

Original Research Article

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Isolation, Screening and Characterization of Efficient Drought Stress Tolerant Fluorescent Pseudomonad Isolates from Groundnut (*Arachis hypogaea* L.) Rhizosphere

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ABSTRACT

Fluorescent pseudomonad bacteria, a major constituent of Rhizobacteria, enhance the plant growth through diverse mechanisms. The association between plant and fluorescent pseudomonad bacteria contributes to the successful thriving of plants in extreme environments featured by moisture stress. The enzyme has been noticed in a few numbers of bacteria and which helps the plant growth and development during biotic and abiotic stress situations with the depletion of stress levels of ethylene production in plant system. Fluorescent pseudomonads drawn broad attention as production of secondary metabolites, phytohormones, siderophores, enzymes, antibiotics, HCN and volatile compounds. They can be promoted in low input sustainable agricultural practices. The present experiment revealed that fifty-one efficient bacterial isolates obtained from drought prone rhizosphere soils of groundnut. Isolated strains were also tested for further drought stress screening by poly ethylene glycol 6000 at 0 % (-0.05 MPa), 10 % (-0.65 MPa), 20 % (-1.57 MPa), 30 % (-2.17 MPa) and 40 % (-2.70 MPa). Thirty-seven bacterial isolates were further found to have an enzyme 1-aminocyclopropane-1-carboxylate (ACC) deaminase activity which improved plant growth during stress conditions. Twelve isolates were found to be positive for IAA production. The positive isolates taken and screened for the presence of Fluorescent pseudomonads using King's B media.

Keywords

Rhizobacteria, ACC deaminase, α -ketobutyrate, Abiotic stress, Biocontrol, Pseudomonads, Phytohormones

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Introduction

The abiotic stress affects the plant-water relations at both cellular and whole-plant level, causing both specific and non-specific reactions and injury. Bacteria can withstand during stress conditions because of production

of exopolysaccharides, during water stress conditions (Bashan *et al.*, 2004). Field application of microorganisms having drought-tolerant ACC deaminase may influence the plant growth. The subject of PGPR elicited tolerance to abiotic stresses has been reviewed (Venkateswarlu *et al.*, 2008).

Some of the PGPR isolates buildup plant stress endurance by ACC deaminase enzyme can contribute significant conservation in a broad range of plants from the injuries caused by various abiotic stress situations. Ethylene synthesis by 1-aminocyclopropane-1-carboxylic acid breakdown by ACC-deaminase enzyme which decreases the loss of different stress situations by increasing physiological conditions in the plant roots, at an early stages of stress conditions (Ali *et al.*, 2009).

Pseudomonas sp. is present in wide agricultural soils and has many features that shows these bacteria will be best suited as PGPR. Important fluorescent species like *P. aeruginosa*, *P. fluorescens*, *P. putida* (Bhattacharyya and Jha, 2012), production of secondary metabolites like volatile compound hydrogen cyanide (HCN), phytohormones, and siderophores, improves plant growth by the increasing of indole-3-acetic acid (IAA), siderophores and antibiotics (Saber and Ramadan, 2015). Pseudomonads of 16S rRNA group I having the capability to produce soluble yellow-green pigment pyoverdines (PVDs) or pseudobactins (Bultreys, and Maraité, 2003) which act as siderophores. Fluorescent pseudomonads are considered as effective biological control agents across soil-borne plant pathogens because of their colonization with in the plant roots (Lugtenberg, Dekkers, 1999 and Bloemberg, 2001).

Materials and Methods

Soil samples collection

Forty-eight soil samples were collected from different villages of four mandals in the Anantapur (Upland) and Prakasam (Coastal) districts for the isolation of drought stress tolerant bacterial strains. The soil samples were mainly collected from groundnut

rhizosphere fields along with their GPS Coordinates. Crop plants were selected randomly in the field and the intact root system was dug out, carefully taken in plastic bags, labeled well and stored at 4 °C.

Isolation of fluorescent *Pseudomonad* isolates from different rhizosphere soils

For isolation of Rhizobacteria, the method proposed by Vlassak *et al.*, (1992) was followed. In this procedure, 10 g of soil from each soil sample was taken in a conical flask of 90 ml saline. The sample was agitated for 15 minutes on a vortex and serial dilutions of soil suspensions were prepared. 0.1 ml was spread on sterilized petri plates containing Nutrient agar media the petri plates were incubated at room temperatures ($28\text{ }^{\circ}\text{C} \pm 2\text{ }^{\circ}\text{C}$) for 24-72 h. Two replicates were maintained for each dilution. The plates were examined daily up to 3 days for bacterial colonies

Cultural characterization

The plates incubated for a day at $30 \pm 1\text{ }^{\circ}\text{C}$ were observed for the growth of fluorescent *pseudomonas* colonies on Nutrient agar media colonies were further confirmed by using KB plates and the colonies are enumerated manually and recorded. All the bacterial isolates were studied for their colony morphology, cell morphology (Gram reaction), pigmentation, spore production and biochemical characteristics according to the standard methods described in Bergey's Manual of Determinative Bacteriology (Holt *et al.*, 1994 and Cappucino, 1983).

Invitro screening of bacterial isolates for plant growth promoting traits

Drought tolerance

Trypticase soya broth (TSB) with different water potentials (-0.05, -0.15, -0.30, -0.49,

and -0.73 MPa) was prepared by adding appropriate concentrations of polyethylene glycol (PEG 6000) (Michel and Kaufmann 1973; Sandhya *et al.*, 2009) and was inoculated with 1% of overnight raised bacterial cultures in TSB. Osmotic potential of broth media was measured by a psychrometer. Three replicates of each isolate with each concentration were prepared. After incubation at 28 °C under shaking conditions (120 rpm) for 24 h, growth was estimated by measuring the optical density at 600 nm using a spectrophotometer.

EPS production

Bacterial strains grown on YMG agar medium were inoculated in YMG broth and preincubated at 25 °C for 24 h. 200 µl of culture broth was inoculated into 50 ml of YMG broth and incubated at 25 °C for 5 days at 120 rpm. Elimination of cells was followed by centrifugation (10,000 g for 20 min). The culture broth was mixed with 3 volumes of ethanol and after standing at 4 °C for 24 h, it was centrifuged (10,000 g, 4 °C, 20 min). The weight of the precipitated EPS was measured after drying at 80 °C for 3 days (Ali *et al.*, 2013).

Siderophore production

Siderophore production was estimated at qualitatively. Chrome azurol sulphonate (CAS) agar medium (Schwyn and Neilands, 1987) was used for the detection of siderophores, isolates were grown in synthetic medium, containing 0.5 µM of iron and incubated for 24 hrs on a rotary shaker at room temperature. CAS assay is used to detect the siderophores. The CAS plates were used to check the culture supernatant for the presence of siderophores. Culture supernatant was added to the wells made on the CAS agar and incubated at room temperature for 24 hrs. Formation of yellow to the orange colored

zone around the wells indicates siderophore production.

Indole acetic acid production

The production of Indole acetic acid was done according to Duby and Maheswari, 2012. LB broth was prepared and 24 hrs old cultures were inoculated into the broth and incubated at 28 °C for 72 h. After the incubation period, the cultures were centrifuged at recommended rpm and time. 2 ml of supernatant was collected into a test tube and two drops of O-phosphoric acid was added. Salkowski reagent was prepared and added into the test tube double the amount of supernatant. To prepare Salkowski reagent 0.4 gms of ferric chloride was added into 5 ml of distilled water and 17.5 ml of perchloric acid was added into 32.5 ml of distilled water and mixed the ratio of 1:150. Incubate the tubes for 30 min in dark. Development of pink color after the respective incubation period indicates the positive test for IAA production.

Screening for 1-Aminocyclopropane-1-carboxylate (ACC) deaminase activity

Screening for ACC deaminase activity of drought-tolerant PGPR isolates was done based on their ability to use ACC as a sole nitrogen source. All drought tolerant PGPR isolates were grown in 5 ml of trypticase soya broth (TSB) medium incubated at 28 °C at 120 rpm for 24 hrs. The cells were harvested by centrifugation at 3000 g for 5 minutes and washed twice with sterile 0.1 M Tris-HCl (pH 7.5) and resuspended in 1 ml of 0.1 M Tris-HCl (pH 7.5) and spot inoculated on petri plates containing modified DF (Dworkin and Foster) salts minimal medium 10 ml and distilled water 990 ml, supplemented with 3 mM ACC as sole nitrogen source. Plates containing only DF salts minimal medium without ACC as negative control and with (NH₄)₂ SO₄ (0.2 % w/v) as a positive control.

The plates were incubated at 28 °C for 72 hrs. Growth of isolates on ACC supplemented plates was compared to negative and positive controls and was selected based on growth by utilizing ACC as a nitrogen source (Honma and Shimomura, 1978).

Phosphate solubilizing activity

Phosphate solubilization activity was determined using Pikovskaya's agar medium containing 0.5 % (W/V) $\text{Ca}_3(\text{PO}_4)_2$ (Pikovskaya, 1948). Pikovskaya's agar plates were prepared and sterilized. The inoculums were spot inoculated on the pikovskaya's plate. 24 hrs old culture was used for the inoculation. The plates were incubated for 72-96 hrs at room temperature. The clear zone was observed around the spotted area after the incubation period.

Potassium releasing ability

Potassium solubilization determined using Aleksandrov medium containing 0.2 % potassium aluminum silicate (Prajapati and Modi, 2012). KMB media was prepared and sterilized. The 24 hrs old culture was spot inoculated on the KMB plates and incubated for 72 hrs at room temperature. Plates were observed for the clear zone around the spotted area after the incubation period.

Ammonia production

10 ml of peptone broth was prepared and sterilized. 24 hrs old culture was inoculated into the broth and incubated for 48 hrs at 30 °C on shaking conditions at 120 rpm. Nessler's reagent was prepared and 0.5 ml of Nessler's reagent was added into each test tube. Observation of faint yellow to the brown color in the tubes indicates the presence of ammonia production (Cappuccino and Sherman, 1992).

Results and Discussion

The serially diluted samples were plated onto Nutrient agar medium further confirmed by using KB media. The colony morphology of the isolates are glistening, smooth, small to medium, convex elevation and were tested further by gram reaction. Among these two bacterial isolates are gram-negative, rods as noticed under a microscope. Among total 12 isolates, two isolates showed yellowish green to light pigmentation, under fluorescent light. Morpho and cultural aspects of the bacterial isolates selected on KB medium and also recorded pigmentation about thirty colonies were selected, purified and were stored in the refrigerator at 4 °C. Our results agreement with Akter *et al.*, 2014 who isolated 325 bacteria and 14 of them were identified as fluorescent pseudomonads by morphological and biochemical characterization. Fifty *Pseudomonas fluorescens* and 28 *Rhizobium* isolates were isolated from rhizospheric soil and root nodules of redgram, identified and characterized biochemically as *Rhizobium* and *Pseudomonas fluorescens* (Basha *et al.*, 2014).

Morphological and cultural characteristics of isolates

Two fluorescent pseudomonas bacterial strains took about 48 h to show their growth on King's B agar media plate. Isolates are small size, round margin, white, dull white and smooth colonies. Isolates producing yellowish green to light green pigmentation, glistening, opaque, convex, viscid colonies. In the microscopic studies, these isolates exhibited gram -ve nature with single, isolated, rod-shaped cells with no endospores.

Biochemical characterization

Among two Fluorescent Pseudomonad bacterial isolates, R-14 isolate showed

positive results for Indole, Ammonia production, Carbohydrate utilization, gelatin hydrolysis, citrate utilization, Indole production, Methyl red test, Starch Hydrolysis and Voges prausker. Isolate K-17 showed positive result- for Ammonia production, Catalase test, Carbohydrate utilization, gelatin hydrolysis, citrate utilization, Oxidase test and Voges prausker (Table 3).

Plant growth promoting properties

In the table 5 among four bacterial isolates, two fluorescent isolates produced exopolysaccharide production. The isolates were also expressing positive results for siderophores production, ACC deaminase and production of indole acetic acid. Among four isolates, two isolates were showed as maximum PGP properties Among four efficient isolates two isolates i.e R-14 showed P-solubilization Index was (380.0 mm), followed by K-7 (433.3 mm), K-10 (412.5 mm) and K-17 (228.6 mm). Data on potassium solubilization activity show that

out of two isolates, R-14 showed highest solubilization zone (350.0 mm), followed by K-10 (316.7 mm), K-7 (283.3 mm) and K-17 (250.0 mm). ACC deaminase enzyme of bacterial origin might be the reduction of ACC deaminase levels in roots, as noticed in previous research. The higher expression of ACS may trigger ACC accumulation in the tissues of the plant, during the initial phase of stress induction. Therefore, the overall result of this cross-talk reveals that the role of IAA to enhancement of plant growth under drought stress. Previous studies revealed that the efficiency of IAA on root length elongation under a drought stress regime.

Recently, Sorty *et al.*, (2016) have reported the influence of bacterial IAA in seed germination and seedling growth in wheat under stress. Further, the phosphate-solubilizing bacterial isolates of drought agrosystems are likely to be more useful for plant health improvement under water deficit conditions.

Table.1 Physico-chemical characteristics of collected soil samples from Anantapur district Andhra Pradesh

S.No	District	Name of the mandal	Name of the village	pH			Electrical conductivity (dS m ⁻¹)		
				S ₁	S ₂	S ₃	S ₁	S ₂	S ₃
1.	Anantapur	Anantapur	Alamuru	6.26	6.70	6.41	0.11	0.13	0.08
			Kateganikalva	5.68	7.24	6.40	0.07	0.16	0.08
		Kambadur	Andepalli	6.10	5.35	8.07	0.11	0.06	0.22
			Pallur	6.18	7.89	8.13	0.06	0.17	0.20
		Gummagatta	Veerapuram	7.06	5.78	6.31	0.10	0.22	0.06
			Pulakunta	5.23	7.99	7.79	0.03	0.18	0.16
		Vidapanakal	Vidapanakal	8.11	8.26	8.36	0.11	0.06	0.22
			V.Kottakota	8.40	8.32	8.13	0.06	0.17	0.20

*Values with in the brackets are total rhizosphere soils collected from each village.

Table.2 Physico-chemical characteristics of collected soil samples from Prakasam district Andhra Pradesh

S.No	District	Name of the mandal	Name of the village	pH			Electrical Conductivity (dS m ⁻¹)		
				S ₁	S ₂	S ₃	S ₁	S ₂	S ₃
2.	Prakasam	Chirala	Thotavaripalem	7.49	7.17	6.43	0.27	0.21	0.32
			Kavurivaripalem	6.26	5.36	5.82	0.26	0.62	0.26
		Chinaganjam	Motepalli	7.21	6.82	6.41	0.49	0.33	0.45
			Kadavakuduru	5.99	6.11	6.29	0.30	0.22	0.22
		Kothapatnam	Ethamukkala	7.63	7.51	7.05	0.24	0.18	0.24
			Rajupalem	8.23	6.12	5.81	0.26	0.15	0.16
		Vetapalem	Akiapalem	5.48	6.52	6.53	0.16	0.16	0.17
			Kothapatnam	7.32	7.70	7.92	0.21	0.21	0.22

* Values with in the brackets are total rhizosphere soils collected from each village

Table.3 Biochemical Characteristics of effective drought stress tolerant Fluorescent pseudomonad isolates

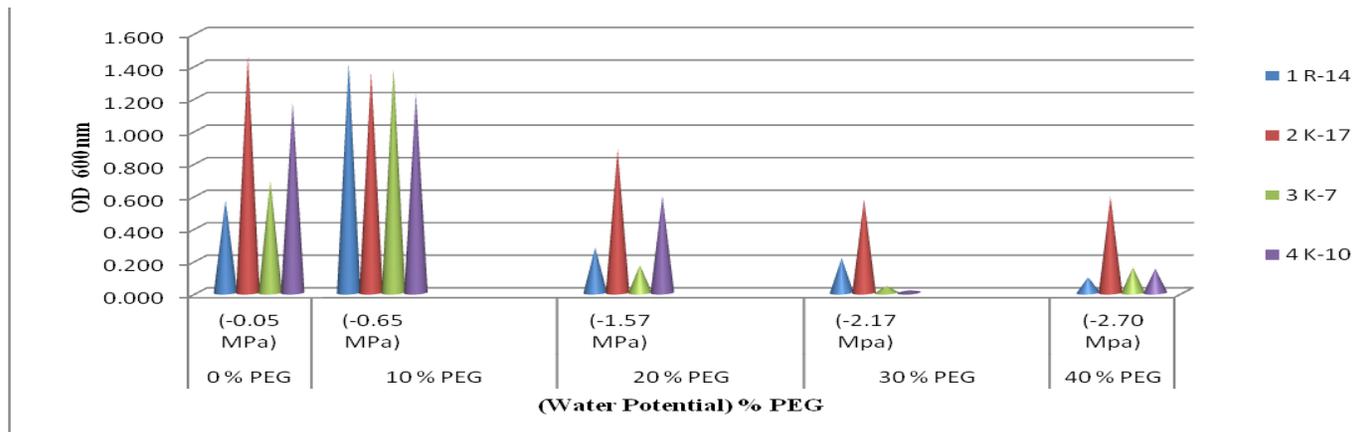
S.No		1	2	3	4
Isolate Name		R-14	K-17	K-7	K-10
Ammonia		+	+	-	+
Carbohydrate Fermentation	Dextrose	+	+	+	+
	Mannitol	-	-	+	-
	Sucrose	-	-	+	-
	Lactose	+	-	-	-
Catalase		+	+	+	+
Citrate		+	+	+	+
Gelatin		+	+	+	+
H₂S		-	-	-	-
Indole		+	+	-	+
Methyl red		+	-	-	+
Nitrate reductase		-	-	-	-
Oxidase		-	+	-	-
Starch Hydrolysis		+	-	+	-
Voges Prausker		+	+	+	+

Table.4 Morphological and cultural characteristics of fluorescent *Pseudomonads*

S.No	Isolate	Size	Margin	Colour of the colony	Elevation	Surface of the colony	Pigmentation	Gram Stain	Shape
1.	R-14	Small	Round	White	Convex	Smooth shiny	Yellowish green	-ve	Rods
2.	K-17	Small	Round	Dull White	Convex	Smooth shiny	Light green	-ve	Rods
3.	K-7	Small	Undulate	White	Crateriform	Glistening	No pigmentation	-ve	Cocci
4.	K-10	Small	Entire	Creamy White	Crateriform	Glistening	No pigmentation	-ve	Small rods

Table.5 Plant growth promoting attributes of efficient drought tolerant fluorescent pseudomonads

S.No	Isolate	Matric Potential					EPS Production (mg/ml)	Siderophore Production	IAA	ACC deaminase			P. Sol		K. Sol	
		(-0.05 MPa)	(-0.65 MPa)	(-1.57 MPa)	(-2.17 MPa)	(-2.70 MPa)				Control	Ab/Control	ACC	SI	% Pi	SI	µg/ml
1.	R-14	0.570	1.415	0.278	0.213	0.095	0.0	+	+	+	+	+	380.0	6.80	350.0	1625.0
2.	K-17	1.462	1.361	0.882	0.577	0.591	1.0	+	+	+	+	+	228.6	4.70	250.0	1375.0
3.	K-7	0.690	1.380	0.170	0.042	0.157	0.0	+	++	+	+	+	433.3	5.95	283.3	1625.0
4.	K-10	1.171	1.224	0.596	0.015	0.149	3.0	+	+	+	+	+	412.5	6.10	316.7	1437.5



Graph.1 Screening isolates with PEG 6000

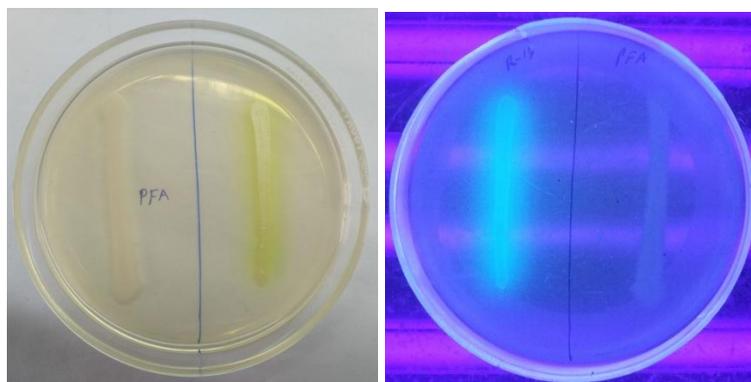


Fig.1 Fluorescent pseudomonas without UV and under UV Transilluminator

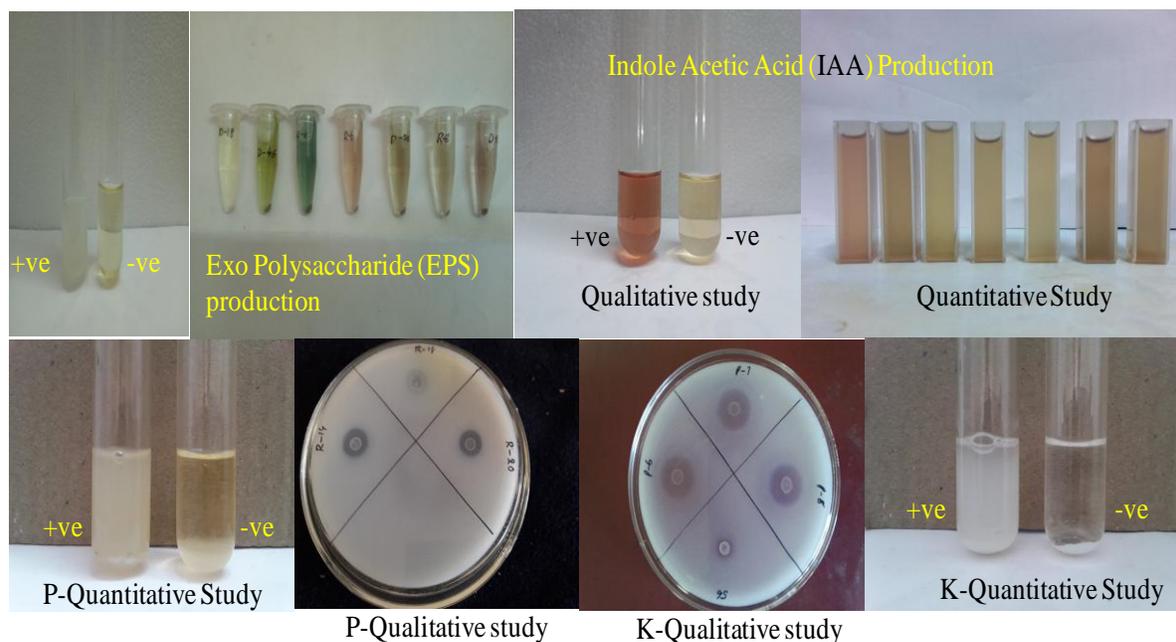


Fig.2 Plant Growth Stimulating Traits

Stress tolerance of fluorescent pseudomonad isolates

Table 5 shows drought tolerance ability of fluorescent *Pseudomonad* isolates revealed that, three isolates showed tolerance to water potential from -0.05 Mpa to -2.73 Mpa (0 % to 40 % PEG) R-14 recorded optical density of 0.570 at 0 % PEG (-0.05 MPa), 1.415 at 10 % PEG (-0.65 MPa), 0.278 at 20 % PEG (-1.57 MPa), 0.213 at 30 % PEG (-2.17 MPa) and 0.095 at 40 % PEG (-2.70 MPa). Isolate K-17 recorded as optical density of 1.462 at 0 % PEG (-0.05 MPa), 1.361 at 10 % PEG (-0.65 MPa), 0.882 at 20 % PEG (-1.57 MPa),

0.577 at 30 % PEG (-2.17 MPa) and 0.591 at 40 % PEG (-2.70 MPa). Isolates screened for drought tolerance (-1.2 MPa) showed positive stress tolerance in *Pseudomonad* strains when compared to other strains (Praveen *et al.*, 2014).

This study suggests that with the application of efficient isolates exhibit a high tolerance to abiotic stress. The peanut roots will have the source for rhizobacteria that are capable of directly protecting plants from drought stress. The above results suggested that positive ACC deaminase bacterial isolates and EPS-producing bacterial isolates are correlated

with groundnut crop could alleviate drought stress, EPS production by ACC deaminase-producing bacteria appears to increase the efficiency as PGP bacteria in stress situations, possibly by improving soil structure and bacteria survival.

The isolation and characterization of stress-tolerant fluorescent pseudomonad bacteria are not only essential for understanding their characteristics with in rhizosphere but also their utilization in eco-friendly and sustainable agro-technologies.

Besides these mechanisms, the inherent PGP traits of individual bacteria may provide an indirect mechanism for water stress alleviation in the tested plants by providing sufficient phosphate, iron, available nitrogen, and cross-protection against pathogen attack.

The use of such microbial consortium/consortia, which can induce drought stress tolerance and also buildup plant growth promotion during the normal condition, might be very much beneficial for sustainable agriculture.

The integrative application of such consortium having the characteristics of a suitable stresses suppressor is very important aspect for drought stress alleviation in other crops as well.

Moreover, the bacterial strain and their consortium formulation require further field evaluation and validation before being confirmed as bio-inoculants to combat various abiotic stresses in the acidic soil based agro-ecosystems.

The effective bacterial strains will go a long way in supporting the plant to develop both intrinsic and extrinsic ability to tolerate stressful conditions and sustain yield. In regard to isolates having PGP properties and

biocontrol activity, both the fluorescent pseudomonads having drought stress tolerance.

Isolates never showed commonly PGP properties, abiotic stress tolerance and biocontrol activities. *fluorescent pseudomonad* isolates screened from the research work presented could be screened further under *in vitro* and *in vivo* conditions from different soils with several crops for confirming their use as bioinoculants.

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