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MINING OF 1-AMINOCYCLOPROPANE-1-CARBOXYLIC ACID DEAMINASE EXHIBITING WATER STRESS TOLERANT NOVEL RHIZOBACTERIAL ISOLATES

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Abstract–An investigation was carried out to isolate, characterize and identify the rhizobacterial isolates of crop plants grown in the arid region of the North-eastern Karnataka.Twenty-one strains out of 120 rhizobacterial isolates that grew vigorously at-0.30Mpa were selected, and strains were biochemically characterized and screened *in vitro* to determine their water stress tolerance activity. Among 21 isolates, APPP-1 and GPPP-1 showed ACC deaminase activity (5.13 μMmg^{-1}/h and 2.69μMmg^{-1}/h, respectively). The isolate BSOP-1 produced higher proline (138.87μMmg^{-1}) and IAA (68.49 $\mu g\ ml^{-1}$); while NONB-2 produced higher exopolysaccharides (23.53mg mg^{-1} protein) at-0.30Mpa. Our study recorded the ACC Deaminase activity of the isolates, APPP-1and GPPP-1 later identified as a novel strain of *Pseudomonas* sp. VG309(NCBI accession number MH664930) and *Corynebacterium kroppenstedtii* (NCBI accession number MH671918) through 16SrDNA molecular sequencing technique.

INTRODUCTION

A Drought is a prolonged period of scanty rainfall. It occurs in arid and semi-arid regions where evapotranspiration exceeds precipitation. Water stress is one of the substantial components of abiotic stresses that limit crop productivity in agriculture. Plants are limited in their ability to protect themselves against drought stress due to their sessile nature. Thus, plants develop a wide range of strategies to cope with the stressful situation. Under conditions of water deficiency, drought escape and drought tolerance are two important mechanisms to ensure plant growth: Under the condition of mild water deficit, the plant could adapt through changes in molecular and physiological mechanisms, but have to pay the price in the form of reduced biomass yields (Osakabe *et al.*, 2014). A severe deficit of water may result in the arrest of photosynthesis, reduction in turgor, water potential, solute concentrations in the cytosol, and an increase in extra-cellular matrices and also lead to the inhibition of cell enlargement (Sakr *et al.*, 2018). Consequently, continuous accumulation of abscisic acid (ABA) and compatible osmolytes, overproduction of reactive oxygen species (ROS) result in wilting and finally plant death (Hossain and Dietz, 2016).

With the gambling nature of rainfall in agricultural crop production and a consistent increasing population, diminishing agricultural land holdings, and a rise in food demand, all these probes drive a new concept for water stress tolerance inducing traits. In this context, some rhizospheric bacteria have been shown to survive under water stress conditions by producing exopolysaccharides (Sandhya *et al*., 2009); this helps the bacteria to attach firmly and colonize the roots due to the network of fibrillar material that connects the bacteria to the surface of a plant, thereby aiding the plant to overcome water stress conditions. It has been observed that some of these rhizospheric bacteria exhibit 1-aminocyclopropane-1-carboxylate

deaminase (ACCD) activity which helps in the degradation of 1-aminocyclopropane -1-carboxylic acid (ACC), the immediate precursor of the plant hormone ethylene, into α-ketobutyrate and ammonia. This enzyme plays a significant role in reducing stress-induced ethylene production in plants and thereby sustaining plant growth. In addition to these properties, some microbes accumulate osmolytes and possess antioxidant properties (Jogawat, 2019). These plant growth-promoting bacteria would play a significant role in inducing water stress-tolerant traits in plants. If we can harness their unique properties of tolerance to extremities, their ubiquity, genetic diversity, and their interaction with crop plants, we can develop methods for their successful deployment in agricultural production.

MATERIALS AND METHODS

Soil sample collection

A total of 60 soil samples were collected from the rhizosphere of crop plants grown in the region of the North-Eastern Dry Zone of Karnataka. Soil samples from the rhizosphere of crop plants were collected by uprooting the plants upright, the bulk soil was removed by shaking the plants gently, and the roots were dipped in sterile normal saline. After bringing the soil samples to the laboratory, they were shaken for 30 minutes on a mechanical shaker.

Isolation of rhizobacteria

Ten grammes of rhizosphere soil were added to 90 ml of distilled water. The soil suspension was serially diluted, and the appropriate dilutions were poured onto T3 Agar plates (Tryptone 3g , Tryptose 2g, Yeast extract 1.5g, Na_2HPO_4 4g, NaH_2PO_4 3g, $MnCl_2$ 0.05g, Agar 20g, Distilled water 1000 ml, pH 7.2) and King's B Agar plates (Protease peptone 10g, K2HPO4 1.5g, MgSO4.7 H_2O 1.5g, Glycerol 15 ml, Agar 20g, Distilled water 1000 ml, pH 7.2) for isolation of *Bacillus* sp., *Pseudomonas* sp., respectively. After inoculation, plates were kept upside down for incubation at 37 °C for 24 hours. For each treatment, three replications were maintained. After a day of the incubation period, King's B plates were subjected to a UV illuminator and colonies of *Pseudomonas* sp. were picked based on their glowing ability under UV light. The colonies were picked up using a sterile loop and streaked in a quadrant fashion on King's B plates. For *Bacillus* sp. colonies were picked up based on morphology shown on T3 Medium and streaked in quadrant fashion on T3 Agar plates and stored at 4 °C for further studies.

In vitro screening for water stress tolerance

Trypticase Soy Broth (TSB) with various water potentials (-0.05,-0.15,-0.20,-0.30Mpa) were created by adding pertinent concentrations of polyethene glycol (PEG 6000). According to Michel and Kaufmann (1973) and Praveen Kumar *et al*. (2014), 326gm PEG 6000 in 1000 ml creates-1.2 Mpa. Based on this information, water potential in the Trypticase Soy Broth (TSB) media was created and inoculated with 1% of 18-hour old cultures of *Bacillus* sp. and *Pseudomonas* sp. After 24-hour incubation at 28 °C under shaking conditions (120 rpm), the growth of the isolates at different stress levels was estimated by measuring the optical density at 600 nm in a spectrophotometer. The growth of the isolates at induced stress levels was recorded.

Morphological and biochemical characterization of water stress tolerant rhizobacteria

All isolates were subjected to morphological characters such as Gram stain reaction and pigment production, whereas biochemical tests such as Catalase, Urease, Citrate utilization, Hydrogen sulphide (H_2S) production tests and starch hydrolysis tests were carried out for all water stress tolerant rhizobacteria.

In vitro screening of water stress tolerant isolates for ACC deaminase activity, exopolysaccharide (EPS) production, endogenous osmolytic accumulation of proline and Indole Acetic Acid (IAA) production was done.

ACC deaminase activity

All water stress tolerant rhizobacterial isolates were grown in 10 ml of TSB medium and incubated at 28 °C for 24 h at 120 rpm. The cells were obtained by centrifugation at 5000 g for 5 minutes, then 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 Dworkin and Foster salts minimal medium (Dworkin and Foster, 1958). Plates containing only DF salts minimal medium without ACC were considered as negative control and with $(NH\text{-}4)_2SO_4$ (0.2% w/v) as the positive control (Shaik *et al.*, 2013; Gupta and Pandey, 2020). The plates were incubated at 28 °C for 72 h. The growth of isolates on ACC

supplemented plates was compared to negative and positive controls and were selected based on growth on ACC supplemented medium.

ACC deaminase activity assay

ACC deaminase activity is determined by measuring the production of alpha ketobutyrate and ammonia produced by the cleavage of ACC by ACC deaminase (Tiwari *et al.,* 2018). Bacterial cells were obtained by centrifugation at 3,000 g for 5 minutes, then repeatedly washed with 0.1 M TrisHCl (pH 7.5) and then resuspended in 200 µl 0.1 M TrisHCl (pH 8.5). Lip the cells by adding 5% toluene (v /v), then vortex at a higher speed for 30 seconds. Incubate 50 µl of lipated cell suspension and 5 µl of 0.3 M ACC in an Eppendorf tube at 28 ° C for 30 minutes. The negative control for the test included 50 µl of lipated cell suspension without ACC, while the blank included 50 µl of 0.1 M TrisHCl (pH 8.5) and 5 µl of 0.3 M ACC. The sample was thoroughly mixed with 500 µl 0.56 N HCl, poured, and lipated at 10,000 g for 5 minutes to remove cell debris. Transfer a 500 µl aliquot of the supernatant to a glass test tube and mix with 400 µl 0.56 N HCl and 150 µl DNF solution (0.1 g 2,4-dinitrophenylhydrazine in 100 ml HCl 2 N), and then incubate the mixture at 28 °C for 30 minutes. Before measuring the absorbance at 540 nm, 1 ml of 2N NaOH was added to the sample.

The concentration of α-ketobutyrate in each sample was determined by comparison with a standard curve generated as follows: 500 µl α-ketobutyrate solutions of 0, 0.01, 0.05, 0.1, 0.2, 0.5, 0.75 and 1 mM were mixed respectively with 400 µl of 0.56 N HCl and 150 µl DNF solution. After adding one ml of 2N NaOH, the absorbance at 540 nm was measured as described above. The values for absorbance and α-ketobutyrate concentration (mM) were compared to construct a standard curve.

Exopolysaccharide (EPS) production.

The ability of test cultures of rhizosphere bacteria that can grow under higher stress levels to produce EPS was analysed (Niu *et al.,* 2018). In the absence of stress and a higher stress level (0.30 MPa), EPS was extracted from a 3-day culture raised in TSB (108.66 g PEG 6000 was added to 1liter of TSB to induce stress), and the test cultures were lipated at 10,000 g for 10 minutes and the supernatant was collected. Highly viscous cultures were diluted with 0.85 per cent potassium chloride before centrifugation. The pellet was washed twice with 0.85 per cent potassium chloride to obtain a complete extraction of EPS. Bradford's reagent was used to calculate the protein concentration in the supernatant (Bradford 1976). Then, the supernatant was then filtered through a 0.45 µm nitrocellulose membrane and dialyzed extensively with water at 4 °C. The dialysate was lipated at 10,000 g for 10 minutes to remove any insoluble material and mixed with 3 volumes of ice-cold absolute alcohol and kept overnight at 4 °C. The precipitated EPS was obtained by centrifugation (10,000 g for 10 minutes), suspended in water and further purified by repeated dialysis and precipitation processes.

Endogenous osmolytic accumulation of proline

In order to screen the accumulation of endogenous osmolyte proline, test cultures were grown in Luria Bertani broth (LB) containing different concentrations of PEG (0.05, 0.15, 0.20, and 0.30 MPa). The bacterial culture grown overnight in the broth was harvested by centrifugation at 2000 g for 5 minutes. The cells were weighed and suspended in 1 ml of sterile distilled water (SDW) and boiled at 100 °C for 20 minutes. The endogenous proline accumulation from the cell is determined by standard protocol (Grieve and Grattan, 1983; Sarma and Saikia, 2014).

Indole Acetic Acid (IAA) production

One hundred ml of King's B broth and T_3 broth supplemented with 5mM L-tryptophan and PEG 6000 to induce water stress and without adding PEG 6000 was prepared to compare the production of IAA. Each content was sterilized in 250 ml Erlenmeyer flask. The overnight grown test cultures were inoculated into those flasks and incubated at 37°C for seven days in the dark. For each isolate, three replications were maintained. The cultures were lipated at 6000 rpm in a refrigerated centrifuge at 4 °C and the supernatant was collected in a Erlenmeyer flask and used for estimation of IAA. The intensity of the pink colour developed was read at 535nm on a spectrophotometer. From a standard graph prepared with a known concentration of IAA, the Indole acetic acid produced by the water stress tolerant isolate was estimated by following the method described by Madhuri (2011) and Kang *et al.* (2019) and expressed as micrograms ml^{-1} of the medium.

Molecular identification of ACCD exhibiting water stress tolerant rhizobacterial Isolates

DNA was isolated from the ACC Deaminase

positive isolates. The sequence of the 16S rDNA gene sequence was performed by XcelrisTM, and the gene sequence was analysed by BLAST and a phylogenetic tree constructed using Clustal W.

RESULTS AND DISCUSSION

Isolation of rhizobacteria from rhizosphere soil of crop plants

A total of 120 rhizobacteria were isolated from 60 rhizosphere soil samples collected from different crop plants onT_3 media and King's B media, respectively. Discrete bacterial colonies on these media were picked up and purified on their respective growth media. These isolates were further streaked on T_3 and King's B slants, respectively, and stored in the refrigerator for further studies.

In vitro screening for water stress tolerance of rhizobacterial isolates

Out of 120 isolates, 80 isolates could grow in higher water potential (-0.30Mpa) conditions. Among 80 isolates, 21 isolates recorded a higer OD value at -0.30 Mpa (Fig. 1). The 21 isolates were coded as,ACOP-3, APPP-1, NONP-2, GCOP-1, GPPP-1, GAMP-1, GSOP-1, GPOP-1, GCHP-1, DAMP-1, DBIP-1, DBRP-1, DRAP-2, GPPP-2, BPPP-1, BPPP-2, BSOP-1, YGGP-1, APPB-2, NONB-2, YCOB-1

Morphological and biochemical characteristics of water stress tolerant rhizobacterial isolates

All the 21 water stress tolerant rhizobacterial isolates were subjected to morphological and biochemical studies (Table 1).

Plates containing DFsalts minimal mediumwith only $(NH_4)_2SO_4$ (0.2% w/v) as positive control. Without ACC, $(NH_4)_2SO_4$ (0.2% w/v) was considered as negative control.

ACC deaminase activity assay

The isolates which were positive for ACCD were subjected to quantification of ACC by measuring the α-ketobutyrate concentration (mM) at 540 nm in a spectrophotometer. The higher α-ketobutyrate activity of 5.13 µM mg^{-1} protein was recorded with the isolate APPP-1 (Fig. 2), whereas GPPP-1 recorded α-ketobutyrate activity of 2.69 µMmg^{-1} protein.

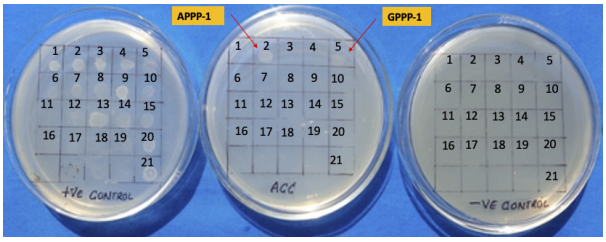

Fig. 2. ACC deaminase positive water stress tolerant rhizobacterial isolates

The accumulation of ethylene in stressed plants is well documented (Jackson, 1997; Gibbs *et al.*, 2015). Under stress conditions, the endogenous ethylene production is accelerated substantially, which adversely affects the root growth and, consequently, the growth of the plant as a whole. Besides, ethylene also induces plant defense responses which help to

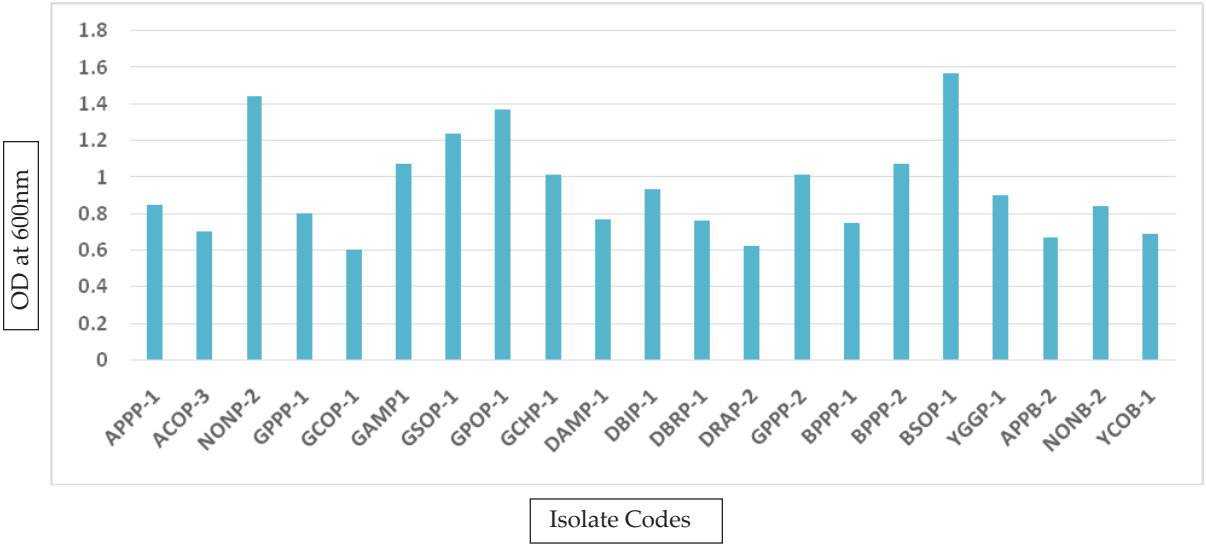

Fig. 1. *In vitro* screening of rhizobacterial isolates for water stress tolerance at -0.30 Mpa

enhance the survival of the plant under adverse conditions (Abeles, 1973; Verma *et al*., 2016). The relationship between this gaseous hormone and microbial mediated water stress alleviation is provided by the enzyme 1-aminocyclopropane-1-carboxylate (ACC) deaminase, which cleaves ACC, the precursor molecule of ethylene, and therefore lowers the level of ethylene in a stressed plant (Honma and Shimomura, 1978).

ACC deaminase is a multimeric enzyme with a monomeric subunit molecular mass of approximately 35–42 kDa. It is a sulfhydryl enzyme that utilizes pyridoxal 5-phosphate as an essential cofactor (Singh *et al*., 2015). ACC deaminase has been found in a wide range of Gram-negative bacteria (Saleem *et al*., 2007; Orozco-Mosqueda *et al*., 2019), Gram-positive bacteria (Saghafi *et al*., 2020), endophytes (Afridi *et al*., 2019), and fungi (Nascimento *et al.,* 2014).

Exopolysaccharide (EPS) production

The isolate NONB-2 produced a higher EPS both under non stress conditions (5.81 mg mg^{-1} protein). and stress (39.20 mg mg^{-1} protein) Whereas, NONP-2 recorded least EPS of 1.29 mg mg^{-1}protein under non stress and 8.70 mg mg^{-1} protein at higher stress conditions (Fig. 3).

The EPS has a high affinity for water content. Hence, it doesn't allow the soil to dry up easily and it has a significant effect on the formation of biofilms, whereby aggregation of microorganisms and soil particles takes place (Banerjee *et al.,* 2019). By holding the water content in its surrounding soil media, it creates a congenial micro environment for the soil ecosystem and this could be the mechanism through which an efficient EPS producing bacteria significantly enhances the water stress tolerant capacity of the crop plants (Costa *et al*., 2018; Vardharajula, 2021).

During our study, we observed curiously the pattern of EPS production both in stressed conditions and non-stressed conditions. We came to the conclusion that the EPS production is directly proportional to the stress in the media

Endogenous accumulation of proline

At-0.30 Mpa water potential, the isolate BSOP-1 accumulated higher proline of 138.87 $\mu g g^{-1}$ followed by the isolate NONP-2 with 97.57 $\mu g g^{-1}$ of proline (Fig 4).

The ability of microbial endurance under a stressed environment is primarily due to the

Table 1. Morphological and biochemical characteristics of water stress tolerant rhizobacterial isolates

Sl. No.	Isolates	Gram reaction	Pigment production	Catalase activity	Urease activity	Citrate utilization	Starch hydrolysis	Gelatine liquefaction	H_2S production
1	ACOP-3	-ve	+	+	-	+	-	-	-
2	APPP-1	-ve	+	+	-	-	-	+	-
3	NONP-2	-ve	+	+	-	-	-	-	+
4	GCOP-1	-ve	+	+	-	-	-	-	-
5	GPPP-1	-ve	+	+	-	+	-	+	+
6	GAMP-1	-ve	+	+	+	+	+	+	+
7	GSOP-1	-ve	+	+	+	+	-	+	+
8	GPOP-1	-ve	+	+	-	+	-	-	+
9	GCHP-1	-ve	+	+	-	+	-	+	+
10	DAMP-1	-ve	+	+	-	+	-	+	-
11	DBIP-1	-ve	+	+	-	+	-	+	-
12	DBRP-1	-ve	+	+	-	+	-	-	-
13	DRAP-2	-ve	+	+	-	+	-	-	+
14	GPPP-2	-ve	+	+	-	+	-	-	+
15	BPPP-1	-ve	+	+	-	+	-	+	+
16	BPPP-2	-ve	+	+	-	-	-	-	-
17	BSOP-1	-ve	+	+	-	+	-	-	+
18	YGGP-1	-ve	+	+	-	+	-	+	-
19	APPB-2	+ve	-	+	-	+	-	-	-
20	NONB-2	+ve	-	+	-	+	-	+	+
21	YCOB-1	+ve	-	+	-	+	-	-	-

Note: +; Positive for the test -; Negative for the test***ACC deaminase activity***
Two out of 21 isolates showed positive for ACCD activity **(Fig. 2)**

production efficiency of different osmolytes, such as proline, glycine betaine, as well as L-amino acids and their D-isomers (Shahjee *et al.*, 2002). The production of different osmolytes formulates a microenvironment inside the bacterial cell mass and thereby protects the bacterial cell from further desiccation during a stressful environment (Mukhtar *et al.*, 2020; Rupak and Ratul, 2013). Similar to the microbial synergy during stressful environments, plant systems also respond to water-deficit conditions by stimulating their osmolyte production and thus maintaining the osmotic potential inside their cellular environment (Farooq *et al.*, 2009). Enhancing the rate of glycine betaine production in the plant due to PGPR was reported by earlier workers (Yuwono *et al.*, 2005).

Indole Acetic Acid (IAA) production

Nine out of 21 isolates were positive for IAA production. Among the nine isolates, BSOP-1 produced the highest amount (68.49 µg ml^{-1}) of IAA (Fig. 5) whereas thelowest was recorded by DBIP-1 (12.40 µg ml^{-1}).

Naturally occurring substances with an indole

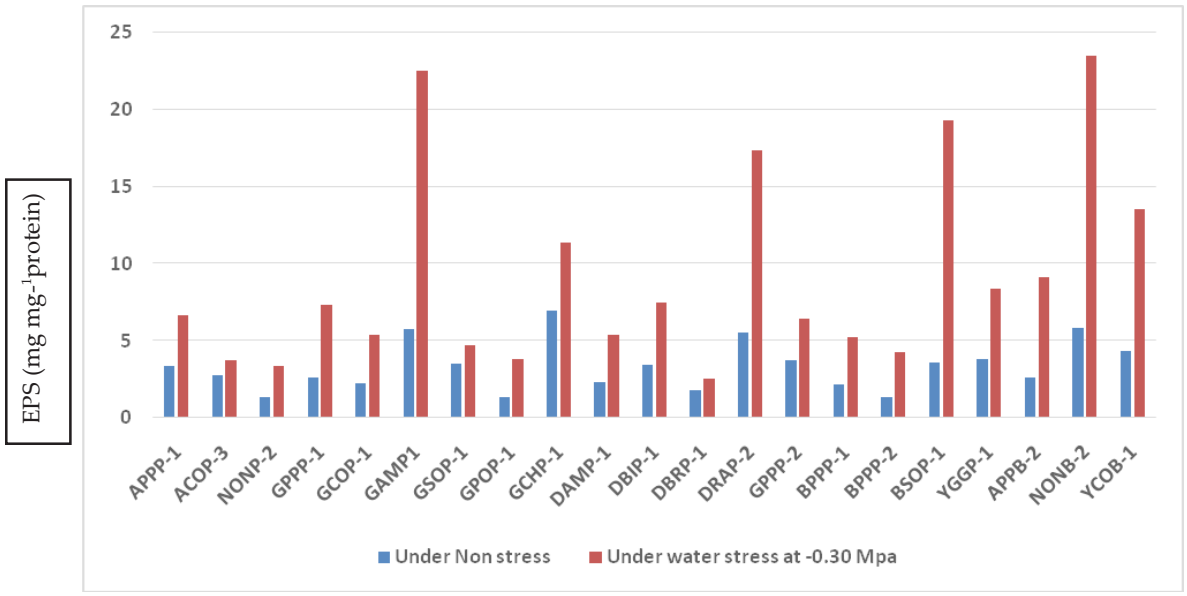

Fig. 3. Exopolysaccharide production potential of water stress tolerant rhizobacterial isolates

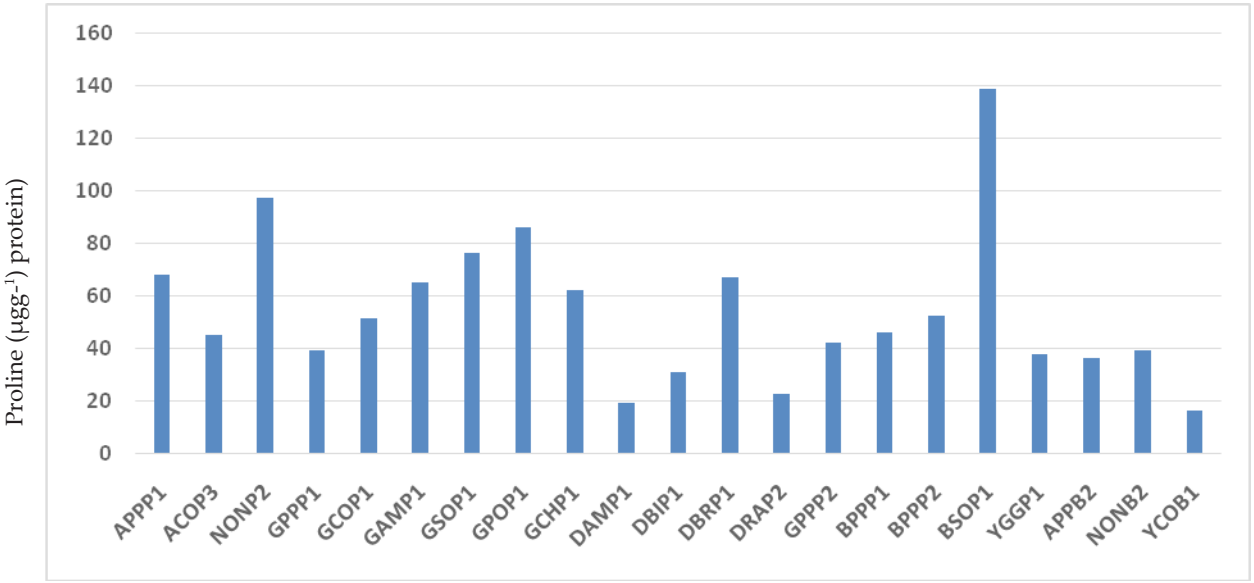

Fig. 4. Endogenous prolineaccumulationin water stress tolerant rhizobacterial isolates

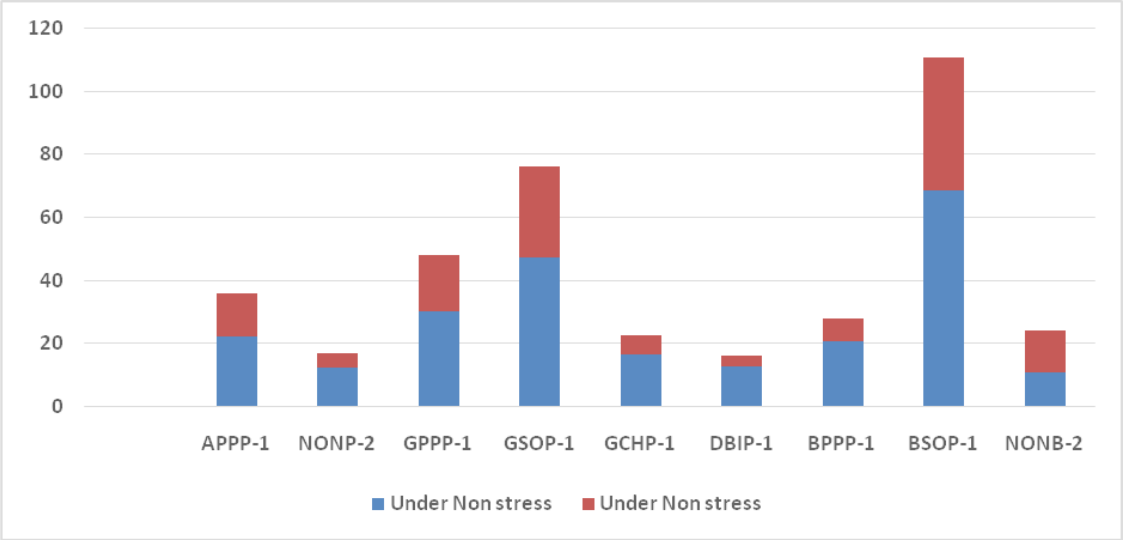

Fig. 5. *In vitro* IAA production potential of water stress tolerant rhizobacterial isolates

nucleus possessing growth-promoting activity are referred to as auxins. Chemically, it is Indole acetic acid that has the ability to synthesize plant hormones and is widely distributed among plant associated bacteria. Eighty per cent of the bacteria isolated from the plant rhizosphere produce IAA (Mwajita *et al.*, 2013). According to Halda-Alija (2003), up to 74% of rhizobacteria were identified and tested to produce IAA. The action and interaction of growth regulators regulate most of the physiological activities in plants.

The plant growth regulator Indole Acetic Acid (IAA) has long been postulated to play a key role in one or more aspects of nodule growth and development, and the detection of increased levels of IAA in nodule tissue revealed the hypothesis (Ferguson *et al.*, 2003) that the in vitro performance of the IAA strain produced provides significant results *in vivo* too. In our study, the isolate BSOP-1 produced a significant amount of IAA in both stressed conditions and non-stressed conditions.

Molecular Identification of ACC Deaminase Exhibiting Activity of Water Stress Tolerant Rhizobacterial Isolates

Isolates, APPP-1 and GPPP-1 were identified as novel strain of *Pseudomonas* sp. VG309 (NCBI Accession No. MH664930.1) and *Corynebacterium* sp. identified through sequencing and phylogenetic analysis of the 16S rDNA gene (Fig. 6 and 7). The quality was tested on 0.8% Agarose Gel. A fragment of *16S* rDNA was amplified by PCR using 8F and 1492R. An amplicon band of a single individual PCR of 1500 bp was observed. The PCR amplicons were purified and sequenced using 704F and 907R primers with the BDT v3.1 Cycle sequencing kit on

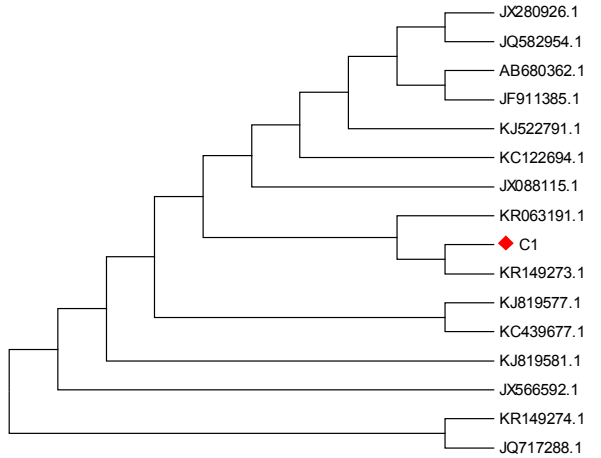

Fig. 6. Phylogenetic relationships among different *Pseudomonas* species along with C1 (*Pseudomonas* sp. VG309)

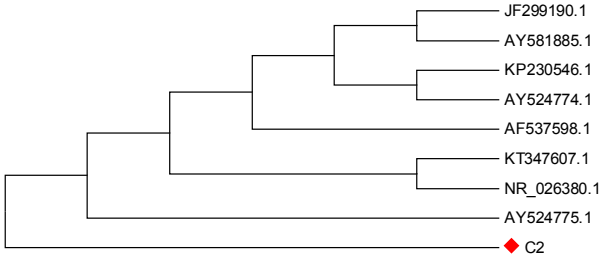

Fig. 7. Phylogenetic relationships among different *Corynebacterium* species along with C2 *Corynebacterium kroppenstedtii*

an ABI 3730xl Genetic Analyzer. 1332 bp 16S rDNA consensus sequences were generated from forward and reverse sequence data using the aligner software. The 16S rDNA sequences were used to carry out the BLAST alignment search tool of the NCBI gene bank database. Based on a higher identity score first Fifteen sequences were selected and aligned using the multiple sequence alignment software programs Clustal W. the distance matrix was generated using the RDP database and the phylogenetic tree was constructed using MEGA5.

Through our research we could able to isolate a novel strain of *Pseudomonas* sp. VG309 and our findings suggest that screening for ACCD positive rhizobacteria along with its multiple plant growth promoting activities under water stress conditions can be a boon for plant growth. Such novel species can be exploited based on further evaluation to reveal the efficiency of rhizosphere bacteria PGP in soil-plant systems.

Conflict of interests: None

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