Original Research Article

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

ABSTRACT:

Plant growth-promoting rhizobacteria (PGPR) that produce 1-aminocyclopropane-1-carboxylate (ACC) deaminase can reduce limits to plant growth due to water-deficient conditions. 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. Among the six strains, only one strain MZ 3-ABF belongs to *Agrobacterium larrymoorie*, according to their 16S rDNA sequencing analysis. A drought tolerance experiment revealed two PGPR strains MZ 3-ABF and MZ 5-ABF with high phosphate solubilization, nitrogen fixation, indoleacetic-3-acid (IAA) and ACC deaminase enzyme secretion potential. Among these two strains only one strains (MZ 3-ABF) was selected for use in a pot experiment to evaluate their growth-promoting effects on chickpea under drought conditions. This PGPR strain inoculation into chickpea was expected to alleviate the comprehensive growth inhibition of chickpea seedlings caused by drought stress. The inoculation was hypothesized to elicit the best growth-promoting effects. Inoculation with strain MZ 3-ABF significantly altered the plant height, root length and dry biomass, and net photosynthetic rate of leaves, enabling chickpea seedlings to better cope with drought. They indirectly affected biochemical and physiological properties of chickpea seedlings to alleviate their drought stress. Taken together, these results demonstrate that the MZ 3-ABF PGPR might be useful for effectively weakening the growth inhibition caused by drought in chickpea. The strain might also be applied as effective bioinoculants to maintain the quality of peas.

Key words: Plant growth promotion, ACC deaminase, drought stress, chickpea

INTRODUCTION:

Global climate change has emerged as an important environment challenge due to potential impact on biological systems of planet Earth **[1]**. It appears more likely that greenhouse gases from human activities were the dominant drivers of these global average temperature changes during the 20th century. In future, increase in greenhouse gases were projected to raise earth's surface temperature between 1.5 to 11 ºC by 2100 **[2]** that would severely reduce crop production. Among abiotic stresses, drought stress is the most destructive abiotic stresses that increased in intensity over the past decades affecting world's food security. Drought stress may range from moderate and short to extremely severe and prolonged duration, restricting the crop yields **[3]**. Worldwide extensive research is being carried out to develop strategies to cope with drought stress through development of drought tolerant varieties, shifting the crop calendars, resource management practices etc., **[4]** and most of these technologies are cost intensive. Recent studies indicate that microorganisms can also help plants to cope with abiotic stress factors **[3]**.

Plant growth promoting rhizobacteria (PGPR) are one class of beneficial bacteria inhabiting the soil ecosystem **[5]**. PGPR are found in association with roots of many different plants. The effects of PGPR on plant growth can be mediated by direct or indirect mechanisms **[6, 7]**. PGPR also enhance the tolerance of plants to abiotic stresses like drought **[8]**, chilling injury **[9]**, salinity **[10]**, metal toxicity **[11]** and elevated temperature stress **[12]**. It has been discovered that certain PGPR strains contain an enzyme ACC deaminase that hydrolyses ACC into ammonia and α-ketobutyrate **[13, 14, 15]**. The uptake and hydrolyses of 1-amino cyclopropane-1-carboxylic acid (ACC) by ACC deaminase enzyme containing PGPR decreases the amount of ACC, as well as ethylene in the roots, thereby acting as a sink for ACC. Decreased levels of ACC results in lower levels of endogenous ethylene, which eliminate 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).

MATERIALS AND METHODS

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 spread plated on YEMA medium **[16]**. 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 **[17]** 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.

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 ^{o}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 **[18, 19]**.

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 **[20]**. The concentration of proteins in the pellet was determined by Bradford method **[21]**, and the amount of IAA produced was expressed as µg/mg cell protein.

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 ^{o}C for five days and checked for development of orange color **[22]**. 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 ^{o}C for 2-4 days. Formation of strips from yellow to brownish orange color confirms positive for HCN production **[23]**.

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 **[24]** alone (negative control), DF media supplemented with 3 mmol ACC as the sole source of nitrogen and DF media amended with $(NH_4)_2SO_4$ (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+ $(NH_4)_2SO_4$ 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 0.1mol 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 **[25]**.

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 (10^8 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 ^oC (heat stress) and the remaining 12 pots (inoculated (6 No.) and uninoculated (6 No.) maintained at 26/16 ^oC 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 (350µmol 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) **[19]**.

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 ^{0}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 **[26]**. Free proline content was determined by the method of Bates *et al.* **[27]**. The leaf samples homogenized in 3% sulphosalicylic acid were centrifuged 8,815 xg and the supernatant was heated at 100 ^{o}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 ^{o}C for 4 h. The absorbance of the solution was then read at 645, 663 and 480 nm **[28]**. The experiment was replicated three times to see the variability among the replicates of same treatment. The membrane injury index (MII) of leaf tissues was determined by recording electrolyte leakage in deionized water at 50 ^{o}C and 121^{o}C **[29]**. Leaf samples (0.1 g) were cut into discs of uniform size and submerged in 10 ml of deionozed water in test tubes and heated at 50 ^{o}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 ^{o}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 sorghum 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 **[30]**. 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 **[31]**. The assay mixture contained 50 mM potassium phosphate buffer (pH 7.0), 0.5 mM ascorbic acid, 0.1 mM EDTA, 0.1 mM H_2O_2, 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.* **[32]**. 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 H_2SO_4). 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.

Molecular characterization of selected strains:

For molecular characterization, bacterial genomic DNA was extracted according to Chen and Kuo **[33]** and 16S rRNA gene was amplified by polymerase chain reaction (PCR) using universal forward 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and reverse 1425R (5′-AAGGAGGTGATCCAGCCGCA-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).

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.

RESULTS

A total of six *Agrobacterium* spp. was isolated from rhizosphere soil of different crops grown in arid and semiarid regions. All the isolates were screened for drought stress tolerance using PEG 6000, among six, only two isolates MZ 3-ABF and MZ-5-ABF were able to grow at maximum water potentials of -1.03 MPa. Isolates able to grow at maximum drought stress (-1.03 MPa) (Figure 1) level was screened for PGP traits under non-stress and drought stress condition. Two isolates produced IAA, P-solubilization, siderophore and hydrogen cyanide (HCN) production under non-stress and drought stress condition. However, a significant reduction in PGP traits was observed under drought stress (Table 1). Isolate MZ 3-ABF produced the maximum amount of IAA (52.6±0.12 µg/mg protein) under non-stress followed by MZ 5-ABF (45.02±0.16 µg/mg protein) and MZ 2-ABF (39.5±0.18 µg/mg protein). Similarly, under drought stress, isolate MZ 3-ABF was the maximum producer of IAA (47.7±1.4 µg/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).

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

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

	IAA	Phosphate solubilization	Siderophore	HCN
	(μg mg^{-1} protein)	(μg ml^{-1})		Total cyanogen content (ppm)

Isolates	NS	DS	NS	DS	NS	DS	NS	DS
MZ 1-ABF	29.6±0.11	42.4±0.12	34.6±1.2	+	-	-	-	-
MZ 2-ABF	39.5±0.18	30.6±0.11	41.9±1.2	+	-	-	-	-
MZ 3-ABF	52.6±0.12	47.7±1.4	54.4±2.1	46.2±3.0	-	-	-	-
MZ 4-ABF	28.4±0.11	22.6±0.16	30.9±1.1	+	-	-	-	-
MZ 5- ABF	45.02±0.16	40.6±0.12	44.6±1.4	34.5±1.9	-	-	-	-
MZ 6-ABF	25.6±0.014	18.4±0.11	33.3±1.2	+	-	-	-	-

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

Screening and characterization of ACC deaminase

Two isolates utilized ACC as sole source of nitrogen however, variation in efficacy to utilize ACC was observed (Figure. 2 - A, B, C & Table 2) between the isolates. MZ 3-ABF showed higher growth (2.121±0.123) under non-stress followed by MZ 5-ABF. Similarly, under drought stress condition isolate MZ 3-ABF showed significantly higher growth (0.132±0.125) whereas, no growth was observed with other isolates (Table 2). The ACC deaminase activity was assayed under both non-stress and drought stress conditions by quantifying the amount of α-ketobutyrate produced during the deamination of ACC by the enzyme ACC deaminase. Isolate MZ 3-AB utilized ACC as the sole source of nitrogen by producing ACC deaminase enzyme and showed 3.06±0.38 ACC µmol/mg protein/h α-ketobutyrate under non-stress and 1.19±0.16 µmol/mg protein/h α-ketobutyrate under drought stress condition.

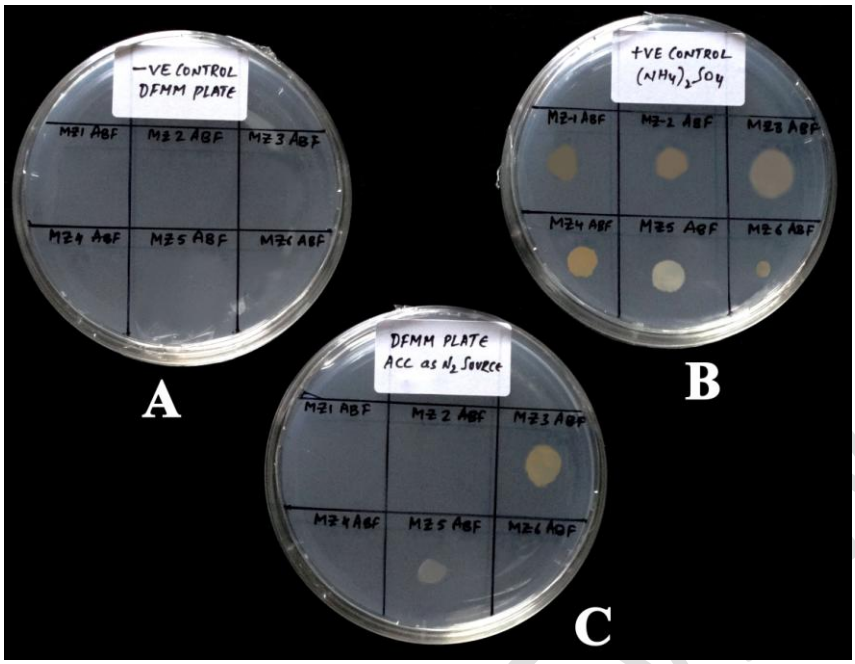

Figure 2: Screening of test bacterial strains for ACC deaminase enzyme production on DF minimal media plate assay. A - negative control plate without any nitrogen source, B – positive control plate with $(NH_4)_2SO_4$ as nitrogen source and C – ACC media plate as sole source of nitrogen

Table 2: ACC deaminase activity of *Agrobacterium* spp. isolates, negative control (without N-source), ACC (ACC as N-source) and positive control ($(NH_4)_2SO_4$ as N-source)

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

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

Plant growth studies

Inoculation of chickpea seedlings with drought tolerant PGP *Agroba*cterium sp. containing ACC deaminase had significant effect on root elongation, shoot length and root dry biomass under ambient and drought stress conditions (Figure. 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).

Figure 3: Thirty days old chickpea seedlings under drought stress conditions.

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

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 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.

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

	Chlorophyll	Proline	Total sugars	Total protein

	(mg g -1 DW)		(µmol g^{-1} DW)		(µmol g^{-1} DW)		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

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.

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 larrymoorie,* and the nucleotide sequence was submitted to NCBI GenBank under accession No. KU885896.1

DISCUSSION

Rhizobacteria are well known for their ability to colonize the root tissues of wide crop plants and promote the plant growth by the production of phytohormones, antagonistic substances and enzymes **[34]**. The stress-induced phytohormone, ethylene has been known to inhibit root growth and be responsible for senescence in crop plants **[35]**. It has been suggested that bacteria containing ACC deaminase activity reduces the levels of stress ethylene and thus confer resistance to various stresses **[36]**. Indeed, this concept is supported by the results of present experiment and those reported previously demonstrating increased resistance to salt stress **[37]**, drought stress **[38]**, flooding stress **[39]**, heavy metal stress **[40]**

and pathogen stress **[41]**. 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 **[36]**. ACC deaminase gene encoding ACC deaminase has been isolated from different soil bacteria **[42, 43, 44, 45, 46, 47, 41]**.

Plants are constantly exposed to abiotic stresses such as drought, temperature, floods, salinity etc. leading to poor performance and yield loss **[48]**. Drought being a major abiotic stress may cause huge productivity losses in arid and semi-arid regions where the agriculture totally depends on rains **[49]**. The extent of a plants ability to withstand such stress is determined by metabolic alterations **[50]**. 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 **[36]**. It was also observed that total chlorophyll content increased due to inoculation with strain MZ 3-ABF containing ACC deaminase. According to Arshad and Frankenberger **[51]**, accelerated ethylene synthesis is known to cause senescence; *A. larrymoorei* strain MZ 3-ABF in this study might have suppressed ethylene synthesis because of its ACC deaminase activity that resultantly slowed down chlorophyll decay. Glick *et al.* **[52]** also found that *Pseudomonas putida* strain GR12-2 increased the chlorophyll content in the shoots of canola plant because of ACC deaminase activity. The increase in chlorophyll content may also be the result of increased photosynthetic leaf area of plant by inoculation compared to uninoculated control where the leaf area was reduced due to drought stress **[53]**.

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 **[54]** and plays multiple roles in plants adaptation to stress **[55, 56]**. 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 **[57]**.

McRae *et al.* **[58]** reported that reactive oxygen species (ROS) such as singlet oxygen ($^1O_2$), superoxide radical (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radical (OH^-) may convert ACC to ethylene, the higher the ethylene production resulting in more cellular damage. 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 combination of O_2^- and H_2O_2 is highly toxic which can damage chlorophyll, protein, DNA, lipids and other important macromolecules, thus affecting plant metabolism and limiting growth and yield **[59]**. The expression of antioxidants such as SOD converts O_2^- to H_2O_2 which is further removed by APX or CAT. Inoculation with ACC deaminase containing *A. larrymoorei* strain MZ 3-ABF lowered the levels of ROS making the chickpea seedling to survive under 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.* **[60]** observed that dicotyledonous plants are more susceptible to the effects of ethylene especially under stress conditions such as flooding **[39]**, salt **[37]**, drought **[38]** and phytopathogens **[41**. These findings are more appropriate with our results wherein chickpea seedlings showed more positive response to the activity of ACC deaminase from the strain MZ 3-ABF. In addition, the present results are in conformity with the findings of Burd *et al.* **[40]**, who reported that plant growth promoting bacterium *Kluyvera ascorbate* SUD165, showed more resistance to the toxic effects of heavy metals and displayed ACC deaminase activity which lowered the levels of stress ethylene.

CONCLUSION

The present study shows that ACC deaminase-producing rhizobacteria exhibit high tolerance to drought stress. Therefore, roots of healthy plants from drought prone areas can be selected as a resource to isolate these type bacteria that might be used to protect plants from drought stress impacts. Our study suggests that inoculations with *A. larrymoorie* strain MZ 3-ABF isolated from rhizosphere soil of Chickpea (*Cicer arietinum*) could effectively alleviate drought stress damage and promote the growth chickpea seedlings upon inoculation showing the best promotion effects. The mechanisms by which PGPRs alleviate environmental stress and promote plant growth rely on complex combination of numerous pathways. The strain MZ 3-ABF improved soil nutrients and then promoted plant growth by contributing to enhanced nitrogen fixation and phosphate solubilization. Additionally, the PGPRs' ability to partly regulate phytohormones and induce the ROS defense system indirectly affects biochemical and physiological properties of chickpea, ameliorating the drought stress incurred by plants. Therefore, the findings suggest the possible role of microorganisms in mitigating adverse effects of climate changes on crop growth and may lead to development of microbial products to mitigate such effects. However, further studies are required under greenhouse and field conditions and the mechanism of protection must be elucidated.

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