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Screening and Plant Growth Promoting Activity of Drought Tolerant Endophytic Bacteria Isolated from Wild *Poaceae*

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A total 26 unique bacterial endophytic strains were isolated from roots, stems and leaves of *Dactyloctenium sindicum, Cenchrus biflorus* and *Chloris barbata* plants and eleven isolates were screened as a drought tolerant endophytic bacteria. The CEB 9 and CEB 76 drought tolerant isolates were positive for phosphate solubilisation, seven endophytic isolates were positive to siderophore and eight isolates were positive to ACC deaminase activity. The CEB 76 isolate produced 47.96 μgml^{-1} Indole acetic acid at 30% PEG 6000 concentration. While the CEB 9 isolate produced 154.68 μgml^{-1} of exopolysaccharide at 30% PEG6000. The CEB 12, CEB 15 and CEB 75 isolates were successfully inhibited growth of *Alternaria triticina* and *Helminthosporium sativum*. The CEB 76 isolate only inhibited growth of *Helminthosporium sativum.* This study evaluates the drought tolerant capacity of endophytic bacteria, plant growth promoting activity and biocontrol mechanism of drought tolerant bacteria.

Key words: Drought tolerant endophytic bacteria, PGPR, Antagonistic potential.

Water deficit is the most common stress affecting plant growth and yield in arid and semiarid regions. Therefore, it is necessary to improve the level of efficiency in plant capture and use of water and nutrients. Inoculation of plants with native beneficial microorganisms may increase the drought tolerance of plants[1]. The *Poaceae* plants are the world's single most important source of food. Xerophytes plant species have mechanisms to overcome drought stress and these mechanism could be considered the endophytic association and interaction between plant and rhizobacteria able to improve the plant growth under abiotic stress conditions[2].

Plants constitute vast and diverse niches for endophytic organisms which occupy internal tissues of plants without causing damage to their hosts. Many bacteria closely interacting with plants produce secondary metabolites as agents needed for nutrient uptake. Plants produce several classes of phytohormones including auxins, cytokinins, brassino steroids, gibberellins, abscisic acid, ethylene, jasmonates and strigolactones playing roles in development and stress responses[3,4].

Although there are several reports on PGPB and biocontrol agents of endophytic bacteria, the induction of drought tolerance in maize by drought-tolerant plant growth-promoting *Bacillus* spp., *Pseudomonas fluorescens* AK1 and *Pseudomonas aeruginosa* AK2 [5,6]. The exopolysaccharide and indole acetic acid production was observed for the strains from Brazilian cacti rhizobacteria for plant growth promotion under drought[7]. The application of plant growth promoting rhizobacteria (PGPR) as crop inoculants for biofertilization, phytostimulation, and biocontrol would be an attractive alternative to decrease the use of chemical fertilizers which also effect environmental pollution.

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The crop growing environment in the semi-arid tropics is highly variable due to erratic spacing and timing of seasonal rainfall. The crops grown under semi-arid lands require drought tolerant bacteria for effective plant growth. The present study aimed to isolate drought tolerant endophytic bacteria and to characterize their performance under drought conditions.

MATERIALS AND METHODS

Location and Sample collection

The experimental materials were consisted of various endophytic bacterial strains which were isolated from species of *Poaceae* family. The *Poaceae* wild plants- *Dactylactenium sindicum, Cenchrus biflorus, Chlorius barbata* were used for isolation of drought tolerant endophytic bacteria. The sample was carried out from banni region (Kutch, Gujarat) an internationally recognized unique grassland stretch of western India. The banni refers to an arid region in the western most end of the Gujarat state in India.

Isolation of endophytic bacteria from poaceae plants

The root, stem and leaves of *Dactyloctenium sindicum* (Madhanu), *Cenchrus biflorus* (Bharat) and *Chloris barbata* (Siyaar puccha) plants(2g) were washed with water and surface sterilized with 0.1% $HgCl_2$ for 3 min and subsequently wash two times with distilled water. The same plant materials again wash with 90% ethanol for 3 min and wash with sterile distilled water. It was then suspended in 0.05 M PBS and ground with a sterilized mortar and pestle for 1 to 3 min. Undiluted 0.1ml aliquot was then inoculated onto nutrient agar media[8]. The plates were incubated at 28°C for 5 to 7 days under observation. Colonies on nutrient agar were selected for further studies.

Screening of drought tolerant endophytic bacteria

Nutrient broth with different water potentials -0.15 Mpa, -0.49Mpa, -1.03 Mpa was prepared by adding the appropriate concentrations of 10%, 20% and 30% Polyethylene glycol (PEG 6000), respectively. A bacterial cultures cultivated overnight in nutrient broth were added in different PEG6000 concentration of N-broth and incubate it at 28°C under 120 rpm in shaking conditions for 24 hour, growth was estimated by measuring the optical density at 600 nm using a spectrophotometer. The growth of the isolates at various stress levels was recorded[9,10].

Biochemical studies of drought tolerant endophytic bacteria

In the biochemical studies, KB002 HiAssorted Biochemical Test kit (Himedia) was used for every bacterial isolates which contents citrate utilization, lysine utilization, Ornithine utilization, urease, phenylalanine deamination, nitrate reduction, H_2S production, glucose, adonitol, lactose, arabinose and sorbitol utilization. In addition fructose, maltose, sucrose, manitol and xylose utilization were conducted on phenol red medium. Serine, arginine and proline utilization were also conducted in decarboxylase broth base, Moller (Himedia).

Plant growth promoting activity of drought tolerant endophytic bacteria

Phosphate solubilization activity

A loopful bacterial culture were spotted on Pikovaskya's medium (g/L—glucose 10, tri-calcium phosphate 5, ammonium sulphate 0.5, sodium chloride 0.2, magnesium sulphate heptahydrate 0.1, potassium chloride 0.2, ferrous sulfate heptahydrate 0.002, yeast extract 0.5, manganese (II) sulfate dehydrate 0.002, agar 20, pH 7.0). The plates were incubated in an incubator at 28 ± 2°C. The plates were then examined daily for seven days for appearance of a transparent halos[11].

Siderophore production

Siderophore production was determined on Chrome-azurol S (CAS) medium[12]. The 24 h old bacterial cultures were spotted on the center of Chrome Azurol S (CAS) agar media and incubated at 28 ± 2^0C for five days. When the present blue colored CAS media was showed orange or yellow halos around the colonies indicate the siderophore production.

ACC deaminase activity

A loopful of 2 days old growth of the endophytic bacterial cultures were spotted on to DF salts minimal medium(potassium dihydrogen phosphate 4 g/L, disodium hydrogen phosphate 6 g/L, magnesium sulfate heptahydrate 0.2 g/L, ferrous sulfate heptahydrate 0.1 g/L, boric acid 10 lg/L, manganese(II) sulfate 10 lg/L, zinc sulphate 70 lg/L, copper(II) sulfate 50 lg/L, molybdenum (VI)

oxide 10 lg/L, glucose 2 g/L, gluconic acid 2 g/L, citric acid 2 g/L, agar 12 g/L) amended with 3mM ACC [19]. The growth of bacterial isolates on the plates were recorded after 4 to 5 days of incubation at 28 ± 2°C. The bacterial cultures showing good growth on ACC supplemented medium plates and capable of utilizing ACC as nitrogen source were scored as ACC^{+}.

Protease activity

The two days old bacterial culture was spotted on milk agar medium consist casein.. The plates were incubated at 28 ± 2^0C for two days. A clear halo zones around the colonies showing the positive results.

Quantification of IAA production

For the quantification of IAA production 100ml nutrient broth was prepared with L-tryptophan (1 $mgml^{-1)}$as a precursor for IAA synthesis. The bacterial cultures were cultivated at 28 ± 2^0C and 120 rpm shaking condition for two days incubation in medium supplemented with 0%, 10%, 20% and 30% of PEG6000 to induce drought stress [9,13]. For spectrophotometrical analysis, Bacterial cultures were centrifuged at 8,000 rpm for 10 min. Two millilitres of freshly prepared Salkowski reagent (1 ml of 0.4 M $FeCl_3$ in 50 ml of 35 % Perchloric acid) was added to 1 ml of culture supernatant. The reaction mixture was incubated at 30 °C for 30 min. Development of pink colour indicates the production of IAA and OD was measured at 530nm.

Quantification of EPS production

For exopolysaccharide determination, 250 ml flasks containing 100 mL of N-broth with D-glucose were supplemented with varying PEG 6000 concentrations (0%, 10%, 20% and 30%). A medium was inoculated (1000 µl) with 24-hours old bacterial culture and incubated at 120 rpm shaker for 48 h at 28°C. The EPS production was measured by 5% phenol and sulphuric acid reagents. The developed

Table 1. Screening of the drought tolerance endophytic bacteria isolated from Poaceae plants

Isolates	N broth supplemented with PEG 6000 (O.D.at 600nm after 24 hr)			
	0 % (0 Mpa)	10%(-0.15Mpa)	20%(-0.49Mpa)	30%(-1.03Mpa)
DS 1	1.851	1.779	0.688	0.345
DS 2	1.789	0.738	0.532	0.200
DS 3	1.963	1.349	0.796	0.567
DS 4	1.123	1.098	0.582	0.185
CEB 5	1.713	1.041	0.706	0.137
CEB 6	2.358	1.726	1.256	0.616
CEB 7	1.823	1.452	0.867	0.401
CEB 8	2.063	1.468	0.885	0.095
CEB 9	1.796	1.310	1.300	1.226
CEB 10	1.623	1.022	0.560	0.218
CEB 11	1.790	1.497	0.641	0.613
CEB 12	1.837	1.744	1.741	1.063
CEB 13	1.639	1.610	0.595	0.545
CEB 14	2.301	2.114	1.820	1.285
CEB 15	2.381	1.969	1.478	1.440
CEB 16	1.138	0.823	0.555	0.510
CEB75	2.114	2.070	2.057	1.467
CEB76	1.900	1.990	1.776	1.498
CHB54	2.241	2.114	1.753	1.236
CHB55	1.665	1.143	0.252	0.015
CHB 56	2.142	2.101	1.360	0.547
CHB 57	0.816	0.112	0.001	0.005
CHB 58	2.401	2.370	2.063	1.803
CHB 59	1.901	1.850	1.702	1.274
CHB 60	1.969	1.896	1.884	1.373
CHB 61	1.694	1.423	1.355	1.040

yellowish orange colour was measured at 490nm[14].

Antagonism of endophytic bacteria against plant pathogenic fungi

The antagonistic action of endophytic bacteria was tested against phytopathogenic fungi *Alternaria triticina* and *Helminthosporium sativum.* Bacterial isolates were streaked at a distance of 4 cm from the rim of individual Petriplates containing potato dextrose agar medium. A 4 mm mycelial disc from 7 day old PDA culture of fungal pathogens was then placed on the other side of the Petriplate and the plates were incubated at 28°C for 12 days[15].

The per cent inhibition was calculated by using the formula:

$$I = C\text{-}T \times 100/C$$

Where, I= Antagonism index, C=Area of test fungus in control (mm^2), T=Area of test fungus in respective treatment (mm^2).

RESULTS AND DISCUSSION

Isolation of endophytic bacteria from *poaceae* grasses

All plants of poaceae family yielded the endophytic bacteria from leaves, stems and roots. A total 26 unique bacterial endophytic strains were isolated from plants. The *Dactyloctenium sindicum* yielded four colonies, *Cenchrus biflorus* yielded 14 colonies and *Chloris barbata* yielded eight colonies on N-agar medium.

Screening of drought tolerant endophytic bacteria

The table 1 showed that among twenty six endophytic strains tested, the best eleven drought tolerant strain were CEB 9, CEB 12,CEB 14,CEB 15,CEB75,CEB 76, CHB54,CHB 58, CHB 59,CHB 60 and CHB 61 which grow at 30%PEG6000 and gave 1.226, 1.063, 1.285,1.440, 1.467, 1.498, 1.236, 1.803, 1.274, 1.373 and 1.040 OD at 600nm respectively. While moderately drought tolerant strain were CEB 6 (0.616) followed by CEB 11, DS 3,CHB 56,CEB 13 and CEB 16 at 30% PEG6000 concentration. So, PEG provides a means of quantifying a water stress. A twenty one *Rhizobium leguminosarum* biovar *trifolii* strains and seven *Rhizobium meliloti* strains were characterized for their nodulation efficiencies and their growth performance against salinity, drought and heavy metals[16]. A 30 bradyrhizobial isolates were tested under drought conditions, Among the 30 isolates, 4 isolates were screened as potential drought tolerant isolates [13]. The growth and persistence of drought tolerant bacteria are positively impacted in drought condition. The drought tolerant bacterial isolates obtained in this study are excellent models to study the mechanisms of resistance and to elucidate the role of genetics of drought tolerance.

Biochemical studies of candidate endophytic bacteria

Table 2 showed Gram staining, sugar fermentation and amino acid utilization tests of eleven completely drought tolerant endophytic bacteria. In that All the isolates were positive for H_2S production and glucose fermentation.

Plant growth promoting activity of drought tolerant endophytic bacteria

The isolates tested for their PGP activities such as phosphate solubilisation, siderophore production, ACC deaminase activity and protease activity.

Phosphate solubilization activity

The results (table 3) revealed that among 11 drought tolerant endophytes CEB 9 and CEB 76 were positive for phosphate solubilisation. These bacteria could convert tricalcium phosphate in the medium from insoluble to soluble forms [17].

Siderophore production

The seven endophytic isolates (table 3) were able to produce siderophore. The formation of orange halo around the colonies due to the chelation of iron was the indication for production of siderophore. The formation of orange halo is as a result of the production of siderophore, which removes the iron from the dye complex that changes the colour of the medium from blue to orange [12]. Siderophores producing bacteria can sequestrate the limited iron and thereby reduce its availability for growth of phytopathogens. Thus, they enable the plant growth promotion indirectly[18].

ACC deaminase and Protease activity

The eight and nine isolates(table 3) were positive to ACC deaminase and protease activity, respectively. Some microbes can utilize the ACC as nitrogen source from the exudates of roots or seeds. This decrease in the levels of ACC and ethylene may prevent the ethylene mediated plant growth inhibition. Endophytic microbes with these capabilities residing inside the host plants can benefit the host by reducing the stress and

Table 2. Biochemical characterization of drought tolerance endophytic bacteria

Tests	CEB9	CEB12	CEB14	CEB15	CEB75	CEB76	CHB54	CHB58	CHB59	CHB60	CHB61
Citrate Utilization	+	-	-	-	-	+	-	+	-	-	-
Lysine utilization	-	-	-	+	-	-	+	-	+	+	-
Ornithrine utilization	+	+	+	+	+	+	+	+	+	+	+
Serine utilization	+	-	+	+	+	+	-	-	+	+	+
Proline utilization	-	-	-	+	+	-	-	-	-	+	+
Arginine utilization	+	+	+	+	+	+	+	+	+	+	+
Urease	-	+	+	-	-	-	-	+	-	-	-
Phenylalanine Deamination	-	-	-	-	-	-	-	-	-	-	-
Nitrate reduction	+	-	+	+	+	+	+	+	+	+	+
H_2S production	+	+	+	+	+	+	+	+	+	+	+
Glucose	+	+	+	+	+	+	+	+	+	+	+
Adonitol	+	-	-	-	+	-	+	-	+	-	-
Lactose	-	-	-	-	-	-	-	-	-	-	-
Arabinose	+	-	+	+	-	+	+	+	+	-	-
Sorbitol	+	-	+	+	-	-	+	+	+	-	-
Fructose	+	-	+	+	-	+	-	+	+	+	-
Maltose	+	-	+	-	-	+	-	+	-	+	-
Sucrose	+	-	+	+	-	+	-	+	+	+	-
Mannitol	+	-	+	+	-	-	+	+	+	-	-
Xylose	+	-	+	+	-	-	+	+	+	-	-
Galactose	-	-	+	-	-	+	+	-	+	-	-
Dextrose	-	-	+	-	-	-	+	-	+	-	-
Raffinose	-	-	+	-	-	-	+	+	+	-	-
Cellobiose	-	-	+	-	-	-	+	+	+	-	-
Inositol	-	-	+	-	-	-	+	+	+	-	-
Gram reaction	-	+	+	+	+	-	+	+	+	+	-
Cell shape	R	R	C	SR	SR	R	C	C	C	C	R

R= rod, C= cocci, SR= short rod, CB= -cocci bacilli. ,+ indicated positive and – indicate negative results

Table 3. Plant growth promoting activities of drought tolerance endophytic bacteria

Tests	CEB 9	CEB12	CEB14	CEB15	CEB75	CEB76	CHB54	CHB58	CHB59	CHB60	CHB61
Phosphate solubilization	+	-	-	-	-	+	-	-	-	-	-
Siderophore production	+	+	-	+	+	+	-	-	-	+	+
ACC deaminase activity	+	-	+	+	+	+	-	+	-	+	+
Protease activity	+	+	+	+	+	-	+	+	+	+	-

increasing the plant growth [19].

Quantification of IAA and EPS production

All the isolates grew well in nutrient broth under normal condition and nutrient broth supplemented with PEG 6000 in different values either increased or decreased. Varying results were recorded in table 4 (IAA production) and table 5 (EPS production).

The CEB 76 isolate (47.96 µgml^{-1}) was the highest IAA producer at 30% PEG 6000 concentration followed by CHB 59 (28.51 µgml^{-1}) and CEB 9 (25.55 µgml^{-1}). The enhancement of root growth by bacterial inoculation could be due to IAA produced by bacteria. Moreover, the ability

Table 4. Indole acetic production of drought tolerant endophytic bacteria

Isolates	IAA production (µgml^{-1}) under different concentration of PEG6000			
	0%	10%	20%	30%
CEB 9	21.24	21.66	25.29	25.55
CEB 12	16.07	19.51	21.18	22.14
CEB 14	7.44	8.81	10.14	10.37
CEB 15	14.29	15.51	17.85	19.48
CEB 75	9.11	13.18	4.40	4.40
CEB 76	15.55	26.48	32.22	47.96
CHB 54	8.14	16.70	18.51	19.29
CHB 58	12.85	28.44	23.74	22.48
CHB 59	13.00	28.07	28.29	28.51
CHB 60	18.18	28.03	15.62	14.74
CHB 61	18.25	27.14	20.29	19.29

Table 5. Exopolysaccharide production of drought tolerant endophytic bacteria

Isolates	EPS production (µgml^{-1}) under different concentration of PEG6000			
	0%	10%	20%	30%
CEB 9	88.59	150.0	153.75	154.68
CEB 12	85.62	67.5	41.87	36.87
CEB 14	27.34	29.84	31.40	33.59
CEB 15	79.37	94.21	57.03	10.93
CEB 75	65.78	66.87	48.75	34.21
CEB 76	96.71	109.53	117.18	118.90
CHB 54	97.65	136.25	137.65	139.06
CHB 58	74.84	113.28	104.37	95.62
CHB 59	41.56	48.59	50.78	67.03
CHB 60	112.18	81.87	78.75	78.75
CHB 61	113.90	71.40	68.75	62.65

of these endophytes to increase the production of IAA as much as the increased osmotic stress (PEG) in the growing medium would account for their osmotic tolerance. Most importantly IAA produced by these endophytes appears to be dependent on the L-tryptophan pathway [9]. The significant effects of four strains tested for IAA under drought condition and they were produced desirable amount of IAA with range 0.10 to 6.10 μgml^{-1} [13].

The CEB 9 isolate was produced 154.68 μgml^{-1} of exopolysaccharide at 30% PEG6000 followed by CHB 54 (139.06 μgml^{-1}) and CEB 76 (118.90 μgml^{-1}) (table 5). Bacteria can survive under water stress due to the production of exopolysaccharide, which protects microorganisms from water stress by enhancing water retention by regulating the diffusion of organic carbon sources [20]. EPS also helps the microorganisms to irreversibly attach and colonize the roots due to involvement of a network of fabrillar material that permanently connects the bacteria to the root surface. Better EPS production leads to making of better biofilm development. Reducing sugars are major components of EPS that are increased in the presence of higher stress and increases the biofilm stability of bacterial cells[14].

Antagonism of endophytic bacteria against plant pathogenic fungi

The results of dual culture technique indicated in table 6. Among the 11 isolate, only three endophytic bacterial strain CEB 12, CEB 15 and CEB 75 could successfully inhibited growth of *Alternaria triticina* and their growth inhibition was 53.84, 47.25 and 53.84 per cent, respectively. While The four isolates CEB 12, CEB 15, CEB 75 and CEB 76 inhibited growth of *Helminthosporium sativum* which gave 57.33, 62.66,53.33, 53.33 and 57.33 per cent growth inhibition, respectively (table 6). A various mechanisms have been attributed to bacterial antagonistic activity namely, different hydrolytic enzymes, chitinases, HCN, and siderophore production and production of antibiotics like phenazines, DAPG, pyrrolnitrin, pyoluteorin, and other secondary metabolites make endophytic bacterial isolates an ideal biocontrol agent [11]. Endophytes may contribute to their host plants by producing a plethora (an excessive) of substances that provide protection and ultimately gave survival value to the plant [21].

Table 6. Antagonist activities of drought tolerance endophytic bacteria

Isolates	Growth inhibition of pathogen over control (%)	
	Alternaria triticina	*Helminthosporium sativum*
CEB 9	28.57	33.33
CEB12	53.84	57.33
CEB14	40.65	32.00
CEB15	47.25	62.66
CEB75	53.84	53.33
CEB76	28.57	57.33
CHB54	20.87	12.00
CEB58	35.16	28.00
CEB59	23.07	37.33
CEB60	31.86	16.00
CEB61	28.57	49.33

CONCLUSION

It was considered that endophytic microbes may have an active role in drought stress condition. Such results suggest that the great diversity of the bacterial world has enough resources to provide functional redundancy in drought stress. This study revealed that many bacteria isolated from *poaceae* plants have characteristics that suggest the potential to promote plant growth, in particular bacteria isolated from the inside of grasses roots, stem and leaves. The ability to antagonise fungal pathogens due to antibiotic production or the release of fungal cell wall-degrading enzymes by the endophytic bacteria and it can be used as a biocontrol agent.

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