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Isolation, screening and identification of moisture stress tolerant Rhizobacteria from xerophyte *Prosopis juliflora* (Sw)

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Abstract
Plant growth and productivity are adversely affected by various abiotic (high temperature, moisture stress and salinity) and biotic stresses (Pest and Disease). To overcome this problem various strategies are followed *viz*., modified cultivation practices, improved breeding methods and application of stress tolerant/resistant microorganisms. Present investigation was designed with the view to address the moisture stress by exploring autochthonous microflora of xerophyte *Prosopis juliflora* (Sw). Ten different isolates were obtained from southern agro-climatic zone of Tamil Nadu and screened *in vitro* by artificial induction of drought in solution using PEG 6000. Of which two potential isolates (MLSB 2 & MLSB 6) were obtained from the rhizosphere of *Prosopis juliflora* collected based on significant anount of IAA and proline produced during moisture stress condition and withstand upto (-1.03 M Pa) osmotic potential.

Keywords: Xerophyte, PGPR, Prosopis, PEG 6000, moisture stress

Introduction
Plant growth and productivity are adversely affected by various abiotic and biotic stress factors. Amongst all the stresses, drought is a major hindrance to crop production. Growing agricultural crops under dry conditions is achieved through utilization of xerotolerant microorganisms associated to the xerophytic crops. Xerophytic microflora can be found in environments where they are constantly exposed to water stress over a long span of time. Extremophilic plants and their associated micro biota involves in beneficial microbe-plant interactions (Jha *et al*., 2012; Pampurova *et al*., 2014) [8, 14] by boosting plant growth and productivity under harsh conditions such as soil salinity and water shortage (Cherni *et al*., 2019; Egamberdieva *et al*., 2011) [4, 6].

Prosopis (*Prosopis juliflora*) is a xerophytes, belongs to the family *Fabaceae* profusely occurs in many tropical regions including Southeast Asia, South Asia, North-eastern Brazil, Australia and Africa. They also called as phreatophyte-deep rooted plants that depend for their water supply upon ground water that lies within reach of their roots. This deep rooted bush/tree and widely propagated in Asia, particularly in India and Pakistan (Benata *et al*., 2008; Qureshi *et al*., 2014) [3, 16]. In many parts of the world it is a well-known plant species for its use as a fuel, shade, timber and forage. It also has many ethno medicinal values to human being. They have been reported to be resistant to salinity, heat and drought. Moreover, being a leguminous plant it can also help to fix the atmospheric nitrogen in the soil and improves the soil nutrient status. In spite of their allelopathic and phreatophytic effect upon other plant sp., potentiality of the tree against various a biotic stresses is explored recently.

PGPR or stress homeostasis-regulating bacteria (PSHB) (Sgroy *et al*., 2009) [20] enhanced the growth of many different crops even under stressed agricultural environment (Dood *et al*., 2004) [5]. These microbes confers drought tolerance to all plant species than those they were isolated originally from (Marasco *et al*., 2013) [11], and in some circumstances they enhance plant growth only under moisture limiting conditions (Wang *et al*., 2014; Rolli *et al*., 2015) [22, 18]. There were many evidences reported that inoculation of beneficial rhizobacteria imparts drought tolerance in plants (Niu *et al*., 2018) [13]. Drought resistance in plants were induced by PGPR, through the elicitation called Rhizobacterial-Induced Drought Endurance and Resilience (RIDER) process (Khalid *et al*. 2006) [14] which promotes modifications in phytohormonal content and antioxidant defense of plants.

Many evidences reported that inoculation of beneficial rhizobacteria imparts drought tolerance in plants (Bandeppa *et al*., 2015; Niu *et al*., 2018) [2, 13]. The mechanism behind drought tolerance of rhizobacteria is associated with the production of osmolytes, antioxidants, volatile compounds, stress proteins, exopolysaccharides and up-regulation or down-regulation of stress- responsive genes (Vanderlinde *et al*., 2010; Kaur and Asthir, 2017) [21, 9]. With the available knowledge on the moisture stress mitigation, the present investigation was planned to explore potential xerophytic microflora that could survive under severe moisture stress conditions and as a result abiotic stress tolerant microorganisms were isolated from the rhizosphere of *Prosopis juliflora*, xerophytic tree species of Fabaceae.

Materials and Methods

Isolation and screening of drought tolerant xerophytic microflora

Rhizosphere soil samples were collected from young saplings/bushes of *Prosopis juliflora* located in Melur, Madurai (10° 1' N, 78° 20' E,). Soil samples were serially diluted up to 10^{-6} dilution and bacteria were isolated using Tryptic soy agar (TSA), nutrient agar (NA) and Luria Bertani agar (LB) by pour plate method. Isolates differed in morphology were purified. Moisture stress was induced artificially using PEG 6000 in nutrient broth and the isolates were screened *in vitro* based on their drought tolerant potential. Rhizosphere isolates were inoculated in nutrient broth containing different concentrations of PEG 6000 (0, 2, 4, 6, 8 &10%) and allowed for incubation for five days at RT. Concentration of PEG 6000 and Osmotic Potential (M Pa) in terms of negative water potential created in solution were given below.

PEG 6000 Concentration (%)	Bars of OP at 25^{0}C (M Pa)
0	-
2	-0.14
4	-0.36
6	-0.66
8	-1.03
10	-1.48

The Osmotic Pressure (OP) of PEG 6000 solutions was calculated using the formula (Michel and Kaufmann, 1973)
$OP= (-1.18 \times 10^{-2}) \times C - (1.18 \times 10^{-4}) \times C + (2.67 \times 10^{-4}) \times CT + (8.39 \times l0^{-7}) \times C^2T$,
Where C=PEG concentration; T=Temperature

Characterization of rhizobacteria and identification

The bacterial isolates (MLSB 2 & MLSB 6) exhibiting high drought tolerant in PEG 6000 amended nutrient broth were screened and identified by Phenotypic, biochemical characterization. For molecular characterization, genomic DNA of the isolate was extracted following Green and Sambrook (2012) [7]. The 16S rRNA amplification of the bacterial isolates was done using universal primer set consisting of fDl 5′AGAGTTTGATCCTGGCTCAG3′ and rDl 5′AAGGAGGTGATCCAGCC3′ (Weisburg *et al*., 1991) [23] and the amplicons were purified and sequenced. Obtained bacterial sequences were compared with 16S rRNA gene sequences available in the GenBank databases of NCBI by BLASTn search. The 16S rRNA sequence was submitted to GenBank Database and accession numbers were assigned *Bacillus altitudinis* (MLSB 2) -MT729974, *Bacillus pumilus* (MLSB 6)-MT729998.

Plant growth promoting properties

Estimation of Indole acetic acid using Salkowski method

The bacterial isolates were grown in LB broth for 24h. The isolates were inoculated to 10 mL of LB broth without and with PEG (4, 6 and 8%) concentration amended with 0.1% with tryptophan. Then incubated for 72 h in a shaker at 28 ± 2ºC (120 rpm). The IAA production was determined using the Salkowski method (Rahman *et al*., 2010) [17]. After 72 h of growth, the isolates were centrifuged and cell-free supernatants were used for IAA determination. To the 10 mL of supernatant, 2 mL of Salkowski reagent was added and incubated for 10 min. The blank was prepared using sterile broth with Salkowski reagent. Then the samples were read for absorbance at 530 nm. IAA standard graph curves were calibrated using Indole acetic acid (Himedia) in LB broth at different concentrations (5, 10, 20, 50 and 100 µg mL^{-1}) and sample IAA concentration was calculated by plotting the values against standard graph.

Estimation of endogenous osmolytes production by bacterial isolates

The major osmolytes produced from bacterial isolates were estimated under both stressed and non-stressed condition (Qurashi and Sabri, 2013) [15]. A day old drought tolerant bacterial culture were inoculated in 10 mL LB broth at 4, 6 and 8% PEG and without PEG (0%). The cultures were incubated for 48 h at 32°C on a shaker (180 rpm) and the OD of respective cultures were taken at 600 nm. The cells were extracted by centrifugation at 5000 rpm for 5 min. The cells were resuspended in sterile distilled water. To estimate the endogenous proline, the bacterial cells were boiled for 20 min and the respective cell extracts were used for osmolyte estimation.

Endogenous Proline content of drought bacterial isolates

For proline estimation, 2 mL of cell free culture extract were transferred to separate tubes and kept in a water bath at 100ºC for 20 min. To each tubes, 2 mL of acid ninhydrin and 2 mL of glacial acetic acid were added and mixed gently and placed in a water bath at 100°C for 1 h. Appearance of red colour indicates the presence of proline in the sample. Red chromophore of proline from the culture samples were separated by the addition of 4 mL of toluene and mixed vigorously. Within few seconds, the proline content was transferred to the toluene layer. The concentration of proline was determined by checking the absorbance at 520 nm in UV-Vis spectrophotometer. Standards curves were prepared by pure L-Proline in sterile distilled water (0.2, 0.4, 0.6, 0.8 and 1 mg Proline mL^{-1}) and the sample proline concentration was calculated using standard curves.

Result and discussion

Isolation and screening of moisture stress tolerant rhizobacteria from *Prosopis juliflora*

Ten isolates were obtained from *Prosopis juliflora* rhizosphere soil samples collected from Melur, Madurai using three different media *viz*., MLSB1, MLSB2, MLSB3, MLSB4 in Tryptic Soy agar; MLSB6, MLSB7, MLSB8 in nutrient agar: MLSB9, MLSB10 in Luria Bertani Agar (table 1). These isolates were tentatively identified as *Bacillus* sp. based on morphological and biochemical characterization. Further the isolates were subjected to artificial drought induction using PEG 6000 *in vitro* and their drought tolerant potential were assessed based on their growth in PEG amended (0, 2,4,6,8 & 10%) nutrient broth. Similarly study conducted by

Sandhya *et al.* (2011) [19], isolated 65 *Bacillus* spp., of which10 potential sp. grow at minimal water potential (-0.73 M Pa) and were screened *in vitro* for PGP traits under stressed and non-stressed conditions.

In the present study, among the 10 isolates, isolates MLSB2 and MLSB6 recorded growth up to 10 per cent (-1.48 M Pa) PEG concentration. MLSB2 register profuse growth up to 10 per cent, moderate growth in 6 (-0.66 M Pa) and 8 per cent (-1.03 M Pa) and tolerate 10% PEG concentration. Similarly isolate MLSB6 has highest growth in 2 per cent PEG concentration (-0.14 M Pa) and moderate growth in 4, 6 and 8 per cent PEG concentration and tolerate 10% PEG concentration. Isolate MLSB4 withstand up to 8 per cent PEG and MLSB9 was grown up to 6% PEG concentration. Isolate MLSB8 is highly prone to moisture stress; it doesn't grow in PEG solution irrespective of the concentration. Hence best performing isolates MLSB2 and MLSB6 were taken for further studies. Similar results were recorded by Sandhya *et al.* (2011) [19] reveals that *Bacillus* spp. tolerate minimal water potential (-0.73 MPa).

Table 1: Isolation of rhizospheric bacteria on different media

S. No.	Isolates	Growth at various levels of PEG 6000					
		0%	2%	4%	6%	8%	10%
1.	MLSB1	+	+	+	-	-	-
2.	MLSB2	+++	+++	+++	++	++	+
3.	MLSB3	++	+	+	-	-	-
4.	MLSB4	+++	++	++	+	+	-
5.	MLSB5	++	++	+	+	-	-
6.	MLSB6	+++	+++	++	++	++	+
7.	MLSB7	++	+	+	-	-	-
8.	MLSB8	+	-	-	-	-	-
9.	MLSB9	+++	++	++	+	-	-
10.	MLSB10	+	+	-	-	-	-

OD_{600}	Indication
>1.00	+++
0.5-1.00	++
<0.5	+
Negative	-

Identification of moisture stress tolerant rhizobacteria from xerophytes *Prosopis juliflora*

Rhizobacterial isolates obtained from *Prosopis juliflora* were screened *in vitro* by PEG 6000 and two potential isolates (MLSB2 & MLSB6) were obtained. Isolates were identified and confirmed by 16 S r RNA method as *Bacillus altitudinis* (MLSB2) and *Bacillus pumilus* (MLSB6). Sequences were submitted to GenBank Database and accession numbers were assigned to MLSB 2- *Bacillus altitudinis* (MT729974) and MLSB 6 - *Bacillus pumilus* (MT729998) (table 2).

Table 2: Identified drought tolerant isolates and its NCBI accession number

S. No.	Isolates	Organism	Accession number
1	MLSB 2	*Bacillus altitudinis*	MT729974
2	MLSB 6	*Bacillus pumilus*	MT729998

Estimation of Indole acetic acid production in moisture stressed and non-stressed condition

Effect of the strains *Bacillus altitudinis* (MLSB 2) and *Bacillus pumilus* (MLSB 6) on plant growth hormone IAA was measured under stressed and non-stressed conditions compared with standard culture *Bacillus megaterium* (MTCC 453). In general, *Bacillus altitudinis* produces highest hormone production (63.22 µg mL^{-1}) compared to Standard (58.66 µg mL^{-1}) and *Bacillus pumilus* (56.78 µg mL^{-1}) under non-stressed condition. Study of Sandhya *et al.* (2011) [19] reveal *Bacillus* Isolate HYD-17 produced highest amount of IAA (16.2 and 32.5 µg mL^{-1} protein under stressed and non-stressed conditions, respectively).

IAA production was reduced during artificial induction of moisture stress by PEG 6000 @ 4, 6 and 8 compared to non-stressed condition (0% PEG). Fig 1. Shows IAA production was higher at 6% concentration and reduced at 8% PEG concentration irrespective of the isolates. Per cent reduction of IAA was low in *Bacillus pumilus* (59.9% @6% PEG), followed by *Bacillus altitudinis* (62.9% @6% PEG). Standard strain recorded higher per cent reduction (69.46% @6% PEG) compared to two isolates (table 3, fig 1)

Table 3: Impact of xerophytic microbes on IAA production under moisture stressed condition

Isolate	Strain	0%PEG	4% PEG	6% PEG	8% PEG
		Per cent reduction in IAA			
MLSB 2	*Bacillus altitudinis* (MT729974)	100	18.06	62.90	80.57
MLSB 6	*Bacillus pumilus* (MT729998)	100	20.55	59.91	79.64
MTCC 453	*Bacillus megaterium* (Standard)	100	34.21	69.46	79.64

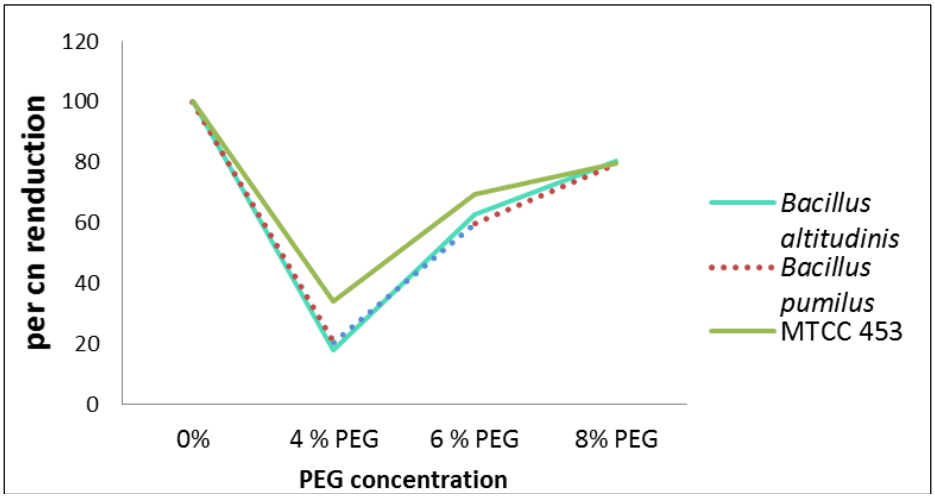

Fig 1: Impact of xerophytic microbes on IAA production under moisture stressed condition

Estimation of proline in moisture stressed and non-stressed condition

Proline is an osmactant produced when a plant subjected to a stress conditions like drought, high temperature and salinity and also an adaptive mechanism provided to the plant to withstand the harsh stress conditions. Similarly plant growth promoting rhizobacteria subjected to induced drought in a solute by PEG 6000, it also produces osmactants like proline, glycine betaine, trehalose etc., provides stress tolerance to the rhizobacteria. Estimation of internally produced proline concentration of rhizobacteria during moisture stress conditions comparing with non-stress condition give insight about the potentiality of the microorganisms to counteract the effect of abiotic stress.

In the present investigation bacterial strain *Bacillus altitudinis* MLSB2 produces significant amount of proline (698.1µg mL^{-1}) at -0.66 M Pa osmotic potential compared to standard strain *Bacillus megaterium* MTCC 453 (685.2 µg mL^{-1}). Proline concentration was increased in 4 & 6% and reduced in 8 per cent PEG concentration compared to non-stressed condition (table 4, fig 2). Similarly in the study conducted by Sandhya *et al*. (2011) [19] recorded significant increase in the concentration of proline and total soluble sugar content was observed under stressed conditions as compared to non-stressed conditions. Study conducted by Aswathy *et al*. (2017) [1] registers that *B. altitudinis* FD48 was found to produce Indole acetic acid (IAA) (2.82 µg/ml) compared to other two isolates *(Bacillus pumilus* FS20 and *Bacillus aquimaris* MD02) even under PEG induced drought conditions. However, under normal conditions, B. altitudinis FD48 produced 8.0 µg/ml.

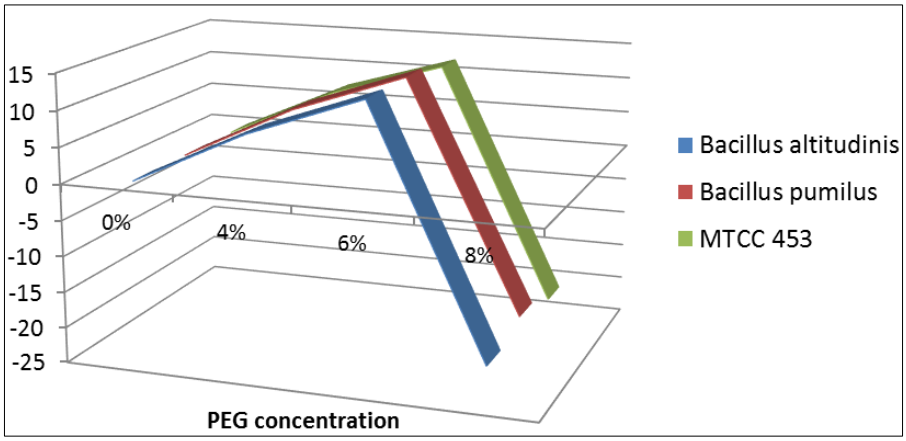

Fig 2: Impact of xerophytic microbes on proline production under moisture stressed condition

Table 4: Impact of xerophytic microbes on proline production under moisture stressed condition

Isolate	Strain	0%PEG	4% PEG	6% PEG	8% PEG
		Per cent reduction in proline			
MLSB 2	*Bacillus altitudinis*(MT729974)	0	7.64	13.36	-20.26
MLSB 6	*Bacillus pumilus*(MT729998)	0	7.73	13.32	-18.62
MTCC 453	Bacillus megaterium (Standard)	0	7.18	11.95	-21.02

Conclusion

Moisture stress is one among the major abiotic stress pose a serious problem to crop cultivation and several mitigation & adaptation strategies were followed worldwide to resolve this problem. Inoculation of PGPR is one amongst the technology to alleviate the ill effects created by the moisture stress. In the present study an attempt was made to explore xerophytic microflora from *Prosopis juliflora* to alleviate moisture stress and two potential strains were isolated (*Bacillus altitudinis* -MT729974 and *Bacillus pumilus* -MT729998) from rhizosphere of *Prosopis juliflora* having essential adaptive mechanism against moisture stress.

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