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Alleviation of Drought Stress Using ACC (1-amino cyclopropane -1-carboxylate) Deaminase Producing Plant Growth Promoting Rhizobacteria (PGPR) in *Cyamopsis tetragonoloba*

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Abstract

Use of efficient PGPR isolates with ACC deaminase activity is an added advantage for mitigating adverse effects of drought. Two selected isolates, *Acinetobacter pitti* and *Rhizobium leguminosarum* was used for evaluation of drought stress alleviation in *Cyamopsis tetragonoloba*. Results indicate that both the isolates were able to make the plant survive even under severe drought conditions (12.5% water holding capacity) as evidenced from different plant growth parameters evaluated. Photosynthetic machinery including chlorophylls, leaf's, leaf area and relative water content (RWC) was found significantly increased in plants treated with the isolates. Antioxidants, both enzymatic and non-enzymatic were evaluated as indicators for alleviation of drought. Total proline and sugars, Ascorbate peroxidase (APOX), Guiacol peroxidase (GPOX), superoxide dismutase (SOD), catalase (CAT), Malondialdehyde (MDA), Nitrate reductase (NR), Glutathione reductase (GR) was estimated for their active involvement in scavenging reactive oxygen species (ROS).

***Key words*:** ACC-deaminase, PGPR, Cyamopsis, Drought stress-alleviation, ROS

Plant rhizospheres are naturally inhabited by plant-specific microorganisms in response to root exudates [1]. Rhizosphere is an active zone of many metabolic activities that influence plant growth and health [2]. The amendment of the rhizosphere with potentially beneficial microorganisms (PGPR) can help in overcoming natural disasters like drought. Drought is shown to be the most influential disaster effecting on agricultural productivity [3-4]. Drought in crop plants results in severe consequences like decreased leaf size, stem elongation, root development, cell division, water and nutrient uptake, decreased productivity and efficiency of water usage [5]. This may still increase in the coming years owing to global climate change [4-6].

Plant ethylene levels are important for plant development and stress response for normal plant development. An optimum level of ethylene is required. However, under stressed conditions, ethylene levels become high, leading to stunted root elongation and leaf senescence. Certain rhizobacteria produce an enzyme, ACC Deaminase, to curb the ACC (1 amino cyclopropane 1 carboxylate) precursor of ethylene to α- keto butyrate and ammonia. These bacteria therefore arrest the adverse effects of high levels of ethylene. Certain rhizosphere bacteria have the ability to enhance plant growth, called plant growth promoting rhizobacteria (PGPR) [7]. They are found to have traits like the production of hormones, hydrolytic enzymes, siderophores, HCN etc., PGPRs with the ability to alleviate drought stress are an added advantage.

Further, recent reports indicate that these bacteria could also make the plant scavenge reactive oxygen species (ROS) produced during drought stress, as evidenced by the production of peroxidases Nitrate reductases, catalase, malondialdehyde etc. [8]. An attempt was made in the present work to evaluate drought stress amelioration in *Cayamopsis tetragonoloba* using ACC deaminase-positive PGPRs. *Cayamopsis tetragonoloba* (cluster bean) is a tall herb, bushy, hardy annual legume that grows in sandy, arid/semiarid soil with deep rooted system and has a natural resistance to drought and salinity [9]. This plant is known for its various applications due to galactomannan from endosperm in industries [10]. The rhizosphere of this plant may be the vicinity of many potential drought-resistant PGPRs.

MATERIALS AND METHODS

Isolation of PGPR

In view of earlier studies, bacteria were isolated from the Rhizosphere soils of cluster beans from different locations in the Mahabubnagar district of Telangana state. Three different media: Yeast Extract Mannitol Salt agar (YEMA), Jensen's agar, and Kings - B media were used.

Evaluation of PGPR traits

Purified isolates were evaluated as per standard methods for PGP traits: The production of Indole acetic acid (IAA) was estimated by using salkowaski method [11]. The phosphate solubilisation was determined by the formation of a hallow zone around the bacterial colony on Pikovskaya's medium [12]. The siderophore activity was detected by using CAS (Chrome azurol S agar) medium [13]. The HCN production ability was identified by colour change of filter paper reaction [14].

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Gibberellic acid estimation was done as per the method given by Graham and Thomas [15]. Nitrogen fixation can be known by growing the bacterial cultures on nitrogen free medium.

Evaluation of ACC deaminase activity

The selected isolates with more number of PGP traits were inoculated on DF salt agar medium amended with 1-Aminocyclopropane – 1- Carboxylate (ACC) as nitrogen source. Quantitative estimation of ACC Deaminase activity assessed under drought stress conditions (-1.03 Mpa, -1.76Mpa) by measuring the production of α-keto butyrate. The enzyme activity was expressed in µmoles of KB / g/mg of protein/hr [16].

Evaluation of drought resistance

The selected isolates with a higher number of PGP traits along with ACC- Deaminase activity was checked for their ability to grow in 40% PEG-6000 [17-18]. Broth cultures of 24 hours were inoculated into nutrient agar amended with different percentages (10%, 20%, 30% and 40%) of PEG-6000. Optical density at 600nm was recorded.

Molecular characterization

The selected isolates are obtained in pure cultures and grown in nutrient broth (30-32°C/ 24 h). The log phase broth is set for isolation of genomic DNA by using Genomic DNA Extraction kit (Thermo-scientific) by using standard protocol [19]. By using universal primers (27F and 1492R) of 16 S r RNA amplification of gene (~ 1500bp) is carried out by PCR and a similarity search was done in NCBI database by using BLAST search tool to calculate pair wise sequence similarities for identifying its closest relative.

Plant experiments

Plant experiments were done in a greenhouse as a factorial experiment. A complete randomised block design with 3 replications of each treatment was maintained. Experimental treatment includes 75, 50, 25 and 12.5% water holding capacity (WHC) of soil used. The water-holding capacity of the ground was previously measured as per standard procedures [20]. Two controls were maintained. Control 1: Without PGPR + drought and 2: With PGPR + Drought. Seeds of cluster beans coated with respective bacterial cultures using carboxy methyl cellulose, as adhesive were sown in pots of size (6-inch diameter) and 4 kgs of soil. Initially, pots were well watered up to WHC until 4 leaf stage was obtained. At this stage, pots were subjected to drought treatments as per [21]. Plants were carefully excavated after 50 days after sowing and plant morphological, non-enzymatic and enzymatic, physiological parameters were measured for analysis.

Morphological traits

After 50 days of sowing the plants were harvested, the morphological traits (Shoot length, root length, number of leaves, leaf area, fresh shoot weight, dry shoot weight, fresh root weight, dry root weight, root volume) were measured and dry weights were obtained by drying samples in hot air oven at 70°C for 24 - 48 hrs.

Chlorophyll content

Estimation of Chlorophyll a, b and total was done by the method proposed by [22]. One gm of fresh leave sample cut into small discs was placed in test tube with 5 ml of dimethyl sulphoxide (DMSO). After incubation the absorbance was measure in a spectrophotometer at 645nm and 663nm with DMSO as Blank.

$$\text{Chlorophyll a (mg/g)} = (12.7 \times A663) - (2.59 \times A645)$$
$$\text{Chlorophyll b (mg/g)} = (22.9 \times A645) - (4.7 \times A663)$$
$$\text{Chlorophyll total (mg/g)} = (8.2 \times A663) + (20.2 \times A645)$$

Soluble carbohydrates

One gram of fresh leaves was ground with ethanol-chloroform- water (60:25:15v/v) mixture and incubated at 60^0C/2 hours. The samples were centrifuged at 10000 rpm/ 30mins. Supernatant (0.2ml) made up to 1 ml with distilled water and mixed with 1 ml of 5% phenol and 5 ml of 96% H_2So_4. Properly mixed aliquots were measured for OD at 490 nm against glucose standard curve [23].

Relative water content (RWC)

One gram of fresh leaves cut into discs and soaked in 25 ml of distilled water for 6 hours. The leave samples were blot dried and weighed. Leaves were then oven dried (70^0C for 24 hours) and weight was recorded [24].

$$\text{Relative water content} = \text{Fresh weight} - \text{Dry Weight} / \text{Saturated weight} - \text{Dry weight} \times 100$$

Ammonia production

Nessler's reagent (0.5 ml) is added to each tube of freshly grown broth cultures. Appearance of light-yellow colour (+), Deep yellow to brown colour (++) indicates production of ammonia [25].

Protease production

Formation of a halo zone surrounding the cultures plated onto the Skim milk agar medium and incubated at 30°C for 4-5 days indicate protease production [26].

β – 1, 3 – glucanase assay

The isolates were grown in 250 ml conical flask containing peptone medium with 0.2% laminarin and incubated for 4 days at 28°C. Later the cultures were centrifuged at 10000 rpm/30 min at 4°C. Supernatant (0.75 ml) is mixed with 0.3 ml of phosphate buffer (pH 5.5) and 0.5 ml of 2% laminarin and incubated (40°C, 2 hrs). Glucanase activity is determined by spectrophotometric evaluation [27].

Chitanase production

The isolates were grown in 250 ml conical flask containing 50 ml of chitin - peptone medium and incubated for 4 days at 28°C. After incubation the cultures are centrifuged (10000 rpm, 30 min) at 4°C. Supernatant (0.25 ml) was mixed with 0.3 ml of sodium acetate buffer (pH 5.3) and 0.5 ml of 0.1% Colloidal chitin and incubated at 50°C / 4 hours in water bath. Development of purple colour on addition of DNS reagent and Rochelle salt solution indicates the presence of chitinase enzyme [28].

Total proline

The acid ninhydrin method as described by Bates *et al*. [29] was used for estimation of total proline accumulated in leaves of cluster bean [29].

Lipid peroxidation or malondialdehyde (MDA)

Davenport et al developed a method for estimation of malondialdehyde (MDA). One gram of fresh leaves of cluster bean was grinded with 10 ml of 0.25% thiobarbutric acid (TBA) in 10% TCA. The mixture was incubated at 95°C, 30 min in water bath and immediately kept in ice bath, centrifuged (10000 rpm, 15 min) at 25°C. Optical density was read at 532 nm and 600nm [30].

MDA (µ moles/g of FW) = (Abs 532 – Abs 600) / Extinction coefficient × 1000

Evaluation of antioxidant enzyme activities

In a pre- chilled mortar add one gram of liquid N_2 – treated leaves a homogenate was made with 5 ml of 50 mM potassium Phosphate buffer (pH- 7) added with EDTA, $MgCl_2$, β – mercaptoethanol and 1% polyvinylpolyrrolidone (PVPP). Homogenate was centrifuged at 6000 rpm/20 mins at 4°C and the supernatant collected for estimation of Super oxide dismutase (SOD) by Beauchamp and Fridovich [31]. The APX was measured by Nakano and Asada [32] method. Guaicol peroxidise was assayed by Castillo *et al*. method [33].

Catalase

The estimation of catalase is carried out by a method proposed by Aebi. To 0.1 ml of enzyme extract, 1.5 ml of 100 mM Phosphate buffer (pH 7.0), 0.5 ml of 75mM H_2O_2 and 950 µl of distilled water. OD at 240 nm was measured [34].

Nitrate reductase

Plant leaf (0.5g) was crushed in 1 ml of 50mM potassium phosphate (pH8.0) buffer consisting of 1mM EDTA, 25 mM cystein, and 3% (w/v) BSA. The mixture was grinded and centrifuged at 12000 rpm at 40°C / 20 min. The supernatant was used as enzyme sample. A mixture of 200µl of 50mM potassium nitrate, 200ul of 0.5 mM NADH, 400µl enzyme extract 1200ul 50mM potassium phosphate buffer (pH 7.0) was made and incubated for 15 min/ 25°C. The absorbance was read at 540 nm after pink colour complex has formed [35].

Glutathione reductase

A mixture of 10 mM potassium phosphate buffer, 0.5 mM 5, 5 dithiobis-2-nitrobenzoic acid (DTNB), 2.0 mM NADPH, 0.33 mM EDTA, 0.1 ml of enzyme extract, and 20mM GSSG (Oxidised Glutathione) was made. Double distilled water was used to make up the solution to 3 ml. Optical density was read at 412 nm after development of red colour in tubes [36].

Statistical analysis

All experiments were repeated twice with at least three replicates for each treatment. The data pertaining was subjected to statistical analysis using Fischer's one-way ANOVA at $P \leq 0.05$. Values in the data was compared with each other using LSD at $p \leq 0.05$.

RESULTS AND DISCUSSION

A total of fifteen isolates were isolated from different rhizosphere soils of *Cyamopsis tertragonoloba* growing in Mahabubnagar district, Telangana State. These isolates were obtained on three different media Congo red YEMA, Jenson's agar and King's B agar. Later all the isolates were evaluated for the presence and absence of plant growth promoting (PGPR) traits along with ACC deaminase activity. A perusal of the (Table 1) shows that most of the selected isolates were positive for ACC deaminase activity. Out of 15 isolates IS-5, IS-6, IS-11, IS-12, IS-17 IS-18, IS-22 and IS-28 were observed to be having more number of PGPR characters. More the number of traits is taken as criterion for efficient PGPR.

Table 1 Characterization of PGPR traits of selected isolates from *C. tetragonoloba*

Rhizospere soil location of Mahabubnagar	Media	Isolates No.	IAA	Siderophore	N_2 fixation	P' solubilization	HCN	GA	ACC deaminase
Ammapalli	CR-YEMA	IS-04	--	+	--	--	--	--	+
	Jenson	IS-5	+	+	+	+	+	+	+
	Kings - B	IS-6	+	+	--	+	--	--	+
Avancha	CR-YEMA	IS-10	--	+	--	--	--	--	+
	Jenson	IS-11	+	+	+	--	--	--	+
	Kings - B	IS-12	+	+	--	+	--	--	+
BRR degree	CR-YEMA	IS-16	--	--	--	--	--	--	--
college	Jenson	IS-17	+	+	+	+	--	--	+
	Kings - B	IS-18	--	--	--	--	--	--	+
Burgula	CR-YEMA	IS-22	+	+	+	+	-	+	+
	Jenson	IS-23	--	--	--	--	--	--	--
	Kings - B	IS-24	+	--	--	--	--	--	+
Gangapur	CR-YEMA	IS-28	+	+	--	--	--	--	+
	Jenson	IS-29	--	--	--	--	--	--	--
	Kings - B	IS-30	--	+	--	--	--	--	+

Table 2 Optical densities (600nm) of selected rhizosphere isolates grown in media amended with PEG

Isolate No	Polyethyleneglycol				
	0%	10%	20%	30%	40%
IS-5	1.305	0.845	0.297	0.108	**0.020**
IS-6	1.125	0.573	0.218	0.098	0.018
IS-11	1.237	0.978	0.318	0.048	0.000
IS-12	1.370	0.746	0.426	0.125	0.015
IS-17	0.998	0.734	0.170	0.040	0.000
IS-18	0.845	0.643	0.193	0.051	0.015
IS-22	1.523	0.933	0.306	0.162	**0.092**
IS-28	0.901	0.732	0.405	0.058	0.000

Selected 8 isolates were further evaluated for their ability to withstand the presence of 40% polyethylene glycol (PEG 6000). Optical densities of bacterial growth at different percentages of polyethylene glycol (PEG) are shown in (Table 2). Data shown in the table indicates that all the selected isolates were able to grow at 30% PEG though the readings were low.

Comparatively IS-5 and IS-22 were found withstanding even 40% PEG.

IS-5 and IS-22 selected for their PGPR characters and resistance to 40% PEG were further evaluated for retention of PGPR traits even under 40% PEG. Table 03A depicts the results pertaining to efficacy of these strains to express PGPR traits even in the presence of 40% PEG. Both the isolates at 40% PEG were able to show PGPR traits IAA, Gibberellic acid, 'P' Solubilization and ACC deaminase. The quantity produced was comparatively less than control. However, both the strains could not show production of HCN, Ammonia, Chitinase, Protease and β-1,3 glucanase as represented in (Table 3B). Both the isolates were also evaluated for their plant growth promotion activity in plant experiment.

Table 3A PGPR traits expressed at 40% PEG by selected isolates

PGPR trait	IS-5			IS-22				
	Control	30% PEG	40% PEG	Control	30% PEG	40% PEG	CD at 0.05	CD at 0.01
Indole acetic acid (mg/ml)	11.54	6.48	0.68	12.96	3.86	0.31	5.40	7.47
Gibberellic acid (mg/L)	112.6	9.86	2.21	22.6	2.86	0.64	43.82	60.60
P Solubilization (mg/100ml P_2O_5)	30.46	12.86	5.24	6.42	0.26	0.04	11.49	15.89
ACC deaminase activity in µmol α-KBA (mg/h)	8.76	ND	1.96	7.23	ND	0.96	3.88	5.37

Table 3B PGPR traits expressed at 40% PEG by selected isolates

PGPR trait	IS-5		IS-22	
	Control	40% PEG	Control	40% PEG
HCN	++	+	--	--
Siderophore	+	+	+	+
Ammonia production	+	+	--	--
Chitinase	+	--	--	--
Proteaase	+	--	--	--
b-1,3 glucanase	--	--	--	--

Data depicted in (Table 4) shows data pertaining to the drought experimentation using IS-5 and IS-22. A critical perusal of the data indicates that both the isolates were able to make the plants withstand severe drought stress (12.5% FC). Recent studies in corn [37] wheat, bean, lettuce [38-39] reported the usage of microbial fertilizers with drought tolerance could enhance nutrient uptake of plant and thereby help in amelioration of plant parameters. Control plants subjected to drought stress and without IS-5/IS-22 could not survive drought beyond 50% FC. It clearly indicates the ability of isolates to make the plant survive drought conditions. In general, with the increase in drought condition there is a significant decrease in different plant parameters. There was no significant difference between the control plants and plants subjected to drought with IS-5 and IS-22 up to 50% WHC. Later in 25% and 12.5% FC significant decrease of plant parameters was observed. The root to shoot ratio is directly proportional to nutrient supply and fertilization. High values of this ratio indicate a possibility to absorb more nutrients and thereby increase the resistance to stress [40-41]. Highest root shoot ratio was seen in plant treated with IS-5 isolate compared to IS-22. Compared to control plants, significant decrease in plant parameters was not observed up to 50% WHC in plants treated with IS-5 and IS-22.

Table 4 Effect of selected PGPR isolates for plant growth promotion in *Cyamopsis tetragonoloba* subjected to drought stress

Isolate No.	WHC %	Shoot height (mm)	Root length (mm)	Shoot wt. wgt. (gm)	Shoot dry wt. (gm)	Root wt. wgt. (gm)	Root dry wgt. (gm)	Root volume (cm^3)	Root shoot ratio
IS-5	75	14.7^{a}	12.2^{ab}	8.12^{a}	1.06^{a}	0.414^{abc}	0.176^{ab}	0.39^{a}	$0.16(23.58)^{b}$
	50	13.4^{ab}	8.4^{c}	6.26^{abc}	0.94^{ab}	0.268^{cd}	0.134^{b}	0.3^{ab}	$0.14(21.97)^{b}$
	25	8.7^{dc}	4.6^{d}	$4.52c^{cd}$	0.56^{c}	0.204^{ed}	0.047^{c}	0.24^{b}	$0.08(16.43)^{b}$
	12.5	5.2^{d}	2.2^{d}	1.83	0.24^{c}	0.096	0.036^{c}	0.12^{c}	$0.15(22.79)^{b}$
IS-22	75	13.9^{a}	9.4^{cb}	7.63^{ab}	1.07^{ab}	0.422^{ab}	0.144^{ab}	0.36^{a}	$0.13(21.13)^{b}$
	50	12.8^{a}	4.6^{d}	5.28^{bc}	0.82^{bc}	0.314^{bcd}	0.118^{b}	0.3^{ab}	$0.14(21.97)^{a}$
	25	9.6^{cb}	3.9^{d}	2.86^{d}	0.62^{c}	0.102^{e}	0.056^{c}	0.21^{bc}	$0.09(17.46)^{b}$
	12.5	4.2	2.4^{d}	1.08	0.32^{c}	0.014	0.008^{c}	0.14^{c}	$0.02(8.13)^{ab}$
Control	FC	16.2^{a}	15.6^{a}	8.26^{a}	1.24^{a}	0.524^{a}	0.198^{a}	0.6	$0.15(22.79)^{b}$
C.D at 0.05		4.02	3.54	2.56	0.32	0.15	0.06	0.10	5.03
C.D at 0.01		5.48	4.82	3.49	0.44	0.20	0.09	0.13	6.85

*Values are significant at P<0.05 as per Fisher's test, values super scribed by same alphabet are not significantly different P<0.05, Values in the parenthesis are properly transformed values before subjecting to ANOVA analysis

Similar pattern was also seen in number of leaves, leaf area, chlorophyll, total sugars and relative water content (Table 5). Earlier studies indicate that net photosynthetic rate, leaf relative water content, shoot water potential, starch and soluble proteins, total chlorophyll, nitrate reductase activity decreased significantly with the increase in drought [42]. From the data it can be inferred that use of both the isolates IS-5 and IS-22 were able to ameliorate the effect of drought significantly. Compared to control chlorophyll content was high in isolate treated plants subjected to drought. Even severe drought has less effect on chlorophyll content in presence of both IS-5 and IS-22. Total soluble sugars observed more in plants inoculated with the isolates and gradually decreased with the increase in severity of drought. With the increase in stress, amino acid synthesis also decreases and plants have reduced ability to synthesize new proteins and mitigate stress effects. In general, proline content

increases in plant to overcome damage to membranes in tissues [43]. In the present investigation a gradual increase of proline content was observed with the increase in drought severity due to inoculated isolates. There was no significant difference among proline content compared to control plant indicating the effect of isolates in overcoming adverse effects of damage due to drought. Relative water content in general decreased with the increase in drought severity. IS-5 and IS-22 could make the relative water content retain in even severe drought (12.5% FC) conditions 12.85% and 14.83% respectively. Similar observations were made earlier where relative water content (RWC) is considered as most effortless variable to monitor drought tolerance in plants [44].

Table 5 Effect of selected PGPR isolates for plant growth promotion in *Cyamopsis tetragonoloba* subjected to drought stress

Isolate No.	WHC %	No. of leaves (Plt^{-1})	Leaf area (mm^2)	Chlorophyll A (mg g^{-1} FW)	Chlorophyll B (mg g^{-1} FW)	Total chlorophyll (mg g^{-1} FW)	Total soluble carbohydrates (mg g^{-1} DW)	Relative water content (%)
IS-5	75	30.23^{a}	2964.32^{a}	9.88^{a}	3.42^{a}	31.64^{a}	69.28^{ab}	$56.28\ (34.25)^{ab}$
	50	28.64^{a}	2733.86^{ab}	7.64^{ab}	1.64^{bc}	20.28^{b}	52.14^{cd}	$43.52\ (25.80)^{bcd}$
	25	26.28^{ab}	2289.62^{bc}	4.28^{c}	1.02^{bc}	15.42^{bc}	38.92^{de}	$18.32\ (10.26)^{ef}$
	12.5	16.66^{d}	1093.28	1.87^{c}	0.76^{c}	10.62^{c}	30.74^{e}	$12.85\ (7.38)^{f}$
IS-22	75	29.46^{a}	2968.32^{a}	9.44^{ab}	2.64^{a}	30.87^{a}	65.66^{ac}	$52.24\ (31.49)^{abc}$
	50	22.32^{bc}	2487.6^{abc}	6.28^{bc}	1.27^{b}	20.76^{b}	59.18^{bc}	$41.86\ (24.75)^{cd}$
	25	20.47^{cd}	2066.84^{c}	4.21^{c}	1.08^{b}	15.28^{bc}	44.82^{d}	$30.72\ (17.89)^{de}$
	12.5	18.62^{cd}	1897.96^{c}	1.32^{c}	0.95^{c}	9.64^{c}	39.64^{ed}	$14.83\ (8.53)^{ef}$
Control	FC	45.64	4768.24	8.96^{ab}	1.87^{b}	32.06^{a}	76.28^{a}	$64.68\ (40.30)^{a}$
C.D at 0.05		5.15	618.18	3.18	0.93	8.20	13.53	10.32
C.D at 0.01		7.01	841.38	4.33	1.26	11.16	18.42	14.05

*Values are significant at P<0.05 as per Fisher's test, values super scribed by same alphabet are not significantly different P<0.05, Values in the parenthesis are properly transformed values before subjecting to ANOVA analysis

Plants exposed to most of the abiotic stresses result in increase of oxidation stress by highly toxic reactive oxygen species which cause damage to DNA, protein, lipid and carbohydrate [45]. To scavenge reactive oxygen species formed during drought condition, several antioxidant enzymes could be observed. Earlier reports indicate that there was a gradual increase in SOD, APX, CAT and GR with the increase in drought [46]. SOD is able to convert O_2^- to H_2O_2 and O_2; H_2O_2 is reduced further to water by APOX/GPOX using the substrate as electron donor in chloroplast. Both SOD and APOX/GPOX are considered most significant antioxidant in plants to scavenge ROS [47-48]. In the present investigation a gradual increase in APOX, GPOX was observed with the increase in drought. No significant difference was observed between control APOX, GPOX and plants treated with isolates IS-5 and IS-22 at 50% WHC (moderate drought) (Table 6).

Table 6 Effect of selected PGPR isolates on antioxidants of *Cyamopsis tetragonoloba* subjected to drought stress

Isolate No.	WHC %	APOX (Unit mg^{-1} protein)	GPOX (Units mg^{-1} protein)	SOD (Units mg^{-1} protein)	Catalase (Units mg^{-1} protein)	MDA (n mol g^{-1} FW)	NR (mg$N0_2$ g^{-1} h^{-1})	GR (m mol NADPH $min^{-1}g^{-1}$FW)	Total proline (uMg^{-1})
IS-5	75	3.97^{a}	0.94^{b}	4.47^{a}	1.8^{c}	16.9^{ab}	1632^{c}	4.2^{b}	584.29^{a}
	50	4.22^{ab}	1.26^{b}	4.92^{a}	1.64^{bc}	18.4^{b}	743^{a}	3.3^{b}	592.1^{a}
	25	5.62^{b}	1.3^{c}	5.36^{b}	1.08^{a}	18.8^{b}	525^{a}	1.5^{a}	620.16^{a}
	12.5	ND	1.39^{c}	5.82^{c}	0.94^{ab}	19.2^{b}	298^{a}	ND	640.28^{a}
IS-22	75	3.54^{a}	0.92^{b}	4.31^{a}	1.27^{b}	14.7^{a}	1528^{c}	4.6^{bc}	570.84^{a}
	50	5.17^{b}	1.1^{b}	4.78^{a}	1.05^{b}	17.8^{b}	1044^{b}	2.9^{b}	592.27^{a}
	25	6.8^{b}	1.28^{c}	5.2^{b}	0.93^{a}	18.3^{b}	978^{b}	1.6^{a}	620.42^{a}
	12.5	4.16^{a}	ND	5.48^{bc}	0.62^{a}	17.4^{b}	826^{ab}	0.9^{a}	680.76^{b}
Control	FC	2.68^{a}	0.47^{a}	4.26^{a}	1.97^{c}	16.7^{a}	1424^{bc}	6.8^{c}	576.28^{a}
C.D at 0.05		1.99	0.49	0.51	0.38	1.39	449.74	1.63	34.98
C.D at 0.01		2.70	0.66	0.69	0.52	1.89	612.13	2.21	47.61

*Values are significant at P<0.05 as per Fisher's test, values super scribed by same alphabet are not significantly different P<0.05, Values in the parenthesis are properly transformed values before subjecting to ANOVA analysis

Nitrate reductase (NR) is involved in nitrogen assimilation, acquisition of nutrients by plants under drought stress can be evaluated by measuring NR activity. It is an indicator of physiological status of plant and therefore used to understand plant physiology under drought stress [49]. Control plants were observed to have more NR activity than treated plants. It decreased gradually with the increase of drought but without much significant difference. Similar is the pattern observed with Glutathione reductase. GR preserves the balance of redox potential in cells achieved by interconversion of reduced and oxidized glutathione in plants which in turn helps in scavenging ROS [48]. MDA (intercellular) is considered as stress biomarker, lowest level in control plants and increased levels in stressed plants was earlier reported [61]. A gradual increment of MDA was observed in the present investigation with the increase of drought. Similar results were recently reported in *Phaseolus vulgaris* subjected to salinity stress by using ACC deaminase producing PGP bacteria [8]. The selected isolate IS-5 and IS-22 were identified by 16S rRNA gene sequencing and phylogenetic analysis. The selected two ACC deaminase positive PGPR isolates were showing 95-100% similarity with the known sequences in NCBI gene bank and were identified as IS-5: *Acinetobacter pitti* (submitted sequence accession number: OR593308) and IS-22: *Rhizobium leguminosarum.* The relatedness of these isolates with other strains was evaluated with MEGA X software (Fig 1 a-b).

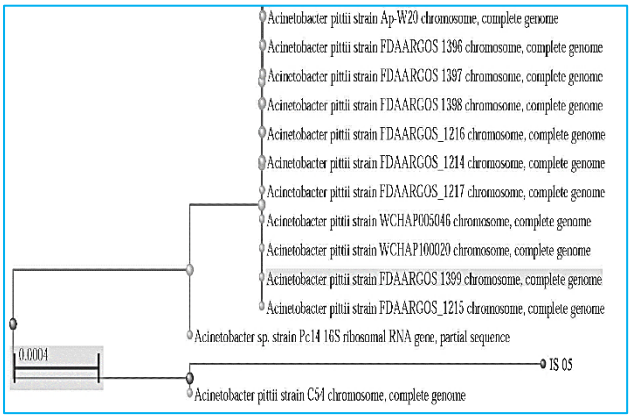

Fig 1 Phylogenetic dendrograms based on 16SrRNA nucleotide partial sequence showing relationship with closely related taxa. IS 05: *Acinetobacter pitti*

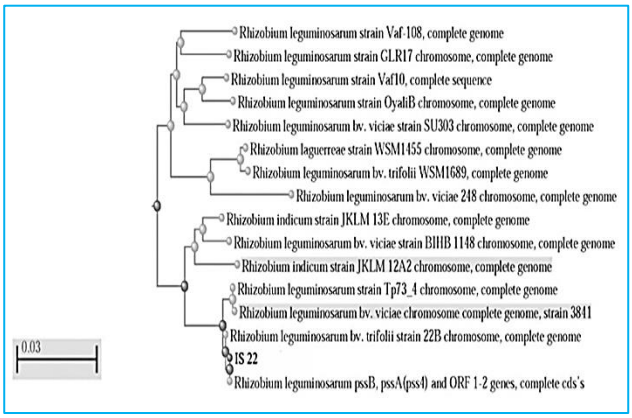

Fig 1 Phylogenetic dendrograms based on 16SrRNA nucleotide partial sequence showing relationship with closely related taxa. IS 22: *Rhizobium leguminosarum*

CONCLUSION

The selected isolates with ACC deaminase and other PGPR traits is an added advantage to mitigate stress conditions. From the data it can be concluded that both these isolates could mitigate the drought stress adversities. With the aim to commercialize both this isolates, further evaluation in the field experimentation is to be done and is underway.

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