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Amelioration of drought tolerant plant growth promoting endophytic bacteria from *Ricinus communis*

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Abstract- In the present study, 26 endophytic bacteria were isolated from different parts of plant *Ricinus communis*. Endophytes were screened for drought stress tolerance, plant growth promoting traits and ACC deaminase activity. Out of 26 isolates, 22 could show drought tolerance up to a minimum water potential of -0.001 MPa, whereas 18 could tolerate up to -0.27 MPa, and 13 up to -0.54 Mpa respectively. Among them 5 isolates showed highest tolerant up to -1.09 matric potential (MPa) and exhibited most of the plant growth promoting traits. Based on the results, 5 promising isolates namely MGT7, MGT9, MGT13, MGT16 and MGT19 were selected and identified using biochemical and 16S rRNA gene sequencing as *Bacillus pumilus* (MGT7), *Paraburkholderia megapolitana* (MGT9), *Achromobacter xylosoxidans* (MGT13), *Alcaligenes faecalis* (MGT16) and *Stenotrophomonas maltophilia* (MGT19). Further, at -1.09 MPa all the five isolates showed PGP traits and ACC activity. Thus, indicating that drought tolerant plant growth promoting endophytic bacteria (PGPE) helps in plant growth under drought stress condition.

Keywords: Endophytes, Drought tolerance, Plant growth promoting activities, ACC deaminase

1. INTRODUCTION

The word endophyte define as "those bacteria and fungi which can be present at a precise moment within the tissues of deceptively healthy plant hosts without producing any kind of negative symptoms" [1]. This definition excludes pathogens and nodule-producing microbes. Only small fractions of rhizosphere and phyllosphere bacteria are able to live inside the plant. Obtaining a sufficient amount of quality food to feed all of the people in the world, both now and future is a serious global concern [2]. By 2050, more than 50% of the world's arable lands are expected to have serious plant growth problems, largely because of issues associated with drought. Moreover, there is a possibility of decreasing production of global food in the future because of global warming [3]. In developed countries, for example, the annual crop yield increase is currently less than 1% while the increase in demand in those countries is around 3% annually [4]. This problem is a consequence of several phenomena, including global warming, that can frequently lead to drought and severe water stress [2]. Inhibition of plant growth is one of the results of these negative stresses in natural environment [5].

To address the above mentioned issue, increasing crop water use efficiency may occur either by better crop management or through the development of drought resistant plants [6]. To reach higher levels of productivity of these crops without any harmful effects to the environment, the soil quality and agricultural practices should be improved [7]. In addition, researchers have to find strategies and technologies to increase crop yield and at the same time decrease the use of potentially harmful chemical fertilizers and pesticides [7]. Currently, one of the biotechnological strategies that is being applied to induce environmental stress tolerance of plants is judicious application of strains of plant growth promoting endophytes (PGPE) [8]. They enhance growth of plant by direct or indirect mechanisms. Direct promotion occurs either by increased acquisition of essential nutrients which involve nitrogen, phosphorus and iron or by modulation of hormone levels synthesizing auxin, cytokinin or gibberellins. In addition, some endophytes cn lower levels of the phytohormone ethylene by synthesizing an enzyme, 1-aminocyclopropane-1-carboxylate (ACC) deaminase that cleaves the compound ACC, the immediate precursor of ethylene in all higher plants. Indirect promotion occurs by inhibition by production

of antagonistic substances against bacterial or fungal pathogens [9].

Ricinus communis is a dicot plant belonging to the fourth largest family called 'Euphorbiaceae' family (spurge family). It is commonly known as castor. Castor is indigenous to India, southeastern Mediterranean Basin and Eastern Africa but is widespread throughout the world [10].

As the plant growth promoting properties of endophytic bacteria can vary, it is important to study such properties from microbial populations associated with economically important and physiologically unique plants. In the current study, endophytic bacterial isolates from *Ricinus* were investigated for plant growth promoting potential. Five bacterial strains out of twenty six belonging to various genera were isolated, identified and studied for the presence of various plant growth promoting compounds. Since studies on such aspects from *Ricinus communis* is very limited, the study is significant and novel in its approach.

2. MATERIALS AND METHODS

2.1. Collection of plant sample

Healthy and mature samples of castor plants were randomly collected from different locations of Ahmedabad, Gujarat, India. Leaves, stem, roots and castor bean specimen were excised with a sterile scalpel and labeled. The samples were brought to the laboratory in air tight zip lock bags and kept cold until processed.

2.2. Surface sterilization of plant sample

Samples were washed under running tap water and then with double distilled water. Samples were cut using knife into small segments of 1.0 x 1.0 cm. the segments were immersed in 70% ethanol for 1-3 minutes, followed by immersion in 4% sodium hypochlorite for 5-7 minutes. The segments were rinsed with 70% ethanol and finally with sterile double distilled water. The segments were dried under aseptic conditions [11].

2.3. Isolation of endophytic bacteria

The appropriate dilutions were plated on nutrient agar and trypticase soya agar (TSA). The plates were incubated at 28^{o}C and morphologically different colonies were picked and purifies on respective media. The pure cultures were maintained on agar slants under refrigeration conditions for further experiments.

In order to screen the isolates for drought stress tolerance, trypticase soya broth (TSB) with different water potentials was prepared by adding appropriate concentration of poly ethylene glycol (PEG) 6000 [12]. Drought stress was created by adding polyethylene glycol-6000 (PEG) at four different concentrations: 0, 5, 10 and 20% of PEG which is equivalent to four osmotic potential levels including -0.001, -0.27, -0.54 and -1.09 MPa, respectively [13]. PEG was used because it has a high molecular weight, it cannot pass through the cell wall and therefore it is used to regulate water potential and to evaluate resistance to drought at germination stage and to create different levels of water potential [13]. They were inoculated with the overnight grown broth cultures adjusted to optical density (OD) of 0.5 at 600nm. Growth of the isolates at various stress levels was estimated by measuring the OD at 600nm after incubation at 28^{o}C for 24 h, under shaking conditions.

2.4. Screening for plant growth promoting activities

2.4.1. Phosphate solubilization by selected endophytes

Isolates which were able to grow at minimum water potential (-1.09 MPa) level were tested for plant growth promoting traits under non-stress and drought stress condition. To determine phosphate solubilization under non-stress, Pikovskaya's medium amended with bromophenol blue was inoculated with 1% of over night culture raised in Nutrient broth and for drought stress Pikovskaya's medium with desired water potential (-1.09 MPa) was inoculated and incubated for 48 to 72 h at 37^{o}C. The cells were harvested by centrifugation at 10,000 rpm for 5 min and the supernatant was used for the quantitative estimation of phosphate [14].

2.4.2. Indole-3-Acetic Acid production by selected endophytes

Auxin production was checked in trypton yeast medium (non-stress and drought-stress) supplemented with 50 mgl^{-1}of L-Tryptophan was inoculated with 1% of overnight culture raised in LB broth and incubated in dark at 28^{o}C for 72 h on orbital shaker. Cells were harvested by centrifugation at 10,000 rpm for 5 min and supernatant was mixed with Salkowsky reagent, followed by incubation for 30 min at room temperature under dark conditions. The absorbance of pink colour was read at 536 nm [15]. The concentration of IAA produced was calculated from standard graph of pure indole acetic acid, study was carried out every 24 h for up to 120 h and the pattern of IAA production was recorded [16].

2.4.3. Siderophore production

To determine siderophore production under non-stress and drought-stress Chrome Azurol S (CAS) plates were prepared, inoculated on agar plate with actively growing bacterial cultures, incubated at 30^{o}C for five days and

checked for development of yellow-orange halo around the colony indicated production and release of siderophores on the agar plates [17]. Quantitative analysis was also carried out using liquid medium broth.

2.4.4. HCN production and Ammonia production by selected endophytes

Overnight broth cultures were inoculated in 10 ml peptone water and incubated at 30^{o}C for 48-72 h. Nessler's reagent was added in each tube. Development of brown colour was recorded as a positive test for ammonia production [18]. HCN production under non-stress and drought-stress was tested on Nutrient agar slants streaked with the test isolates. Whatmann no.1 filter paper strips soaked in 0.5% picric acid in 2% sodium carbonate were hanged in test tubes, sealed with para-film and incubated at 28^{o}C for four days. Conversion of strips from yellow to brown colour is positive for HCN production [19].

2.4.5. 1-Aminocyclopropane-1-carboxylix acid (ACC) deaminase activity

For qualitative analysis of ACC deaminase activity bacterial isolates were grown in Nutrient broth and cell pellets were collected by centrifugation, washed, suspended in sterile water and spot inoculated on Dworkin and Foster (DF) salt minimal medium [20]. Alone (negative control), DF supplemented with $(NH_4)_2SO_4$ (positive control). In order to screen ACC deaminase activity under non-stress and drought stress selected isolates were grown individually in liquid DF minimal medium alone, DF+ACC and DF+ $(NH_4)_2SO_4$ and their growth were measured at 600 nm.

2.5. Biochemical characterization

Endophytic isolates were characterized for Gram staining, Malonate, Voges Proskauer's, Citrate, ONPG, Nitrate reduction, Catalase, Arginine, Sucrose, Mannitol, Glucose, Arabinose, Trehalose was carried out using biochemical kit. (Himedia, India identification Kit)

2.6. Molecular characterization

For molecular characterization, bacterial genomic DNA was isolated [21]. and subjected to polymerase chain reaction (PCR) for amplification on 16S rDNA gene using universal forward (5'-AGAGTTTGATCCTGGCTCAG-3') and reverse (5'-AAGGAGGTGATCCAGCCGCA-3') primers under standard conditions (initial denaturation at 94^{o}C for 5 min, 30 cycles for denaturation at 94^{o}C for 1 min, annealing at 50^{o}C for 40 s, extension at 72^{o}C for 90 s, and final extension at 72^{o}C for 7 min). The PCR product (approximately 1.5 kb) was purified and sequenced. The partial 16S rDNA sequence was compared with the sequences available in the GenBank databases using the gapped BLASTN 2.2.21 program through the National Centre for Biotechnology Information server.

2.7. Statistical analysis

Experiments were repeated thrice and data represents the mean of three experiments. After analyzing statistically, variable results were found in plant growth promoting activities. These results were subjected to standard error.

3. RESULTS AND DISCUSSION

3.1. Surface sterilization of plant samples

Fresh and cleaned castor plants were used for the isolation of endophytic bacteria. The sample was surface strerilized to remove the epiphytic microorganisms. The surface sterilization procedure for the isolation of endophyic bacteria as standardize in the experiment was quiet satisfactory as no growth appeared on the control plate. Surface sterilized root, shoot, and leaf samples incubated on Nutrient agar and TSA for 24 h did not show any microbial growth indicating that bacterial isolates were endophytes.

Figure 1: Site of sampling

3.2. Isolation of endophytic bacteria from castor plant

To understand PGP potentials of endophytic bacteria under drought-stress, isolation of endophytes was done from different parts of plant with main focus on castor as a model crop for further studies. A total 26 endophytic bacteria were isolated from root tissues, leaf tissues and shoot tissues.

Plant Name	Plant Parts	No. of Isolated Bacteria	Name of Isolates
Ricinus communis	Leaves	Six	MGT1, MGT2, MGT3, MGT4, MGT5, MGT6
	Roots	Thirteen	MGT7, MGT8, MGT9, MGT10, MGT11, MGT12, MGT14, MGT15, MGT16, MGT17, MGT18, MGT24, MGT25,
	Stems	Seven	MGT13, MGT19, MGT20, MGT21, MGT22, MGT23, MGT26

Table 1: Endophytic bacterial isolated from different parts of *Ricinus communis*

3.3. Screening for drought tolerance

Growth of endophytic bacteria using PEG 6000 showed that out of 26 isolates, 22 could grow up to a minimum water potential of -0.001 MPa, whereas 18 could tolerate up to -0.27 MPa, and 13 up to -0.54 Mpa respectively. Among them 5 isolates showed highest tolerant up to -1.09 MPa.

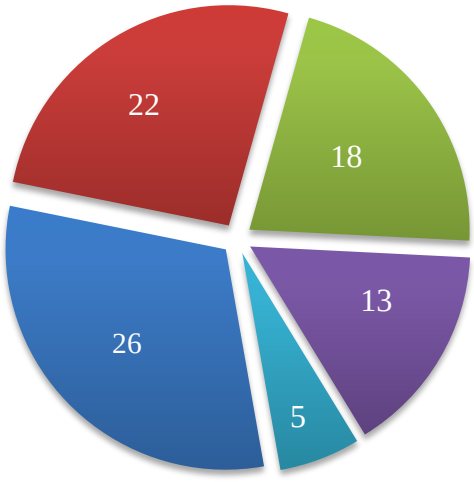

Figure 1: Growth of endophytic bacteria under different drought stress environments

3.4. Plant growth promoting activity under non-stress

Total five isolates which are able to grow under maximum drought stress (-1.09 MPa) are tested for further plant growth promoting activities. These isolates were good phosphate solubilizer and they showed zone of solubilization in the range of 20 mm to 29 mm. Similarly, IAA was produced by these isolates in the range of 37 to 50 µg/ml. The PGP trait ammonia was produced by all five isolates in the range of 27 to 48 µg/ml, where as siderophores was detected in three isolates within range of 27 to 46 µg/ml.

3.5. PGP properties under drought stress

Many literatures described PGP by bacteria under normal conditions but there is a little information on PGP properties under stress conditions particularly drought stress. The 5 promising isolates (MGT7, MGT9, MGT13, MGT16 and MGT19) tested for PGP properties under drought stress showed IAA production in range of 28 µg/ml to 47 µg/ml. P-solubilization in the range of 18 to 25 mm under drought stress (-1.09 MPa). Isolate MGT9 was the highest IAA producer (47 µg/ml) and MGT16 was the highest P-solubiler (27 mm) under drought stress respectively. MGT16 showed the highest ammonia production (44µg/ml). The isolates MGT16, MGT19 could show the siderophore whereas no isolate could show HCN production under drought stress.

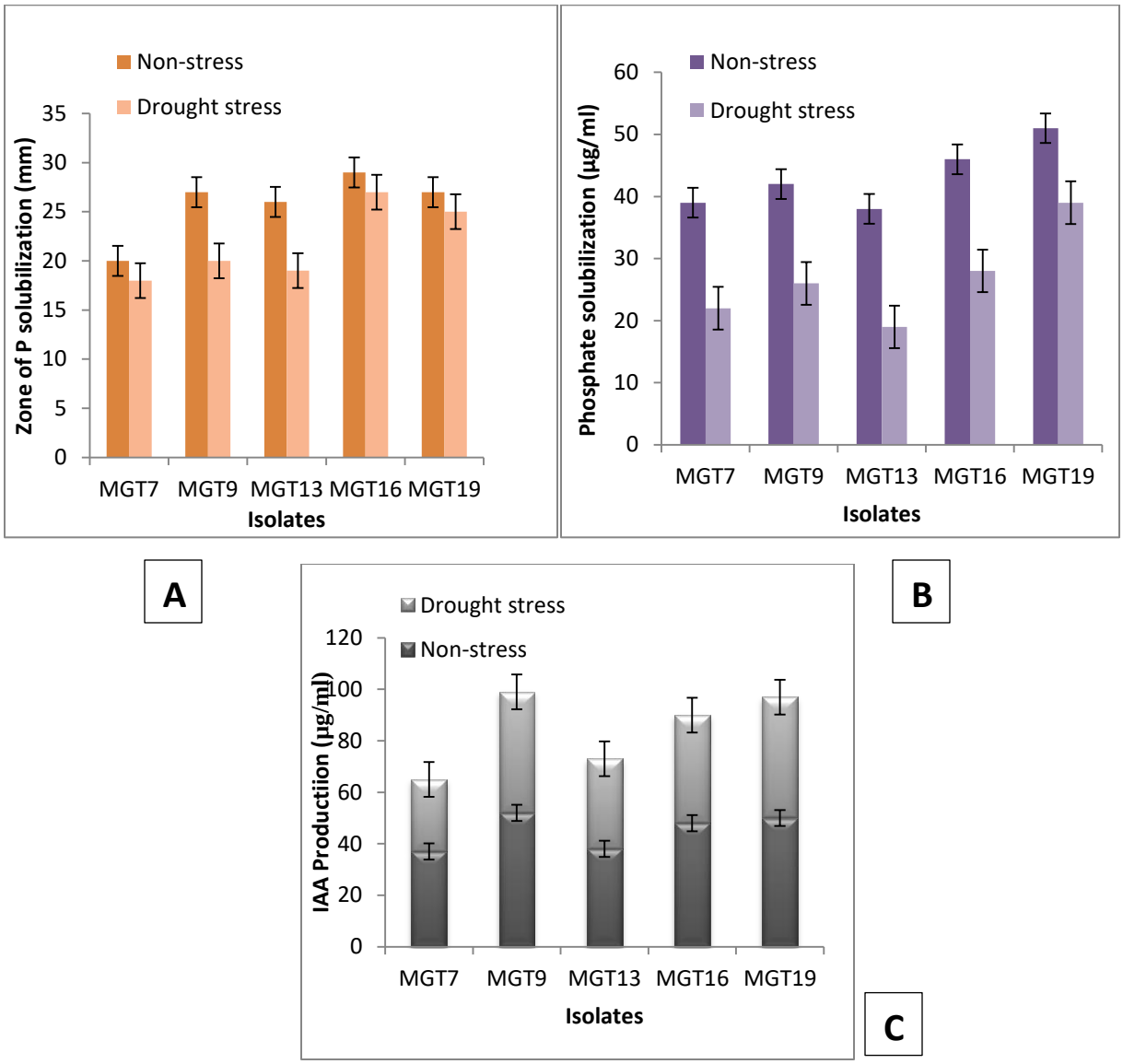

Figure 2: Qualitative study by the selected isolates under non-stress and drought stress condition (-1.09 MPa) (A) Zone of P solubilization (B) Quantitative study of Phosphate solubilization and (C) Indole acetic acid production

Sr. no.	Isolates	Siderophore production (µg/ml)		Ammonia production (µg/ml)		HCN Production	
		Non-stress	Drought stress	Non-stress	Drought stress	Non-stress	Drought stress
1	MGT7	ND	ND	32.68±0.61	27.50±0.41	-ve	-ve
2	MGT9	27.98±1.11	ND	41.95±0.90	32.97±0.59	-ve	-ve

3	MGT13	ND	ND	27.88±0.45	25.22±0.95	-ve	-ve
4	MGT16	46.64±0.36	42.09±0.52	48.77±0.66	44.97±0.89	-ve	-ve
5	MGT19	33.76±0.42	30.14±0.62	38.69±0.34	33.07±1.01	-ve	-ve

Table 2: plant growth promoting activities (siderophore, ammonia and HCN) of selected endophytes under non-stress and drought stress condition (-1.09 MPa); ND – Not Detected; -ve – negative results.

3.6. ACC deaminase activity

Further, the PGP endophytes that has ACC deaminase activity helps the plants to withstand both biotic and abiotic stress under non-stress and drought stress (-1.09 MPa). Isolate MGT9 and MGT16 showed maximum growth in negative, positive control as well as ACC incorporated medium.

Sr. no.	**Isolates**	**Negative control (OD at 600 nm)**		**Positive control (OD at 600 nm)**		**ACC (OD at 600 nm)**	
		Non-stress	**Drought stress**	**Non-stress**	**Drought stress**	**Non-stress**	**Drought stress**
1	MGT7	0.14±0.06	0.08±0.003	1.09±0.02	0.81±0.09	0.75±0.03	0.34±0.05
2	MGT9	0.32±0.005	0.10±0.04	2.88±0.05	1.82±0.02	1.86±0.04	0.99±0.06
3	MGT13	0.12±0.03	0.04±0.002	1.73±0.06	1.34±0.16	0.25±0.04	0.11±0.02
4	MGT16	0.24±0.06	0.13±0.012	2.14±0.04	1.48±0.07	1.50±0.05	0.87±0.05
5	MGT19	0.16±0.17	0.05±0.01	1.14±0.02	0.92±0.02	1.11±0.09	0.48±0.04

Table 3: Screening of bacterial isolates for 1-aminocyclopropane-1-carboxylic acis (ACC) deaminase and enzyme activity under non-stress and drought stress condition (-1.09 M Pa)

3.7. Growth curve

Growth pattern under non-stress and drought-stress (-1.09 MPa) of MGT7, MGT9, MGT13, MGT16 and MGT19 revealed that growth was less under drought compared to non-stress as shown by increase in mean generation time.

Growth curve of these five isolates were determined by spectrotrophic method. It was determined by inoculating early exponential phase culture in 50 ml broth under non-stress and drought stress condition. Sampls were withdrawn after every 4 h. mean growth rate constant (K) was calculated using formula: K=3.322 (logZt-logZ0)/ Dt; where Z0 and Zt are the final cell populations, while Dt is difference in culture time. All isolates were fast growing. K value of MGT7, MGT9, MGT13, MGT16 and MGT19 were 0.78±0.07,1.70±0.04, 1.48±0.09, 1.36±0.01 and 1.46±0.01 h^{-1} respectively. Similarly under drought stress condition, 1.27±0.06, 1.81±0.05, 1.63±0.05, 1.43±0.01, 1.53±0.03 h^{-1} respectivly.

Within this 5 isolates MGT7 and MGT16 have shown faster growth than the other isolates under drought conditions, which might be due to accumulation of osmolytes as osmoprotectants.

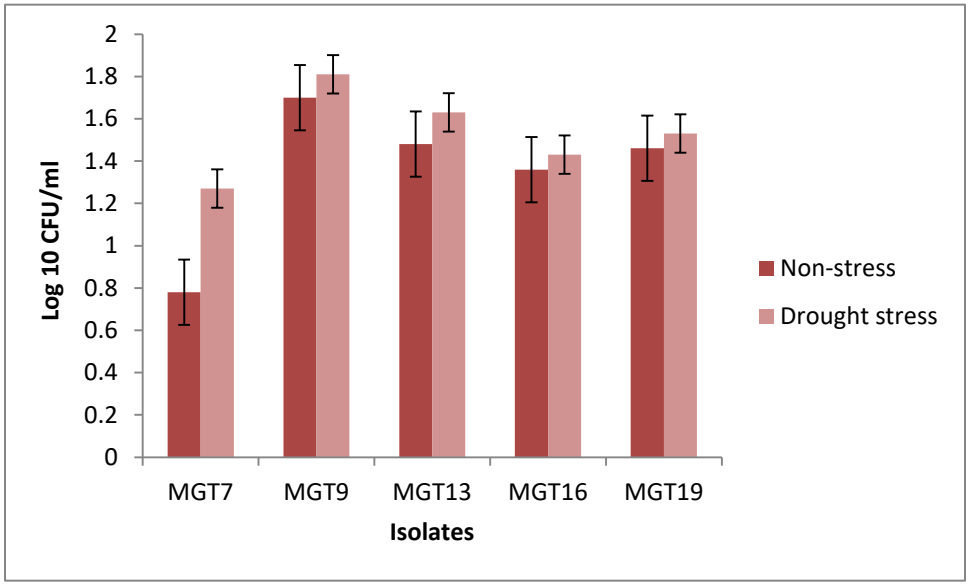

Figure 3: logarithmic growth studies of selected isolates under non-stress and drought stress condition (-1. 09 MPa)

3.8. Biochemical characterization

The morphology of the promising isolates MGT7 was a gram positive rod where MGT9, MGT13, MGT16, MGT19 were rod shaped and gram negative bacteria. According to Bergey's manual of determinative bacteriology, the physiology and biochemical charecteristics of selected isolates was determined.

No	Biochemical media	MGT7	MGT9	MGT13	MGT16	MGT19
1	Melonate	-ve	-ve	+ve	+ve	-ve
2	Voges proskauer's test	+ve	-ve	-ve	-ve	-ve
3	Citrate	+ve	-ve	+ve	+ve	+ve
4	ONPG	+ve	+ve	+ve	-ve	-ve
5	Nitrate reduction	-ve	-ve	+ve	-ve	+ve
6	Catalase	+ve	-ve	+ve	+ve	+ve
7	Arginine	-ve	-ve	-ve	-ve	-ve
8	Sucrose	+ve	+ve	+ve	-ve	+ve
9	Mannitol	+ve	+ve	-ve	-ve	+ve
10	Glucose	+ve	+ve	+ve	-ve	+ve
11	Arabinose	+ve	+ve	-ve	-ve	+ve
12	Trehalose	+ve	+ve	-ve	-ve	+ve
13	Urease	-ve	-ve	-ve	-ve	-ve
14	KOH test	-ve	+ve	+ve	+ve	+ve
15	Oxidase	+ve	-ve	+ve	+ve	+ve
16	Motility	+ve	+ve	+ve	+ve	+ve

Table 4: Biochemical study of selected endophytes

For molecular identification 16S rDNA was amplified and the product (~1.500 bp) was sequenced. Partial 16S rDNA sequences of the strains MGT7, MGT9, MGT13, MGT16 and MGT19 showed more than 90 percent identity to *Bacillus pumilus*, *Paraburkholderia megapolitana*, *Achromobacter xylosoxidans*, *Alcaligenes faecalis*, *Stenotrophomonas maltophilia* of the existing database of National Centre of Bioinformatics respectively (NCBI) and were submitted

to GenBank under the accession nos. MG734138, MG734139, MG734140, MG734141 and MG734142 respectively.

4. DISCUSSION

For endophytic bacterial isolation, surface sterilization is an important step, and sterility can be tested either by dipping the sterile root/seed into 0.85% of saline followed by spread plate method [22] or by incubation of surface sterilized root tissues on to the medium [23].

The density of the endophytic bacteria was more in root tissues, which is similar to the studies showing highest bacterial density in roots of 16 different monocot plants like oats, wheat, maize, rice and broccoli [24]. Typically, higher density of endophytes population is found mostly in plant roots and other below-ground tissues as compared to aboveground tissues [25].

Drought stress tolerance in bacteria has been studied to provide a biological understanding of the adaptation and survival of living microorganisms under extreme environments [26]. Under drought stress, bacterial cells accumulate small compatible solutes called osmolytes that include amino acids like glycine betane and sugars like sucrose, trehalose and polyglucosyl granules that improve cell growth under adverse osmotic conditions serving as osmoprotectants [12, 27].

IAA, an important auxin is required for plants to control important physiological processes including cell enlargement, division and tissue differentiation [28, 29, 30]. Endophytic *Bacillus* sp., *Bacillus subtilis* and *Pseudomonas putida* produced IAA [31, 29], similar to our study where not only isolates belonging to *Bacillus* spp. but also other species such as *Paraburkholderia*, *Alcaligenes*, *Stenotrophomonas* were producing IAA though there was variability in production among the isolates.

In a similar way as 15 endophytes out of 20 from strawberry showed phosphate solubilization in a range of 1-30 µg mg^{-1} protein that belonged to *Bacillus* and *Sphingopyxis* [31]. Endophytic bacteria belonging to the genra *Bacillus*, *Pseudomonas*, *Serratia* and *Enterobacter* solubilize the insoluble phosphate [29]. Phosphate solubilizing bacteria as inoculants increased phosphrous uptake by the plant and crop yield [32]. Endophytes, *Bacillus cereus* and *Achromobacter xyloxidans* from the roots of potato plant showed phosphate solubilization and IAA production when tested as bioinoculants for potato tubers, which significantly increased vegetative growth, photosynthetic pigments and N, P and K concentrations compared with control [33]. Endophytic bacteria, *Pseudomonas*, *Azospirillum*, *Azotobacter*, *Burkholderia*, *Bacillus* and *Serratia* enhanced the plant growth promotion [33, 34, 35] and endophytic *Bacillus* strains from different plants like *Cymoppisis tetragonaloba*, *Trianthema portulacastrum*, *Cyrprus rotaandus*, *Penisetum typhoides*, *Zea mays* and *Cassia angustifola* showed plant growth promoting effect on *Pennisetum typhoides* by producing PGP traits [36].

Siderophores, such as pyochelin and salicylic acid, chelate iron and helps in its uptake by the plants and also contribute to disease control by competing with phytopathogens for trace metals [37].

Similar to *Bacillus* sp., *Pseudomonas* sp., and *Stenotrophomonas* sp. from ginger showing the siderophore activity [38]. In case of HCN production, no isolates could produced HCN. By synthesizing HCN, some bacteria inhbit plant disease development and strengthen the ost's disease resistance mechanism [39, 40]. Some reports indicate that endophytic bacteria have the ability to produce HCN and helping in inhibiting plant disease development.

The ability of endophytic bacteria to produce IAA, HCN, siderphores and to solubilize phosphate as well as to show EPS production indicates that endophytes are important sources as plant growth promoting bacteria and biocontrol agents, though bacterial endophytes activity vary from plant to plant and species to species.

In this study, PGP properties were tested for the isolates at maximum water stress condition (-1.09 MPa). Production of PGP properties were compared both under normal and drought conditions and were found to be lower under drought stress, though certain bacteria were capable of producing higher amouts of PGP traits (IAA) under drought stress [41].

Endophytes *Burkholderia phytofirmans* strain PsJN and *Enterobacter* sp. FD17 showed PGP activity and drought stress tolerance [3]. Plant growth promoting endophytic bacteria *Pseudomonas* and *Bacillus* induced plant growth and resistance to water stress in green gram through plant growth promoting traits [42, 28]. Stress condition can result in the accumulation or increase of ROS level in plants like drought, access of water, high light, cold, salinity and heavy metals [43].

Increased mean generation time has been reported for the strains of *Pseudomonas* [44]. This might be due to the fact that under stress conditions energy flow of the

cells is directed towards protection, which might affect the growth pattern of the cells [44].

5. CONCLUSIONS

With these results we can conclude that all the 5 endophytes, belonging to different genera are potential endophytic strains for the plant growth promotion under drought stress. Isolate MGT9 *Paraburkholderia megapolitana* gives more efficient result in terms of plant growth promoting activities and ACC deaminase activity under non-stress as well as drought stress condition. However, the mechanism of the drought stress tolerance of these strains in plants is needed, for the proper elucidation of endophyte plant interaction under drought stress.

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