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Characterization of Bacterial Endophyte Imparting Drought Tolerance in Rice (*Oryza sativa* L.)

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ABSTRACT

Drought is a major abiotic stress which affects plant growth and productivity. It is proven in recent years, that microorganisms (fungi and bacteria) which can symbiotically inhabit the plant system and impart tolerance against abiotic stresses. In the present study, 58 bacterial endophytes isolated from Himalayan cold deserts were screened *in-vitro* using Polyethylene glycol (PEG MW-8000) against drought tolerance at different concentrations (5%, 10%, 15%, 20%, 25%). Out of 58 isolates, 8 isolates showed drought tolerance up to 20 per cent PEG. The drought tolerance of sensitive crops like, Rice (var. IR-64) seedlings were standardized by paper towel method using pre-germinated seeds. Further, pre-germinated seeds of rice seedings inoculated with the eight-drought tolerant endophytic bacteria were grown at 14.3 per cent PEG in paper towel method, of which one endophyte (CBE 11) significantly showed increased seedling length compared to uninoculated seeds. The endophyte was identified as *Stenotrophomonas maltophilia* by 16S rRNA gene sequence. It was found that this bacterial endophyte can impart drought tolerance in rice.

Keywords : Bacterial endophytes, Polyethylene glycol (PEG MW-8000), Rice (var. IR 64), *Stenotrophomonas maltophilia*

THE term 'Endophyte' is derived from the Greek words 'endon' (within) and 'phyte' (plant)). Endophytes include 'fungi or bacteria, which for all or part of their life cycle invade the tissues of living plants, but do not exhibit symptoms of disease (Kandel *et al*., 2017). Endophytic bacteria have been isolated from roots, leaves, stems, a few from flowers, fruits and seeds (Imran *et al.,* 2019). Endophytic bacteria may accompany certain metabolic properties, such as promoting plant growth, controlling soil-borne pathogens, or helping host plant to defeat stress (Ullah *et al.,* 2019). Further more, the interaction between plants and bacteria aid plants to settle in ecosystem restoration processes. These interactions may enhance the ability of plants to utilize nutrients from the soil by increasing root development, nitrate uptake or solubilizing phosphorus.

The abiotic stress such as drought cause imbalance in natural status of the plant affecting growth and productivity. It is estimated that 50 per cent of world rice production is affected by drought. Drought is a meteorological term that denotes a period without rain which results in water deficit stress. It is an environmental event with water availability below the optimum requirement for the full expression of yield potential (Blum, 2011). It also alters different physiological processes in rice leading to significant crop losses.

Drought tolerance is the ability of a plant to with stand moisture stress conditions. It is an important trait found in crop plants. Plants under drought stress are highly regulated by osmoregulation, antioxidative systems and secondary metabolite contents as reported elsewhere (Takahashi *et al.,* 2020). Drought stress induces an increase in ROS production resulting in various degree of oxidative damage in different genotypes of crops including rice. Therefore, it is important to understand the mechanisms that trigger physiological responses to drought stress and dehydration conditions. In the light of the above, the present study is initiated to screen and characterize bacterial endophytes important to impart drought tolerance in drought sensitive rice variety IR 64.

Material and Methods

Collection of Bacterial Endophytes

Fifty-eight bacterial endophytes isolated from the plants growing in harsh environment of north Himalayan cold deserts of Pangong, Changla and Namika La regions and maintained by the School of Ecology and Conservation (SEC) Laboratory, Department of Crop Physiology, University of Agricultural Sciences (UAS-B), Gandhi Krishi Vignana Kendra, Bengalore - 560 065. These bacteria were rejuvenated on Nutrient agar medium. The physiographical of the cold desert regions are given in Table 1.

Table 1

Geographical information on Himalayan cold desert regions (where plants are collected for endophytes isolation)

Location	Latitude (° N)	Longitude (° E)	Altitude (M)
Pangong	33°43' 2.74"	78 °53' 29.08"	4250
Changla	34°30"	77° 55°	5216
Namika La	34°22'	76° 35	3700

Screening of Bacterial Endophytes for Drought Tolerance

The selected bacterial strains were tested for their drought tolerance in liquid cultures. Five ml of Nutrient broth (NB) supplemented with PEG (MW-8000) at 5, 10, 15, 20, 25 percentage concentrations were inoculated with each bacterial strain. The standard NB was used as control. The cell growth was determined measuring optical density OD_{600} by using a spectrophotometer (Bandeppa *et al*., 2017). All tests were conducted in triplicates.

Standardization of PEG Concentration for Rice

Tolerance level of PEG for rice seedlings was determined by paper towel method. Different concentrations (5, 10, 15, 20 and 25%) of PEG solution was prepared in sterile distilled water (Muddarsu and Manivannan, 2017). These concentrations were corresponding to the osmotic potential -0.04 MPa, -0.14 MPa, -0.30 MPa, -0.51 MPa, -0.77 MPa, respectively. Two germination papers were taken for each concentration and 500 ml each of PEG solutions having different concentrations (5, 10, 15, 20 and 25%) were added to germination paper and excess solution was removed. Control was maintained by soaking the germination paper in distilled water. Then 10 each pre-germinated seeds of rice were placed on germination paper and incubated at 30 °C in the growth chamber. The shoot and root length of seedlings were recorded at 14 days after incubation. Lethal concentration (LC 50) value of PEG - 8000 was calculated for the shoot, root and seedling length using statistical software IBM SPSS statistics 20 (https://www.ibm.com/in-en/analytics/spss-statistics-software)

Inoculation of Bacterial Endophytes to Rice Seedlings

The seeds of rice were surface sterilized and incubated for germination at ambient temperature. Then the sprouted seeds were treated with bacterial suspension having ~$8x10^7$ CFU/ml population for three hours (Walitang *et al.,* 2017). The corresponding control was treated with sterile distilled water. After then the endophytes treated seeds were subjected to drought stress by placing on germination paper amended with 14.3 per cent PEG (LC 50 value) and incubated at room temperature for 14 days. There were two replications for each treatment and each replication comprised of 10 seedlings. Root and shoot length were recorded on 14 days intervals.

Confirmation of Inoculated Endophyte in Seedlings by Re-isolation

The plants were cut into one cm bits (root, stem and leaf) surface sterilized and placed on nutrient agar and incubated at 30 °C for 24 h. The bacterial colony emerged out of cut ends were sub-cultured and confirmed by comparing with the mother culture (Walitang *et al.,* 2017).

Molecular Identification of Bacterial Endophyte using 16S rRNA Gene Sequence

Extraction of Genomic DNA and PCR Amplification : Total genomic DNA of the bacterial endophyte

was extracted by alkaline lysis method (Sambrook and Fristsol Maniatis, 1989) and the DNA concentration was determined by Nano drop. The primers already reported for 16S rRNA sequence from the NCBI (http://www.ncbi.nlm.nih.gov) were custom synthesized by Sigma-Aldrich (Sigma, USA) and diluted accordingly for the PCR reactions (26 bp forward primer 5' GTTAGATCTTGGCTCAGGA CGAACGC 3' and 24 bp reverse primer 5' GATCCAGCCGCACCTTCCGATACG 3'). PCR was performed in 20 µl reaction mixture containing 2.0 µl of 1X PCR Taq buffer with $MgCl_2$ (1.5 mM), 2.0 µl of 10 mM dNTP's mix (200 µM), 0.5 µl of primers (both forward and reverse), 0.3 µl of Taq DNA Polymerase (1U Genei Bengaluru), 1.0 µl of Template DNA, 13.7 µl of Sterile distilled water. Amplification was carried out with an initial denaturation at 96 °C for four minutes followed by 35 amplification cycles consisting of 94 °C for one minute, 60 °C for 30 seconds and 72 °C for one minute and a final extension at 72 °C for 10 minutes. Then the amplified product of DNA was electrophoresed using one per cent agarose gel and documented using gel documentation system. The DNA was eluted by using gel elution kit (The Gene JET™ Gel Extraction Kit, Thermo Scientific) and the amplified product was got sequenced by Chromgene Biotech Pvt. Ltd., Bengaluru, Karnataka. The sequences obtained were analysed for homology using NCBI GenBank.

Statistical Analysis

The data was statistically analysed using WASP: 2.0 (Web Agri Stat Package 2) statistical tool (www.icargoa.res.in/wasp2/index.php) and means were separated by Duncan Multiple Range Test (DMRT).

Results and Discussion

Screening of Bacterial Endophytes for Drought Tolerance

Fifty-eight bacteria isolated from different regions of Himalayan cold desert were screened for drought tolerance using different concentrations of PEG. Of which eight bacterial isolates (PBE 2, PBE 4, PBE 6, PBE 8, PBE 14, CBE 11, CBE 13 and NBE 5) showed tolerance at 20 per cent PEG (Fig. 1). This includes five isolates (PBE 2. PBE 4, PBE 6, PBE 8 and PBE 14) from Pangong region (Table 2), two isolates (CBE 11 and CBE 13) from Changla (Table 3) and one isolate (NBE 5) from Namkila La (Table 4). Other 50 isolates grown up to 15 per cent and did not show any growth at 20 per cent PEG indicating that they are susceptible to increased concentration. This may be due to increased membrane instability and decreased activity of superoxide dismutase (Sun *et al*., 2010) which resulted in decreased growth. Aswathy *et al*., (2020) reported that the bacterial endophytes isolated from leaves of *Ananas comosus* with stand water potential up to -1.5 MPa.

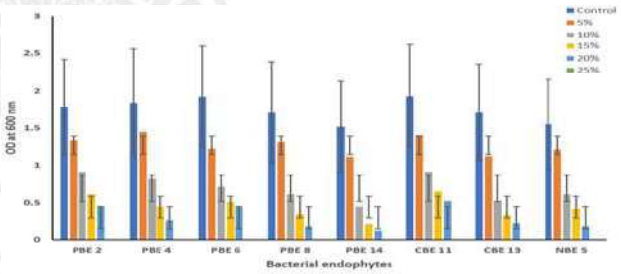

Fig 1. Growth of drought tolerant bacterial endophytes at different concentrations of PEG. Lines over bars indicates standard error mean ± SE(n=3)

Effect of Bacterial Endophytes on Drought Sensitive Rice (IR-64)

Standardization of Polyethylene Glycol (PEG MW 8000) Concentration for Rice : Rice seedlings were screened for drought stress using PEG (MW 8000) at 0, -0.04, -0.14, -0.30, -0.51 and -0.77 MPa concentrations. The length of root and shoot was decreased with increased PEG concentrations (Table 5). The untreated rice seedlings showed highest seedling length whereas lowest seedling length was observed with increased concentrations. LC 50 value of PEG concentration was found to be 14.3 per cent. The results are in agreement with Muddarsu and Manivannan (2017) who reported that eight Chilli cultivars screened for drought stress using PEG showed decreased root and shoot length with increased concentrations.

TABLE 2

Effect of different concentrations of PEG MW- 8000 on growth of endophytic bacteria isolated from Pangong region plants

Bacterial Isolates	Optical density of bacterial growth					
	Control (0%)	5%	10%	15%	20%	25%
PBE 1	0.70	0.38	0.23	0.10	0.0	0.0
PBE 2	1.78	1.34	0.91	0.61	0.45	0.0
PBE 3	0.65	0.58	0.32	0.18	0.0	0.0
PBE 4	1.84	1.45	0.82	0.45	0.26	0.0
PBE 5	0.86	0.68	0.55	0.24	0.01	0.0
PBE 6	1.92	1.22	0.71	0.51	0.45	0.0
PBE 7	1.08	0.34	0.25	0.16	0.00	0.0
PBE 8	1.71	1.31	0.61	0.34	0.18	0.0
PBE 9	0.65	0.48	0.24	0.12	0.0	0.0
PBE 10	0.66	0.54	0.32	0.21	0.0	0.0
PBE 11	0.89	0.52	0.46	0.18	0.0	0.0
PBE 12	0.98	0.55	0.42	0.12	0.0	0.0
PBE 13	0.58	0.44	0.38	0.18	0.0	0.0
PBE 14	1.52	1.11	0.45	0.21	0.12	0.0
PBE 15	0.65	0.48	0.32	0.17	0.0	0.0
PBE 16	0.64	0.45	0.23	0.12	0.0	0.0
PBE 17	0.68	0.44	0.22	0.10	0.0	0.0
PBE 18	0.74	0.33	0.16	0.10	0.0	0.0
PBE 19	0.89	0.58	0.24	0.12	0.0	0.0

Note: PBE = Pangong Bacterial Endophytes

TABLE 3.

Effect of different concentrations of PEG MW-8000 on growth ofendophytic bacteria isolated from Changla region plants

Bacterial Isolates	Optical density of bacterial growth					
	Control (0%)	5%	10%	15%	20%	25%
CBE 1	1.14	0.39	0.38	0.02	0.0	0.0
CBE 2	0.71	0.55	0.35	0.08	0.0	0.0
CBE 3	1.18	0.53	0.37	0.07	0.0	0.0
CBE 4	0.95	0.41	0.42	0.01	0.0	0.0
CBE 5	0.68	0.66	0.69	0.01	0.0	0.0
CBE 6	0.79	0.58	0.66	0.01	0.0	0.0
CBE7	0.58	0.35	0.15	0.01	0.0	0.0
CBE 8	0.78	0.51	0.44	0.10	0.0	0.0
CBE 9	1.10	0.53	0.31	0.10	0.0	0.0
CBE 10	1.01	0.80	0.40	0.18	0.0	0.0
CBE 11	1.93	1.41	0.91	0.65	0.52	0.0
CBE 12	0.50	0.17	0.16	0.11	0.0	0.0
CBE 13	1.71	1.12	0.52	0.33	0.22	0.0
CBE 14	0.35	0.29	0.18	0.12	0.0	0.0

Note: CBE = Changla Bacterial Endophytes

TABLE 4

Effect of different concentrations of PEG MW-8000 on growth of endophytic bacteria isolated from Namika La region plants

Isolates	Optical density of bacterial growth					
	Control (0%)	5%	10%	15%	20%	25%
NBE 1	0.55	0.25	0.12	0.02	0.0	0.0
NBE 2	0.45	0.24	0.11	0.02	0.0	0.0
NBE 3	0.55	0.45	0.12	0.05	0.0	0.0
NBE 4	1.05	0.98	0.50	0.04	0.0	0.0
NBE 5	1.55	1.21	0.61	0.41	0.18	0.0
NBE 6	1.02	0.65	0.45	0.24	0.0	0.0
NBE 7	0.56	0.32	0.21	0.14	0.0	0.0
NBE 8	0.23	0.10	0.08	0.05	0.0	0.0
NBE 9	0.45	0.32	0.22	0.12	0.0	0.0
NBE 10	0.89	0.40	0.32	0.10	0.0	0.0
NBE 11	0.44	0.35	0.25	0.01	0.0	0.0
NBE 12	0.65	0.52	0.44	0.04	0.0	0.0
NBE 13	0.78	0.69	0.58	0.35	0.0	0.0
NBE 14	0.77	0.58	0.44	0.21	0.0	0.0
NBE 15	0.74	0.35	0.25	0.10	0.0	0.0
NBE 16	0.56	0.24	0.12	0.04	0.0	0.0
NBE 17	0.47	0.21	0.11	0.06	0.0	0.0
NBE 18	0.85	0.78	0.54	0.07	0.0	0.0
NBE 19	0.96	0.44	0.24	0.24	0.0	0.0
NBE 20	0.74	0.33	0.22	0.12	0.0	0.0
NBE 21	0.32	0.24	0.18	0.05	0.0	0.0
NBE 22	0.25	0.12	0.08	0.02	0.0	0.0
NBE 23	0.96	0.69	0.34	0.11	0.0	0.0
NBE 24	0.66	0.34	0.07	0.03	0.0	0.0
NBE 25	0.55	0.24	0.14	0.06	0.00	0.00

Note: NBE = Namika La Bacterial Endophytes

TABLE 5

Effect of different concentrations of PEG (MW-8000) on seedling length of Rice (IR-64)

Treatments	Root length (cm)	Shoot length (cm)	Seedling length (cm)
Control	10.0 [a]	14.5 [a]	24.5 [a]
5% (-0.04Mpa)	9.0 [b]	12.5 [b]	21.5 [b]
10% (-0.14Mpa)	7.0 [c]	10.5 [c]	17.5 [c]
15% (-0.30Mpa)	6.0 [d]	9.8 [d]	15.8 [d]
20% (-0.51Mpa)	4.5 [e]	9.1 [e]	13.6 [e]
25% (-0.77Mpa)	3.0 [f]	7 [f]	10 [f]
CD (P<0.05)	0.119	0.154	0.119

Note: Means with same superscript in a column do not differ significantly as per Duncan Multiple Range Test (DMRT)

In-vitro Inoculation Bacterial Endophytes to Drought Sensitive Rice

Out of eight bacterial endophytes, three endophytes (CBE 11, PBE 2 and PBE 4) inoculated seedlings recorded maximum seedling length (32.5, 27.5 and 27.1 cm, respectively). The CBE11 showed significantly highest length which was selected for characterization (Table 6). PEG induced 14.3 per cent drought stress seedlings showed less seedling growth compared to control (without PEG) seedlings. Timmusk *et al.* (2014) reported higher root length and density improve water and nutrient uptake which could allow plants to tolerate drought stress. Similarly, three *Pseudomonas* sp. used for mitigating drought stress showed significant growth performance of finger millet compared to unino culated plants (Chandra *et al.,* 2018). The bacterium was re-isolated from root, shoot and leaves from inoculated rice seedlings and confirmed by morphological and microscopic observations while comparing with mother culture.

Molecular Characterization of Selected Endophyte using 16S rRNA Primer

Bacteria can be identified using morphological as well as molecular tools. The genes encoding for 16S rRNA in prokaryotes have been used extensively for sequence based evolutionary analysis because they are (1) Universally distributed, (2) Functionally constant, (3) sufficiently conserved and (4) Have adequate length to provide a view of evolution

TABLE 6

Effect of inoculation of bacterial endophytes on root and shoot length of Rice (IR-64) after 14 days of incubation

Treatments	Without drought stress			With drought stress at 14.3% PEG MW-8000 concentration		
	Root Length (cm)	Shoot Length (cm)	Seedling Length (cm)	Root Length (cm)	Shoot Length (cm)	Seedling Length (cm)
Control	13.5 [d]	12.0 [d]	25.5 [d]	10.5 [d]	9.2 [c]	19.7 [d]
PBE 2	15.0 [b]	12.1 [c]	27.1 [c]	13.0 [b]	9.0 [d]	22.0 [c]
PBE 4	14.5 [c]	13.0 [b]	27.5 [b]	12.5 [c]	9.9 [a]	22.4 [b]
PBE 6	13.1 [e]	10.0 [e]	23.1 [f]	10.5 [d]	9.2 [b]	19.7 [e]
PBE8	15.0 [b]	9.5 [f]	24.5 [e]	10.0 [f]	6.0 [e]	16.0 [g]
PBE 14	13.5 [d]	9.5 [f]	23.0 [g]	10.0 [f]	5.6 [g]	15.6 [i]
CBE 11	18.5 [a]	14.0 [a]	32.5 [a]	15.0 [a]	9.0 [d]	24.0 [a]
CBE 13	10.5 [f]	9.2 [g]	19.7 [h]	10.0 [f]	5.7 [f]	15.7 [h]
NBE 5	9.0 [g]	10 [e]	19.0 [i]	10.5 [e]	6.0 [e]	16.5 [f]
CD(P<0.05)	0.162	0.144	0.129	0.124	0.119	0.136

Note: Means with same superscript in a column do not differ significantly as per Duncan Multiple Range Test (DMRT)

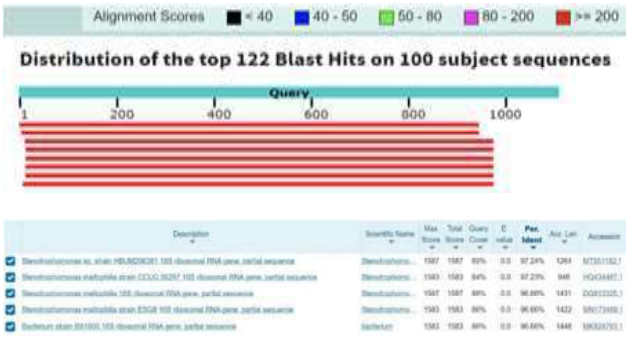

Fig. 2 (a) : 16S rRNA gene partial sequence of CBE 11 isolate showing 97% homologywith *Stenotrophomonas malthophilia* CCUG 50297 (518 bp)

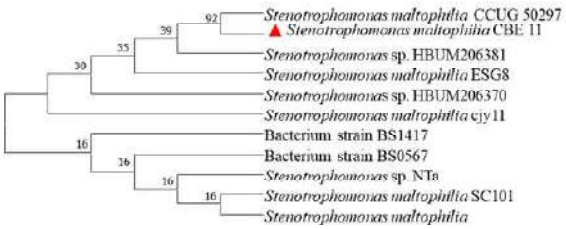

Fig. 2 (b) : Phylogenetic tree of *Stenotrophomonas maltophilia*

encompassing all living microorganisms (Madigan *et al.,* 2009). Molecular methods such as 16S rRNA/ 18S rRNA gene sequence is extensively used for identification of microorganisms (Nandan *et al*., 2021). In the present study, drought tolerant endophyte (CBE 11) was identified by 16S rRNA gene sequence. The amplified product having 1,200 bp was compared with the sequences available at NCBI database and found 97.4 per cent homology with *Stenotrophomonas malthophilia*. The phylogenetic tree constructed with the sequences of 10 *Stenotrophomonas* spp. revealed that the isolate CBE11 is closely related to *S. malthophilia* (HQ434487) Therefore, the bacterium was confirmed as *S. malthophilia* (Fig. 2a and Fig. 2b). Santhosha gowda and Earanna (2017) identified the *Gluconoaceto bacter diazotrophicus* isolated from Maize using 16S rRNA gene sequence.

To conclude, bacterial endophytes isolated from different Himalayan regions were screened and characterized with distinct concentration of PEG, *Stenotrophomonas maltophilia* showed a significant result in Rice (IR-64). Therefore, bio-priming with this endophyte in rice can impart tolerances to drought stress.

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