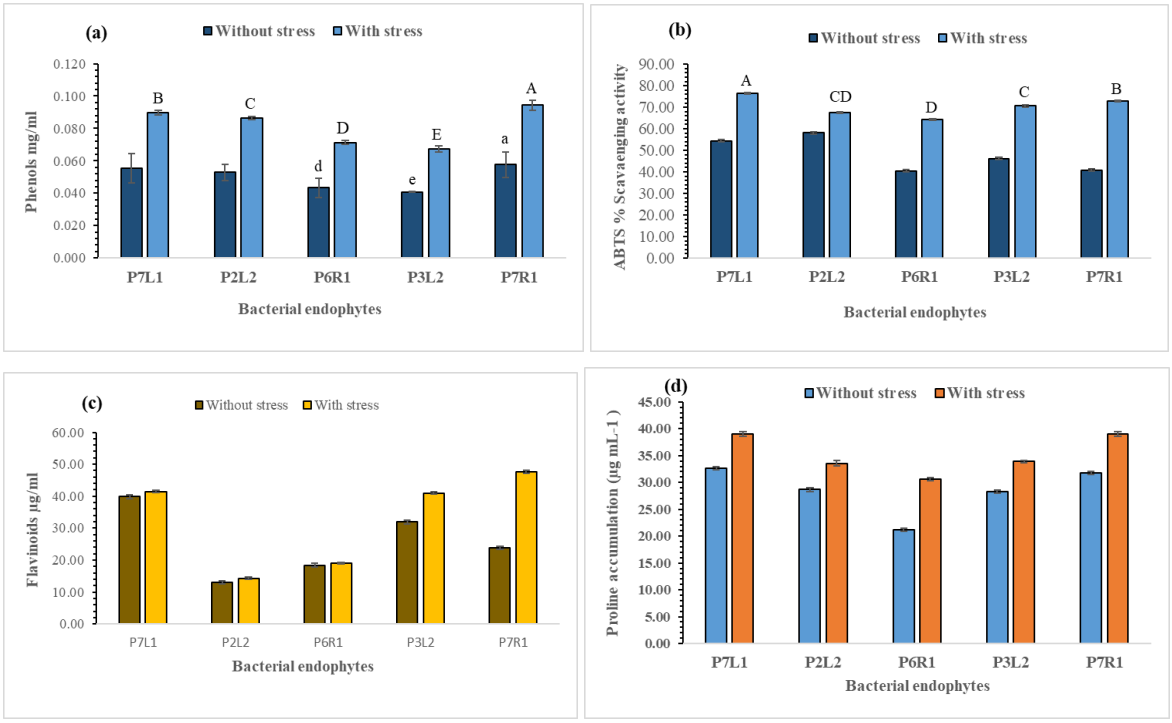

Fig. 2. (a) Phenols, (b) ABTS^{+} scavenging activity (c) flavonoids (d) Proline secreted by bacterial endophytes. Means ± SEM are shown. Bars with same letters do not differ significantly at p≤0.05 as per Duncan Multiple Range Test (DMRT).

CONCLUSIONS

Bacterial endophytes are widely known to impart abiotic stress tolerance in host plants. In this study, bacterial endophytes were able to produce growth hormones both under PEG induced stress and normal condition. Also, they were able to solubilize phosphate and produce siderophores. Further, they showed antioxidant activity and accumulated proline content in under drought stress. Overall, this study gives a comprehensive understanding of the mechanism involved in imparting drought tolerance in host plants by bacterial endophytes.

FUTURE SCOPE

The field evaluation is ought to be carried out to explore the possibility of these bacteria with AM fungus on drought tolerance, growth and yield of maize.

Acknowledgement. Authors are thankful to the Karnataka Science and Technology Promotion Society (KSTePS), Department of Science and Technology, Government of Karnataka for awarding Karnataka DST-Ph.D. fellowship for Science and Engineering (AGR-06: 2021-2022/997) for the Ph.D. research.

Conflict of Interest. None.

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How to cite this article: Nakul Kale, Earanna N. and Neekshitha Shetty (2023). Bacterial Endophytic Approach in drought stress Alleviation and Plant Growth Promotion. *Biological Forum – An International Journal*, *15*(7): 15-20.