



ACC Deaminase Containing Plant Growth Promoting *Agrobacterium larrymoorie* Strain MZ 3-ABF Confers Tolerance to Drought Stress in Chickpea (*Cicer arietinum* L.) Seedlings

Vurukonda Sai Shiva Krishna Prasad ^{a*}, Ningadali Vinay Kumar ^b,
Sriramadas Sravani ^b, Shaik Zulfikar Ali ^a and Vardharajula Sandhya ^a

^aDepartment of Microbiology, Agri Biotech Foundation, PJTSAU Campus, Rajendranagar, Hyderabad, Telangana State-500-030, India.

^bDepartment of Microbiology, Siddhartha P. G. College, Hyderabad, Telangana State-500-070 India.

Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

Plant growth-promoting rhizobacteria (PGPR) that produce 1-aminocyclopropane-1-carboxylate (ACC) deaminase can alleviate plant growth constraints caused by water scarcity. In the present study, six PGPR strains were evaluated to produce several plant growth promoting, and ACC deaminase enzyme isolated from the rhizosphere soil of Chickpea (*Cicer arietinum*) in arid regions of Telangana State, India. According to their 16S rDNA sequencing analysis, only one of the six strains, MZ 3-ABF, belongs to *Agrobacterium larrymoorie*. A drought tolerance experiment revealed two PGPR strains with high phosphate solubilization, nitrogen fixation, indoleacetic-3-acid (IAA), and ACC deaminase enzyme secretion potential were constrained to only MZ 3-ABF and MZ 5-ABF. One strain MZ 3-ABF was chosen for use in a pot experiment to assess their growth-promoting effects on chickpea under drought conditions. This PGPR strain inoculation into chickpea seedlings was expected to alleviate the overall growth inhibition caused by drought stress. The inoculation was thought to have the greatest growth-promoting effects. Inoculation with strain MZ 3-ABF altered plant height, root length, dry biomass, and net photosynthetic rate of leaves, allowing

*Corresponding author: Email: sai.skp@gmail.com;

chickpea seedlings to cope with drought better. They had an indirect effect on the biochemical and physiological properties of chickpea seedlings in order to alleviate drought stress. These findings suggest that the MZ 3-ABF PGPR may be useful for effectively weakening the growth inhibition caused by drought in chickpea. The strain could also be used as effective bioinoculant to maintain pea quality.

Keywords: Plant growth promotion; ACC deaminase; drought stress; chickpea.

1. INTRODUCTION

Because of the potential impact on Earth's biological systems, global climate change has emerged as a major environmental challenge [1]. It appears that greenhouse gases emitted by human activities were the primary drivers of these global average temperature changes during the twentieth century. Increases in greenhouse gases are expected to raise the earth's surface temperature by 1.5 to 11 degrees Celsius by 2100 [2] "that would severely reduce crop production. Drought stress is the most destructive abiotic stress that has increased in intensity over the last decades, affecting global food security. Drought stress can range from moderate and short to extremely severe and long, limiting crop yields" [3]. "Extensive research is being conducted worldwide to develop strategies to cope with drought stress through the development of drought tolerant varieties, shifting crop calendars, resource management practises, and so on" [4] and "most of these technologies are expensive. Recent research suggests that microorganisms can also help plants cope with abiotic stressors" [3].

Increasing temperatures caused by climate change, changes in precipitation regimes, and record-breaking natural disasters caused by meteorological changes every year, which we are beginning to feel more of these days, have a negative impact on human vital activities. Climate change will inevitably have an impact on crop production. Crop production suffers greatly as a result of climate change and associated meteorological disasters. Drought events, which are expected to become more common as a result of climate change, are expected to increase atmospheric evaporation losses [5]. Increased temperatures due to climate change and irregularities in the precipitation regime cause drought to occur more and more every year.

"Global climate change is expected to accelerate in the future as air temperatures and atmospheric

CO₂ levels continue to rise, altering rainfall patterns and distribution" [6]. "Although a lack of water input from rainfall is usually the primary cause of drought stress, water loss from soils due to high temperature events, high light intensity, and dry wind can aggravate an already existing drought stress event" [7]. "Drought stress conditions are common as a result of global climate change across vast areas on a global scale. Along with drought, salinity stress is a major cause of water deficit in plants" [8].

"Plant growth promoting rhizobacteria (PGPR) are one type of beneficial bacteria found in soil" [9]. "PGPR are found in the roots of many different plants. PGPR's effects on plant growth can be mediated by either direct or indirect mechanisms" [10,11]. "Plants' tolerance to abiotic stresses such as drought" [12], chilling injury [13], salinity [14], metal toxicity [15], and elevated temperature stress [16] is also enhanced by PGPR. "Certain PGPR strains have been found to contain an enzyme called ACC deaminase, which hydrolyzes ACC into ammonia and – ketobutyrate" [17,18,19]. The uptake and hydrolysis of 1-aminocyclopropane-1-carboxylic acid (ACC) by the ACC deaminase enzyme, which contains PGPR, reduces the amount of ACC and ethylene in the roots, acting as a sink for ACC. Reduced ACC levels result in lower endogenous ethylene levels, which eliminates the potentially inhibitory effect of stress-induced ethylene concentrations.

In the present study, we tested a known plant growth promoting (PGP) bacterium *Agrobacterium larrymoorei* strain MZ 3-ABF, for its ability to produce ACC deaminase and to enhance drought stress tolerance in chickpea seedlings. The bacterium was isolated from chickpea rhizosphere soil samples collected from drought region Anantapuram, Andhrapradesh India. This bacterium could grow at -1.03 MPa and expressing multiple PGP traits at ambient conditions and at high water deficit conditions (-1.03 MPa).

2. MATERIALS AND METHODS

2.1 Isolation and Screening of Drought Tolerant *Agrobacterium* spp

"*Agrobacterium* spp. were isolated from rhizosphere soil of Chickpea (*Cicer arietinum*) collected from arid and semi-arid regions in India. The crops were grown under rain-fed production system and plants at flowering stage were uprooted and the bulk soil was removed by gently shaking the plants. The root adhering soil (RAS) was collected by dipping the roots in containers containing sterile normal saline followed by shaking for 30 min. The soil suspensions were serially diluted, and the appropriate dilutions were spreadplated on YEMA medium" [20]. The plates were incubated at 28±2 °C and morphologically different colonies were picked and purified on respective media. The pure cultures were maintained on agar slants under refrigerated conditions for further experiments.

"In order to screen the selected isolates for drought stress tolerance, TSB (trypticase soya broth) with different water potentials (-0.05, -0.15, -0.30, -0.49, -0.73, -1.03 MPa) was prepared by adding appropriate concentrations of PEG 6000" [21] and inoculated with the overnight-grown broth cultures adjusted to optical density (OD) of 0.5 at 600 nm. Growth of the isolates at various stress levels was estimated by measuring the OD at 600 nm after incubation at 28 °C for 24 h, under shaking conditions.

2.2 Screening for Plant Growth Promoting Activities

"Isolates which able to grow at maximum negative water potential (-1.03 MPa) level were tested for plant growth promoting traits under control and drought stress condition. To determine phosphate solubilization under control, Pikovskaya's broth (Hi-media, India) was inoculated with 1% of overnight culture (0.5 OD at 600 nm) raised in Luria Bertani (LB) broth and for drought stress Pikovskaya's broth with desired water potential (-1.03 MPa) was inoculated and incubated for seven days at 28 °C on an incubator shaker. The cells were harvested by centrifugation at 2655 g for 5 min and the supernatant thus obtained was used for the quantitative estimation of phosphate" [22,23].

2.3 Indole-3-acetic Acid

"LB broth (control and drought stress) amended with 5 mmol tryptophan was inoculated with 1% of overnight culture (0.5 OD at 600 nm) raised in LB broth and incubated at 28 °C for 3-5 days incubator shaker. Cells were harvested by centrifugation at 2655 g for 5 min and the supernatant was mixed with Salkowsky reagent, followed by incubation for 1 h at room temperature under dark conditions. The absorbance of pink color developed was read at 530 nm" [24]. "The concentration of proteins in the pellet was determined by Bradford method" [25] and the amount of IAA produced was expressed as µg/mg cell protein.

2.4 Siderophore and Hydrogen Cyanide (HCN) Production

"To determine siderophore production under control and drought stress Chrome Azurol S (CAS) broth cultures were prepared, inoculated with 1% bacterial cultures, incubated at 28 °C for five days and checked for development of orange color" [26]. "HCN production under control and drought stress was tested in YEMA broth amended with 0.4% glycine and 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 °C for 2-4 days. Formation of strips from yellow to brownish orange color confirms positive for HCN production" [27].

2.5 Screening for ACC Deaminase Utilization

"For qualitative analysis, bacterial isolates were grown in LB broth and cell pellets were collected by centrifugation, washed, suspended in sterile water and spot inoculated on Dworkin and Foster (DF) salt minimal medium" [28] alone (negative control), DF media supplemented with 3 mmol ACC as the sole source of nitrogen and DF media amended with (NH₄)₂SO₄ (positive control). In order to screen ACC deaminase activity under control and drought stress selected isolates were grown individually in liquid DF minimal medium alone, DF+ACC and DF+(NH₄)₂SO₄ and their growth were measured at 600 nm.

To measure ACC deaminase activity, isolates were grown in 5 mL of LB broth at 28 °C until they reach stationary phase. To induce ACC

deaminase activity under control and drought stress conditions, the cells were collected by centrifugation, washed twice with 0.1 mol Tris-HCl (pH 7.5), suspended in 2 mL of DF minimal medium either supplemented with 3 mmol final concentration of ACC without PEG (control) or with PEG 6000 (drought stress) and incubated at 28 °C with shaking for another 36 – 72 h. ACC deaminase activity was determined by measuring the production of α -ketobutyrate and ammonia generated by the cleavage of ACC by ACC deaminase according to the method of Penrose and Glick [29].

2.6 Plant Growth Studies

The protective effect of inoculated *Agrobacterium* spp. strain MZ 3-ABF on chickpea seedlings exposed to drought stress was studied under sterile soil conditions. Chickpea seeds were surface sterilized with 0.5% NaOCl and 70% ethanol followed by several washes with sterile distilled water and coated with talc-based formulation 10 power 8 cells/g of strain MZ3-ABF using 1% carboxy methyl cellulose as adhesive. The seeds were sown in 500 ml plastic cups filled with 450 g sterile soil and maintained at 2/3 of the field capacity. Soil was collected from college farm, PJTS Agricultural University Campus, Hyderabad, a semi-arid region under rain-fed production system. The soil was air-dried and sieved (< 2mm) before being analyzed for the physicochemical properties. The soil contained 74 % sand, 5 % silt, 24 % clay with 1.40 Mg m⁻³ bulk density, 36.9 % total porosity and 37.9 % water holding capacity; it had pH 7.0 and electrical conductivity of 0.103ms. Organic C, total N and total P content of soil were 0.82 g/kg, 0.16 g/kg and 0.07 g/kg respectively the treatments included seed inoculation with and without *A. larrymoorei* MZ 3-ABF. Two weeks after initial emergence, plants were thinned to three per pot. The pots were watered (sterile water) at 09:30 h daily by weighing the individual pots and supplying each pot a given amount of water that had been pre-calculated based on the water content of dried soil and its water holding capacity. The daily watering brought the water content of the pots to 95% of the field capacity, allowing the plants to grow under conditions free from water stress. After two weeks of seed emergence 12 pots (inoculated (6 No.) and uninoculated (6 No.) were moved into growth chamber 2 at 42/32 °C (heat stress) and the remaining 12 pots (inoculated (6 No.) and uninoculated (6 No.) maintained at 26/16 °C day/night temperature (ambient condition) in the

same chamber 1. In each of the chambers, plants were spaced sufficiently apart to preclude competition effects among treatments. Pots (inoculated and uninoculated) were replicated three times in randomized block method. The growth chamber bench was divided into three sections, one for each of the three replications. Each replication contained 2 pots which were randomly placed within the section on the bench. The three replications were to account for any variability in growing conditions along the bench. Seedlings received 16/8 h light/dark cycle (350 μ mol m⁻² s⁻¹ light intensity). Shoot and root lengths and root dry biomass was determined by harvesting thirty days old seedlings (16 days after transfer to drought stress) [23].

2.7 Plant Biochemical Parameters

In order to study the mechanism of protection of seedlings exposed to drought stress by *Agrobacterium* sp. strain MZ 3-ABF. Thirty days old (15 days after exposure to drought stress) seedlings were harvested and the contents of total sugars, chlorophyll, proline and protein content of seedlings were determined.

“The contents of sugars were determined by incubating 1 g of leaf sample with methanol: chloroform: water (60:25:15 v/v) mixture at 60 °C for 2 h. The samples were centrifuged 8,815 x g and the content of total sugars of the supernatant estimated by phenol sulfuric acid method” [30]. Free proline content was determined by the method of Bates et al. [31]. “The leaf samples homogenized in 3% sulphosalicylic acid were centrifuged 8,815 xg and the supernatant was heated at 100 °C after the addition of acidic ninhydrin. The samples were extracted with toluene and the chromophore containing toluene was aspirated, cooled to room temperature and absorbance was read at 520 nm. The determination of total chlorophyll was done by immersing leaf samples in DMSO and incubating them at 70 °C for 4 h. The absorbance of the solution was then read at 645, 663 and 480 nm” [32]. “The experiment was replicated three times to see the variability among the replicates of same treatment. The membrane injury index (MI) of leaf tissues was determined by recording electrolyte leakage in deionized water at 50 °C and 121°C” [33]. Leaf samples (0.1 g) were cut into discs of uniform size and submerged in 10 ml of deionized water in test tubes and heated at 50 °C for 30 min. The tubes were incubated overnight at room temperature and the conductance was measured using a conductivity

meter. The tubes were then autoclaved for 10 min at 121 °C and the conductance was measured again.

Antioxidant enzymes were estimated after sixteen days (30 days old) of transfer to drought stress. Enzyme extracts for superoxide dismutase (SOD) and catalase (CAT) was prepared by grinding chickpea leaves (1g fresh mass) in a prechilled mortar and pestle first with liquid nitrogen and then with 10 ml of extraction buffer consisting of 100 mM potassium phosphate buffer, pH 7.5 containing 0.5 mM EDTA. For the estimation of ascorbate peroxidase (APX), extraction buffer was further supplemented with 1 mM ascorbic acid and pH was adjusted to 7.5. Extracts were then centrifuged (Sigma 2-16K centrifuge, Germany) at 15000 rpm for 20 min at 4°C and the supernatants analyzed. Enzyme assays were conducted immediately following extraction.

“SOD activity was determined according to method described earlier” [34]. The reaction mixture contained 13 mM methionine, 25 mM nitro-blue tetrazolium chloride (NBT), 0.1 mM EDTA, 50 mM phosphate buffer (pH 7.8), 50 mM sodium carbonate and 0.1 ml enzyme. Activity was determined by adding 2 mM riboflavin and placing the tubes under two 15 W fluorescent lamps for 15 min. A complete reaction mixture without enzyme, which gave the maximal color, served as control. Reaction was stopped by switching off the light and putting the tubes into dark. A non-irradiated complete reaction mixture served as a blank. The absorbance was recorded at 560 nm, and one unit of enzyme activity was taken as that amount of enzyme, which reduced the absorbance reading to 50% in comparison with tubes lacking enzyme.

“APX was estimated by observing the decrease in absorbance due to ascorbic acid at 290 nm” [35]. The assay mixture contained 50 mM potassium phosphate buffer (pH 7.0), 0.5 mM ascorbic acid, 0.1 mM EDTA, 0.1 mM H₂O₂, and 0.1 ml enzyme. The reaction was started with the addition of 0.1 mM hydrogen peroxide. Decrease in absorbance for a period of 30 s was measured at 290 nm in a UV-vis spectrophotometer. Activity is expressed by calculating the decrease in ascorbic acid content by comparing with a standard curve drawn with known concentrations of ascorbic acid.

CAT activity was determined according to method described by Teranishi et al. [36]. “The

reaction mixture consisted of 6 mM hydrogen peroxidase, 0.1 M phosphate buffer pH 7.0, and reaction was started by adding 50 ml enzyme extract. Reaction was terminated after 5 min by adding 4 ml of titanium reagent (prepared by digestion 1 g titanium dioxide with 10 g potassium sulphate and 150 ml of concentrated H₂SO₄). Aliquot was centrifuged at 5000 rpm for 10 min and absorbance of the supernatant was recorded at 415 nm. Reaction mixture without enzyme served as blank”.

2.8 Molecular Characterization of Selected Strains

For molecular characterization, bacterial genomic DNA was extracted according to Chen and Kuo [37] and 16S rRNA gene was amplified by polymerase chain reaction (PCR) using universal forward 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and reverse 1425R (5'-AAGGAGGTGATCCA GCCGCA-3') primers under standard conditions such as initial denaturation, 94 °C for 5 min; 30 cycles of denaturation at 94 °C for 30 s, annealing at 50 °C for 40 s, extension at 72 °C for 90 s; and final extension at 72 °C for 7 min. The PCR product of ~1500 bp was purified and sequenced (SciGenom Labs, India). The sequence obtained was compared with the existing database of 16S rRNA gene using Blast tool on NCBI (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>).

2.9 Statistical Analyses

All data were analyzed statistically by analysis of variance. Plant studies were tested in an experiment using a randomized complete block model with three replications of two independent experiments. Mean comparison among treatments was done by Tukey's multiple comparisons.

3. RESULTS

Six *Agrobacterium* spp. were isolated from the rhizosphere soil of various crops grown in arid and semiarid regions. Using PEG 6000, all six isolates were tested for drought stress tolerance. Only two isolates, MZ 3-ABF and MZ-5-ABF, were able to grow at maximum water potentials of -1.03 MPa. Isolates capable of growing under maximum drought stress (-1.03 MPa) (Fig. 1) were screened for PGP traits in both non-stress and drought stress conditions. Under non-stress and drought stress conditions, two isolates produced IAA, P-solubilization, siderophore, and

hydrogen cyanide (HCN). However, drought stress resulted in a significant reduction in PGP traits (Table 1). Isolate MZ 3-ABF produced the maximum amount of IAA (52.6 ± 0.12 $\mu\text{g}/\text{mg}$ protein) under non-stress followed by MZ 5-ABF (45.02 ± 0.16 $\mu\text{g}/\text{mg}$ protein) and MZ 2-ABF (39.5 ± 0.18 $\mu\text{g}/\text{mg}$ protein). Similarly, under drought stress, isolate MZ 3-ABF was the maximum producer of IAA (47.7 ± 1.4 $\mu\text{g}/\text{mg}$ protein) followed by MZ 5 and MZ 2 (Table 1). The amount of P-solubilization was significantly high in MZ 3-ABF both under non-stress (54.4 ± 2.1 ppm) and drought stress condition (46.2 ± 3.0 ppm) compared to other isolates (Table 1). Siderophore production was not observed in all the isolates under both non-stress and under drought stress. Furthermore, hydrogen cyanide production was negative in all the isolates under non-stress and drought stress (Table 1).

3.1 Screening and Characterization of ACC Deaminase

Two isolates used ACC as the sole source of nitrogen, but there was variation in efficacy (Fig. 2 - A, B, C & Table 2) between the isolates. MZ 3-ABF grew faster (2.1210.123) in the absence of stress than MZ 5-ABF. Similarly, under drought stress conditions, isolate MZ 3-ABF showed significantly higher growth (0.1320.125), whereas other isolates showed no growth (Table 2). The amount of α -ketobutyrate produced during the deamination of ACC by the enzyme ACC deaminase was quantified under both non-stress and drought stress conditions. Isolate MZ 3-ABF produced ACC deaminase enzyme and showed 3.06 ± 0.38 ACC $\mu\text{mol}/\text{mg}$ protein/h α -ketobutyrate under non-stress and 1.19 ± 0.16 $\mu\text{mol}/\text{mg}$

protein/h α -ketobutyrate under drought stress condition.

3.2 Plant Growth Studies

Inoculation of chickpea seedlings with drought tolerant PGP *Agrobacterium* sp. containing ACC deaminase had significant effect on root elongation, shoot length and root dry biomass under ambient and drought stress conditions (Fig. 3). *A. larrymoorei* strain MZ 3-ABF increased root elongation up to 1.32-fold at ambient and 1.29-fold under drought stress over uninoculated control seedlings. Shoot length was also higher in strain MZ 3-ABF inoculated seedlings 1.40-fold at ambient and 1.36-fold under drought stress, similarly significant increase in root dry biomass of chickpea seedlings over uninoculated control was recorded up to 1.52-fold at ambient and 1.75-fold under drought stress respectively. The uninoculated control seedlings started wilting after fifteen days (30 days old) of exposure to drought stress however, seedlings inoculated with ACC deaminase containing *A. larrymoorei* strain MZ 3-ABF survived up to 38 days (53 days old) after exposure to drought stress and started wilting thereafter (Table 3).

3.3 Biochemical Parameters

Drought stress adversely affected the growth of chickpea seedlings. Inoculation with ACC deaminase containing *A. larrymoorei* strain MZ 3-ABF significantly enhanced biochemical parameters of chickpea compared to uninoculated control seedlings. Inoculation significantly enhanced the contents of chlorophyll, total sugars, proline and protein content in chickpea seedlings under ambient

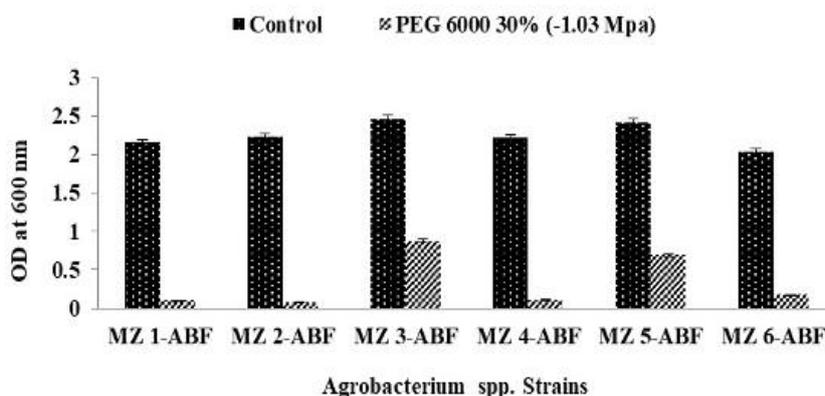


Fig. 1. Optical density (OD) values of *Agrobacterium* sp. strains under drought stress tolerance at -1.03 MPa and non-stress. Error bars Mean of \pm SD (n=3)

Table 1. Plant growth promoting activities of *Agrobacterium* spp. isolates under non-stress and drought stress condition

Isolates	IAA ($\mu\text{g mg}^{-1}$ protein)		Phosphate solubilization ($\mu\text{g ml}^{-1}$)		Siderophore		HCN Total cyanogen content (ppm)	
	NS	DS	NS	DS	NS	DS	NS	DS
MZ 1-ABF	29.6 \pm 0.11	42.4 \pm 0.12	34.6 \pm 1.2	+	-	-	-	-
MZ 2-ABF	39.5 \pm 0.18	30.6 \pm 0.11	41.9 \pm 1.2	+	-	-	-	-
MZ 3-ABF	52.6 \pm 0.12	47.7 \pm 1.4	54.4 \pm 2.1	46.2 \pm 3.0	-	-	-	-
MZ 4-ABF	28.4 \pm 0.11	22.6 \pm 0.16	30.9 \pm 1.1	+	-	-	-	-
MZ 5- ABF	45.02 \pm 0.16	40.6 \pm 0.12	44.6 \pm 1.4	34.5 \pm 1.9	-	-	-	-
MZ 6-ABF	25.6 \pm 0.014	18.4 \pm 0.11	33.3 \pm 1.2	+	-	-	-	-

Numerical values are mean \pm SD of three independent observations; NS, non-stressed; DS, drought-stressed; IAA, Indole acetic acid; HCN, hydrogen cyanide; + positive; - negative

Table 2. ACC deaminase activity of *Agrobacterium* spp. isolates, negative control (without N-source), ACC (ACC as N-source) and positive control ((NH₄)₂SO₄ as N-source)

Isolate	OD values at 600 nm					
	Negative control		ACC		Positive control	
	NS	DS	NS	DS	NS	DS
MZ 1-ABF	0.421 \pm 0.036	0.119 \pm 0.112	0.976 \pm 0.117	0.042 \pm 0.154	2.453 \pm 0.096	1.250 \pm 0.021
MZ 2-ABF	0.352 \pm 0.006	0.089 \pm 0.012	0.954 \pm 0.250	0.082 \pm 0.133	2.117 \pm 0.021	1.271 \pm 0.034
MZ 3-ABF	0.429 \pm 0.038	0.164 \pm 0.014	2.121 \pm 0.123	0.132 \pm 0.125	2.612 \pm 0.001	1.292 \pm 0.022
MZ 4-ABF	0.227 \pm 0.007	0.079 \pm 0.015	0.859 \pm 0.112	0.099 \pm 0.128	2.141 \pm 0.001	1.145 \pm 0.018
MZ 5-ABF	0.321 \pm 0.012	0.099 \pm 0.014	2.001 \pm 0.011	0.089 \pm 0.124	2.548 \pm 0.012	1.047 \pm 0.022
MZ 6-ABF	0.346 \pm 0.011	0.088 \pm 0.015	0.754 \pm 0.102	0.054 \pm 0.112	2.121 \pm 0.102	1.037 \pm 0.011

Numerical values are means \pm SD of three independent observations. NS, non-stress & DS, drought stress

Table 3. Physiological parameters of strain MZ 3-ABF and control under non-stress and drought-stress condition

	Root Length (cm)		Shoot Length (cm)		Dry root biomass (g)	
	NS	DS	NS	DS	NS	DS
Control	28.26±1.12	25.04±1.32	32.25±1.01	28.56±1.00	1.45±0.09	1.25±0.02
MZ 3-ABF	37.56±1.56	32.58±1.54	45.28±1.12	39.12±1.11	2.21±0.00	2.19±0.02

Numerical values are means±SD of three independent observations. NS, non-stress & DS, drought stress; cm, centimeter

Table 4. Biochemical parameters of drought-tolerant isolate MZ 3-ABF under non-stress and drought-stress condition

	Chlorophyll (mg g ⁻¹ DW)		Proline (μmol g ⁻¹ DW)		Total sugars (μmol g ⁻¹ DW)		Total protein mg gm ⁻¹ DW	
	NS	DS	NS	DS	NS	DS	NS	DS
Control	7.42±0.03	4.11±0.11	6.52±0.11	14.45±0.15	8.82±0.09	29.60±0.02	72.06±0.09	49.25±0.02
MZ 3-ABF	8.42±0.03	5.16±0.01	8.82±0.12	19.76±0.12	11.28±0.10	38.21±0.02	88.25±0.00	62.21±0.02

Numerical values are mean ± SD of three independent values. mg-milli grams; NS-non-stress; DS-drought stress; DW-dry weight

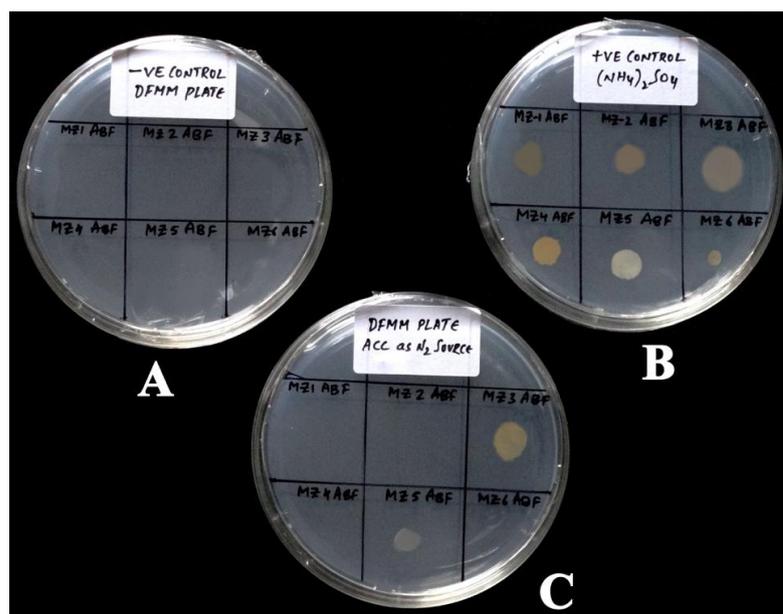


Fig. 2. Screening of test bacterial strains for ACC deaminase enzyme production on DF minimal media plate assay. A - negative control plate without any nitrogen source, B – positive control plate with $(\text{NH}_4)_2\text{SO}_4$ as nitrogen source and C – ACC media plate as sole source of nitrogen



Fig. 3. Thirty days old chickpea seedlings under drought stress conditions

conditions and drought stress (Table 4). Drought stress condition decrease the chlorophyll content of leaves of both inoculated and uninoculated seedlings compared to seedling (inoculated and uninoculated) at ambient conditions. *A. larrymoorei* strain MZ 3-ABF inoculation counteracted the adverse effect of drought on leaf chlorophyll compared to uninoculated control. A significant decrease in the protein and sugar content of uninoculated seedlings was observed on exposure to drought stress, whereas the protein (1.26-fold) and sugar (1.29-fold) content of inoculated seedlings significantly increased on exposure to drought stress

compared to uninoculated seedlings (Table 4). Drought stress significantly increased proline content of leaves as compared to that of ambient conditions. Inoculation of *A. larrymoorei* strain MZ 3-ABF containing ACC deaminase significantly increased (1.36-fold) the leaf proline content compared to the values of the uninoculated control seedlings under drought stress.

Significant reduction in activity of antioxidant enzymes was observed among all three antioxidant enzymes such as Superoxide dismutase, Catalase and Ascorbate peroxidase under drought stress conditions in inoculated plants as compared to uninoculated plants. ACC deaminase containing *A. larrymoorei* strain MZ 3-ABF inoculation counteracted the adverse effect of drought on leaf antioxidant enzymes by lowering the ROS generation compared to uninoculated control. Under drought stress the percent decrease in enzyme activity by strain MZ 3-ABF as 68% SOD, 44% APX and 63% CAT in seedlings is more than uninoculated seedlings such as 20% SOD, 8% APX and 16% CAT respectively.

3.4 Identification of strain MZ 3-ABF

The most prospective strain MZ 3-ABF was selected based on drought stress tolerance and

PGP traits production under drought stressed conditions was characterized based on microscopic, morphological, and molecular studies. Microscopic studies revealed that the isolate MZ 3-ABF was Gram negative, motile, rod-shaped bacteria. On agar plate isolate appeared as white, mucoid and doomed. Based on 16s rRNA gene sequence blast analysis on NCBI, isolate MZ 3-ABF was identified as *Agrobacterium larrymoorei*, and the nucleotide sequence was submitted to NCBI GenBank under accession No. KU885896.1

4. DISCUSSION

“Rhizobacteria are well known for their ability to colonise the root tissues of a wide variety of crop plants and promote plant growth by producing phytohormones, antagonistic substances, and enzymes” [38]. “Ethylene, a stress-induced phytohormone, has been shown to inhibit root growth and cause senescence in crop plants” [39]. “It has been proposed that bacteria with ACC deaminase activity reduce stress ethylene levels and thus confer resistance to various stresses” [40]. “Indeed, the current experiment's findings, as well as those reported previously, show increased resistance to salt stress” [41], drought stress [42], flooding stress [43], heavy metal stress [44] and pathogen stress [45]. “In the present study, the growth of *A. larrymoorei* strain MZ 3-ABF on DF salt minimal medium containing ACC as nitrogen source revealed the secretion of ACC deaminase enzyme” [40]. “ACC deaminase gene encoding ACC deaminase has been isolated from different soil bacteria” [46-51,45].

“Plants are constantly subjected to abiotic stresses such as drought, temperature, flooding, salinity, and so on, which results in poor performance and yield loss” [52]. “Drought, a major abiotic stress, can cause massive productivity losses in arid and semi-arid regions where agriculture is entirely dependent on rains” [53]. “The extent of a plants ability to withstand such stress is determined by metabolic alterations” [54]. “In the present study, we demonstrated that ACC deaminase containing PGP *A. larrymoorei* strain MZ 3-ABF colonizing chickpea roots can significantly influence the seedlings resistance to drought stress. Seed inoculation with strain MZ 3-ABF had a pronounced effect on chickpea growth, development and response to drought stress. Root elongation, shoot length and dry biomass was significantly stimulated by treatment with

ACC deaminase containing strain MZ 3-ABF by lowering endogenous ethylene levels due to hydrolysis of ACC which is the immediate precursor for ethylene synthesis under drought stress when compared to uninoculated seedlings. These results are consistent with the proposed model on the mechanism of plant growth promotion by soil bacteria that lowers ethylene levels” [40]. “It was also discovered that inoculation with strain MZ 3-ABF containing ACC deaminase increased total chlorophyll content. Accelerated ethylene synthesis is known to cause senescence, according to Arshad and Frankenberger” [55], and *A. larrymoorei* strain MZ 3-ABF in this study may have suppressed ethylene synthesis due to its ACC deaminase activity, which slowed chlorophyll decay. Glick et al. [56] discovered that “*Pseudomonas putida* strain GR12-2 increased chlorophyll content in canola plant shoots due to ACC deaminase activity. The increase in chlorophyll content could also be attributed to increased photosynthetic leaf area of the plant as a result of inoculation, as opposed to the uninoculated control, where leaf area was reduced due to drought stress” [57].

“Strain MZ 3-ABF colonization onto chickpea roots also increased the levels of total sugars under drought stress compared to uninoculated seedlings. This, combined with an enlarged root system and improved uptake of nutrients from soil, may have contributed to the stimulation of growth, development and adaptation to drought stress. Proline is a dominant organic molecule that accumulates in many organisms upon exposure to environmental stress” [58] and plays multiple roles in plants adaptation to stress [59,60]. We found a significant correlation between drought tolerance and an increase of proline concentration in chickpea seedling after exposure to drought stress. In this study, *A. larrymoorei* strain MZ 3-ABF significantly increases proline accumulation in chickpea seedlings upon drought stress compared to uninoculated control seedlings. The accumulation of proteins in leaves under drought stress is an adaption mechanism as they bound to membranes, regulating membrane water permeability in cells and influencing water movement among tissue [61].

“Reactive oxygen species (ROS) such as singlet oxygen (1O_2), superoxide radical (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radical (OH^\cdot) may convert ACC to ethylene, the higher the ethylene production resulting in more cellular damage” [62]. Inoculation with *A. larrymoorei* strain MZ 3-

ABF in chickpea seedlings lowers the ACC levels by converting it to ammonia and α -ketobutyric acid making unavailability of ACC for ROS. The OH- radicals formed by the reaction of O₂- and H₂O₂ are extremely toxic, causing damage to chlorophyll, protein, DNA, lipids, and other important macromolecules, affecting plant metabolism and limiting growth and yield [63]. Antioxidant enzymes like SOD convert O₂- to H₂O₂, which is then removed by APX or CAT. Inoculation with ACC deaminase from *A. larrymoorei* strain MZ 3-ABF reduced ROS levels, allowing the chickpea seedling to survive drought stress. Whereas in uninoculated seedlings due to the absence of ACC deaminase enzyme activity the ethylene levels will be high and the generated ROS under drought stress may further converts the ACC to ethylene thereby increasing the concentration of ethylene resulting in the severe damage to cellular components and leads to cell death. In case of antioxidant enzymes ACC deaminase containing *A. larrymoorei* strain MZ 3-ABF inoculated seedlings showed lower activity compared to uninoculated seedlings.

Hall et al. [64] discovered that dicotyledonous plants are more vulnerable to the effects of ethylene, particularly when stressed by flooding [43], salt [41], drought [42], and phytopathogens. [45]. These findings are more consistent with our findings, which showed that chickpea seedlings responded more positively to the activity of ACC deaminase from the strain MZ 3-ABF. Furthermore, the current findings are consistent with the findings of Burd et al. [44], who reported that “the plant growth promoting bacterium *Kluyvera ascorbate* SUD165 displayed increased resistance to the toxic effects of heavy metals and ACC deaminase activity, which reduced stress ethylene levels”.

5. CONCLUSION

The current study demonstrates that ACC deaminase-producing rhizobacteria are drought tolerant. As a result, healthy plant roots from drought-prone areas can be chosen as a resource to isolate these types of bacteria that could be used to protect plants from the effects of drought stress. According to our findings, inoculations with *A. larrymoorei* strain MZ 3-ABF isolated from the rhizosphere soil of Chickpea (*Cicer arietinum*) could effectively alleviate drought stress damage and promote the growth of chickpea seedlings, with the best promotion effects. The mechanisms by which PGPRs

alleviate environmental stress and promote plant growth are a complex network of multiple pathways. The strain MZ 3-ABF improved soil nutrients, which then aided plant growth by increasing nitrogen fixation and phosphate solubilization. Furthermore, the ability of PGPRs to partially regulate phytohormones and induce the ROS defense system indirectly affects the biochemical and physiological properties of chickpea, alleviating drought stress. As a result, the findings suggest that microorganisms may play a role in mitigating the negative effects of climate change on crop growth, which may lead to the development of microbial products to mitigate such effects. However, more research under greenhouse and field conditions is needed, as well as elaboration of the protective mechanism.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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