Int.J.Curr.Microbiol.App.Sci (2016) *5*(11): 275-283

International Journal of Current Microbiology and Applied Sciences
ISSN: 2319-7706 **Volume 5 Number 11 (2016) pp. 275-283**
Journal homepage: http://www.ijcmas.com

Original Research Article http://dx.doi.org/10.20546/ijcmas.2016.511.030

Regulation of Ethylene Level in Mungbean (*Vigna radiata L.*) by 1-Aminocyclopropane-1-Carboxylic Acid (ACC)-Deaminase containing Bacterial Strain under Salt Stress

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Keywords

ACC-deaminase. Ethylene biosynthesis. Mungbean. Pseudomonas. Salinity.

Article Info

Accepted:
16 October 2016
Available Online:
10 November 2016

A B S T R A C T

This study explored several features related to salt tolerance in mungbean plants through plant growth promoting bacteria (PGPB) *Pseudomonas simiae* strain AU. We report the significant effect of 1-aminocyclopropane- 1-carboxylate deaminase on the physical parameters and biomass content of *Vigna radiata* as compared control seedling under salt stress. Control (plants devoid of bacterial strains) and PGPB inoculated mungbean plants were grown in soil: peat (1:1) subjected with saline and non-saline conditions. Results showed that PGPB inoculated plants had superior ability to tolerance against salt stress, as exposed by their enhanced plant biomass (Fresh weight), higher water content, higher photosynthesis activity and lower osmotic stress injury (Table 2). The increased proline accumulation in PGPB inoculated plants root contributed to increased plant tolerance to salt stress (Fig. 1). These results suggest that, in PGPB-inoculation plays a role in mitigate the adverse effect of salt stress. Furthermore, enhanced proline maintain an osmotic balance to keep a positive water potential for water entrance into the roots and reduce oxidative damage by lowering reactive oxygen species level under salt stress condition. Our results indicate that *Pseudomonas simiae* strain AU is multifunctional PGPB strains that can promote plant growth, development and reduce salinity stress by decreasing the stress ethylene level.

Introduction

The plant hormone ethylene is a gaseous hormone which is found in the all higher plants is an important modulator for normal plant growth and developmental process as well as a key feature in the response of different abiotic and biotic stresses (Abeles *et al*., 1992). Ethylene is an inhibitor for plant growth but at very low concentration it may promote plant growth in a large number of ways such as promoting root initiation in many plant species including *Arabidopsis* (Pierik *et al*., 2006). In the presence of wide range of environmental stresses like salinity (Mayak *et al*., 2004a), drought (Mayak *et*

al., 2004b), temperature stress (Ghosh *et al*., 2003) metal stress (Belimon *et al*., 2009) etc., ethylene production may increases, this stress level of ethylene inhibit the root and shoot elongation, inhibiting root nodule formation, decreasing plant-microbe interaction, and inhibiting seed germination (Abeles *et al*., 1992). Hirsch and Fang (1994) reported that leguminous plants produced more ethylene, adversely affects the nodule formation.

Salinity stress extensively associated with elevated release of endogenous ethylene in higher plants which responsible for growth inhibition. Mayak *et al*., (2004a) have been reported the effect of soil salinity on the inhibited growth of plant roots and nodule formation in legumes due to high production of ethylene. Many other researchers also report the concentration dependent effect of salinity on seedling growth, increase in ethylene production with increasing level of salinity (Ahmad *et al*., 2011). The ACC content and ACC oxidase activity were increased due to increasing level of salinity. Many researchers experimentally provide that the induced level of ethylene have been reduced by the using plant growth promoting bacteria (PGPB) containing ACC-deaminase (Glick 2004; Mayak *et al*., 2004a). The use of PGPB-containing ACC-deaminase to improve plant growth in many crops has been increased worldwide. ACC deaminase-containing bacteria significantly decrease the portion of physiological damage in plants due to environmental stresses including exposure to extremes of temperature, high salt, flooding, drought, exposure to metals and organic contaminants. PGPB contain an enzyme ACC-deaminase lowers the stress ethylene level by conversion of ACC into ammonia and α-ketobutyrate in the plants (root and seed) (Glick 2012). Belimov *et al*., (2009) studied that PGPB containing ACC-deaminase enhanced plant growth, particularly under stressed conditions by the regulation of increased ethylene production in response to abiotic and biotic stresses. Glick (2012) was described a scheme to mechanism of action of ACC-deaminase to reduce the stress ethylene level by catalytic conversion of ethylene precursor ACC that includes cyclopropane ring fragmentation, and deamination of ACC to from α–ketobutyrate and ammonia.

Legumes such as *Vigna radiata* (mung bean) serve as a major source of food protein in most of the Asian countries including India. It is also agriculturally important as a rotational crop, as it can fix the unavailable atmospheric N_2 through root nodules in association with *Rhizobia*. Based on the previous findings and reports, we decided the aim of present study that was to investigate the effect of *Pseudomonas simiae* (MTCC-12057) bacterial strain AU on mungbean growth and salt stress tolerance. We found that bacterial inoculum showed better plant growth, decrease ethylene level and improved salt stress tolerance compared to non-inoculated plants.

Materials and Methods

Bacterial strain and growth

Pseudomonas simiae strain AU (NCBI accession no. KJ511869, MTCC no. 12057) was used in the present study and maintained on nutrient broth (NB, Himedia, India) amended with 50% glycerol at –20°C. Bacterial strain was grown in nutrient broth amended with different concentration of NaCl (50mM-300mM) and examined their tolerance level (Jha and others 2010). Inoculum for the seed treatment was prepared by harvesting bacterial cells from 24 hrs cultures on nutrient agar plates at

26°C. The inoculum was suspended in sterile distilled water to yield 10^8 colony-forming units (CFU) per ml. We checked the susceptibility assay for the seeds of mungbean to growth at different salt concentrations and found that seedling growth was retarded at 100mM and 200mM NaCl concentration. On the basis of above described reason we have selected 100mM and 200mM NaCl concentrations for further study.

Quantification of ACC-deaminase activity

ACC deaminase activity was determined by measuring the amount of α-ketobutyrate produced by the cleavage of ACC by ACC deaminase (Penrose and Glick 2003). Both bacterial strains were grown on DF (Dworkin and Foster 1958) salt minimal media supplemented with 3mM of ACC or 0.1M of $(NH_4)_2SO_4$ for 48h. The experiment was arranged into three different culture conditions. These were as following- 1. Bacterial culture and DF salt (N source absent) 2. Bacterial culture + ACC as a nitrogen source and DF salt media. 3. Bacterial culture + ACC as a nitrogen source + NaCl (50mM, 100mM, 200mM and 300mM) and DF salt. The bacterial cells were harvested by centrifugation at 10,000rpm at 4ºC for 10 min, washed twice with 0.1 M Tris-HCl (pH 7.5), and resuspended 0.1 M Tris-HCl (pH 8.5). The cells were labialized by 5% toluene (v/v) and then vortex at the highest speed for 1 min. The mixtures containing 1ml supernatant and 3mM ACC were incubated for 1h at 28°C. The samples were then mixed thoroughly with 500 µL of 0.56 N HCl by vortexing, and the cell debris was removed by centrifugation at 10,000rpm for 10 minutes. A 500 µL aliquot of the supernatant was transferred into glass test tube and mixed with 400 µL of 0.56N HCl and 150 µL of DNF solution (0.1 g 2, 4-dinitrophenylhydrazine in 100 mL of 2N HCl) and the mixture was incubated at 30°C for 30 minutes. One ml of 2N NaOH was added to the sample before the absorbance at 540 nm was measured. The concentration of α-ketobutyrate in each sample was determined by comparison with a standard curve generated as follows: 500 µL α-ketobutyrate solutions of 0, 0.01, 0.05, 0.1, 0.2, 0.5, 0.75 and 1 mM were mixed respectively with 400 µL of 0.56 N HCl and 150µL DNF solution. One ml of 2N NaOH was added and the absorbance at 540 nm was determined as described above. We observed that the *P. simiae* strain AU was showed ACC-deaminase activity at all NaCl stress concentration (Table 1).

Plant growth conditions under salt stress

To check the effect of *P. simiae* strain AU on mungbean plant under drought stress, six experimental groups designed wherein six replication per treatment were used. The bacterial inoculum was suspended in sterile physiological saline (0.9%NaCl) to yield 1×10^8CFU ml^{-1}. Mungbean seeds were used for the pot trial experiment. For surface sterilization, seeds were treated with 70% ethanol for 3 minutes and 0.1% $HgCl_2$ for 1 minute and washed with 3 times milli Q water after each treatment. After surface sterilization seeds were inoculated with *P. simiae* strain AU 0.1% carboxy methyl cellulose (CMC) for 3h according to Ahmad *et al*., (2011). For control, seeds were soaked in sterile water for the same period of time. After 3h incubation period seeds were sown into pots. Mungbean seeds were placed in autoclaved soil: peat (1:1) at equal distance of each other. Seeds were allowed to germinate in the dark in a growth chamber at 28°C for three days. After seed germination, pots were transferred to light with photoperiod of 16h/8h light/dark and temperature 28°C. After 8 days seedlings

were treated with Hoagland's solution containing 100mM and 150mM NaCl and these conditions were maintained for 7 day. Control seedlings were kept in Hoagland's solution without NaCl. Based on the bacterial inoculation and NaCl treatment four experimental groups [Control (T1), 100mM NaCl stress (T2) 200mM NaCl stress (T3), AU-inoculated (T4), AU + 100mM NaCl (T5); AU + 200mM NaCl (T6)] were designed. After treatment for 7 days, the mungbean seedlings were sampled and transferred at -80ºC until the measurement of variables under study.

Monitoring plant growth parameters

Inoculated seedlings and their corresponding non-inoculated control were harvested and root length, shoot length and fresh weights were measured. To measure leaf relative water content (RWC), fresh weighed (FW) 2nd trifoliate leaves were used and floated the leaves tissue in distilled water for 24 h at room temperature in a dark place. Then measured the turgid weight (TW) along with dry weight (DW) for oven dried leaves treated at 80°C for 48 h. RWC was calculated by equation (Mayak *et al*., 2004a): RWC=FW-DW/TW-DW×100

Proline and total soluble sugar analysis

Free proline content was measured by spectrophotometric analysis at 520nm according to modified method of Bates *et al*., (1973) using acid ninhydrin reagent. Amount of proline was determined by using a standard curve of known concentration of L-proline and expressed as µg/gFW. Total soluble sugar(TSS) were analyzed by 0.1 ml of the alcoholic extract of leaves and roots of different treatments reacting with 3 ml freshly prepared anthrone (200 mg anthrone + 100 ml of 72% H_2SO_4) and incubated at 100°C in a boiling water bath for 10 min according to Irigoyen *et al*., (1992). After cooling at room temperature, the absorbance was read at 620 nm in a spectrophotometer and the TSS concentration was measured using standard curve of glucose in the range of 20–400 µg/ ml and concentration of TSS in plant samples was expressed in µg/gFW.

Ethylene production in response to stress

For the detection of ethylene, six seeds for each treatment (T1, T2, T3, T5 and T6) were placed in respective 25 ml flask on sterile filter paper. Seeds were sterilized and then imbibed in water for control and bacterial suspension (0.2 O.D.) of *P. simiae* AU for 2 h before being placed in the flasks. After 6 days, when the cotyledons expanded, 100mM and 150mM NaCl applied for salt stress induction and kept each flask for 24h and then closed for 2 h using a rubber septum. A 1 ml sample of the headspace air from each flask was injected into a gas chromatography (Shimadzu - GC 2010) equipped with flame ionization detector (FID) stainless steel fused capillary column RTX 5 MS (30 m x 320 µm i.d., film thickness 0.25 µM).The amount of ethylene production was calculated by using the standard curve of pure ethylene and the concentration of ethylene in samples was expressed as pmol h^{-1} g^{-1} FW.

Statistical analysis

For statistical analysis, randomized block design (RBD) was employed to investigate error in experimentation with a 2x3 factorial arrangement was used that include: two conditions (well water and drought) and three treatments (control: devoid of bacterial inoculation, AU inoculation, and AU-M4 inoculation).Plant growth parameters data were analyzed by Two way ANOVA analysis followed by Tukey's multiple comparison test. Remaining experiments

such as ethylene detection, stomatal size measurement and bacterial PGP properties were done only under drought condition. Hence, these data were analyzed by One way ANOVA analysis followed by DMRT multiple comparison test. All analyses described were performed in SPSS software (SPSSInc., Version 16.0, Chicago, IL). Results were discussed in terms of percentage variation, with respect to control.

Results and Discussion

Effect of bacterium inoculation on mungbean growth attributes

The ameliorative effect of PGPB on the mungbean plant growth and its allied attributes were assessed under slight (100mM NaCl) and moderate (200mM NaCl) salinity stress. With 100mM NaCl stress the increase for shoot length was 16% and while at 200mM NaCl the increase was 40.35% on the inoculation of AU (Table 2). Similarly, promotion of root length under salt stress was 31% and 70% with strain AU, under 100mM and 200mM NaCl stresses respectively (Table 2). Plant fresh weight was increased by bacterial inoculation by 15.4% and 85.7% at 100mM and 200mM NaCl salt stresses respectively. Exposure to salinity stress adversely influences the plant RWC. The non-inoculated control plants had significantly lower RWC as compared to bacterial strain AU application during normal growth conditions. Relative water content of control plants was 45.9% at no-stress lower than the AU inoculated plants, respectively. However, this effect was more prominent in non-inoculated control plants under salinity.

Free proline and total soluble sugars accumulation

Our findings indicated that under saline stress, bacterial inoculated plants were synthesized proline to a greater extent (Fig. 1). However, the proline content in with slight and moderate salinity was increased in compared to non-stressed conditions. In the present study, bacterial inoculated seedlings root accumulated 35.8% and 60.5% higher proline content under 100mM and 200mM salt stress levels (Fig. 1). Similarly TSS content was also recorded higher to 25.2% and 54.8% for AU inoculated plantlets root tissue than non-inoculated plant roots in 100mM and 200mM NaCl salt stress respectively (Fig. 1).

Effect on ethylene level

In the present study ethylene level was increased in mungbean plantlets under salt stressed conditions. However, bacterial inoculated seedlings exhibited with lower ethylene content during salt stress because of their ACC-deaminase activity that degrade ethylene precursor in mungbean seedlings. *P. simiae* AU inoculated plant showed 41.6% and 50% lower ethylene content than non-inoculated seedlings under 100mM and 200mM NaCl stress respectively (Fig.2).

High salinity in the agriculture sector is worldwide problem. Salinity stress is plant growth inhibitory in many crops. Mungbean is salt sensitive legume food crop; it has been documented that when mungbean plants are exposed to high levels of salt stresses their ethylene production is increased. ACC deaminase-containing plant growth-promoting bacteria have been reported to facilitate plant growth under salt stress by reducing the stress ethylene level that is produced as a consequence of abiotic stress (i.e. salt stress, drought stress etc) (Ali *et al*., 2014). In the present study, one plant growth-promoting bacterial strain *P. simiae* AU is used. As discussed earlier, ACC-deaminase is a key enzyme, produced by

bacteria, which helps in ameliorating the plant stress under a variety of abiotic and biotic stress conditions (Kumari *et al*., 2015). When the mungbean seeds were treated with the plant growth-promoting ACC-deaminase containing bacterial strains (*P*. *simiae* AU), the emerging plant was ready to deal with a number of stresses more effectively and provided better protection against both type of stresses. Even in the absence of any stress, mungbean seeds inoculated with the bacterial strain AU showed better growth when compared to the control plants (no bacterial treatment) (Table 2). In the present study we found that the selected *P*. *simiae* strain AU was positively enhanced the mungbean plant growth parameters and increase the induced systemic salt tolerance. Hence, bacterial inoculation with plants is most effective strategy under stressed environment to increase plant growth and productivity (Ali *et al*., 2014). It has been suggested that containing ACC-deaminase bacteria to maintain stressed ethylene level and confers tolerance to various environmental stresses (Glick *et al*., 1998). This suggestion is supported by the result of the present study (Fig. 2) and also those that were reported previously by many researchers demonstrating induced tolerance level to salt stress, flooding stress, and heavy metal resistance (Mayak *et al*., 2004a). Roots plays major role in salinity tolerance in plants by modifying their anatomical and morphological parameters. Here, plants inoculated with strain AU showed higher shoot and root length compared to control plants (Table 2) under both NaCl salt level.

Table.1 ACC-deaminase activity of selected bacterial strain *P. simiae* strain AU under different NaCl concentrations. Values represent the mean ± SE, n=6

Bacterial strain	**ACC-deaminase (α-ketobutyrate nmol mg^{-1} h^{-1})**				
	0mM NaCl	50mM NaCl	100mM NaCl	200mM NaCl	300mM NaCl
***P. simiae* AU**	98±1.3	93.9±1	86.1.0±0.8	79±.5	71.4±.1.0

Table.2 Effects of different treatment on various plant growth parameters of mungbean seedlings under non-stressed and NaCl salt stressed conditions

Treatment	**Root length (cm)**	**Shoot length (cm)**	**Fresh weight (g)**	**RWC (%)**
T1	9.87±.42	24±.44	3.8±.4	61±3.3
T2	7.1±.33	18±.30	2.6±1.5	57±2
T3	5.0±.26	11±0.26	1.4±0.7	53±4.1
T4	11.0±.48**	25.5±.56**	4.2±0.8**	72±3.5***
T5	9.3±.4***	21±.5***	3±1.4***	60±3.3***
T6	8.5±.5****	16.0±2****	2.4±1.5****	56±3.1***

RWC represents relative water content. Data were analyzed by Two way ANOVA analysis followed by Tukey's multiple comparison test. Values represent the means ±SD, n=6. Asterisks denoting *P* value of significance (** $P \leq 0.01$; *** $P \leq 0.001$; **** $P \leq 0.0001$).

Fig.1 Effect of different treatment on free proline (µg/g FW) and total soluble sugars content (µg/g FW) in mung bean seedlings. Where T1-control; T2- 100mM NaCl; T3- 200mM NaCl; T4- AU inoculated; T5- AU + 100mM NaCl; T6- AU inoculated + 200mM NaCl. Values represent the means±SD, n = 6

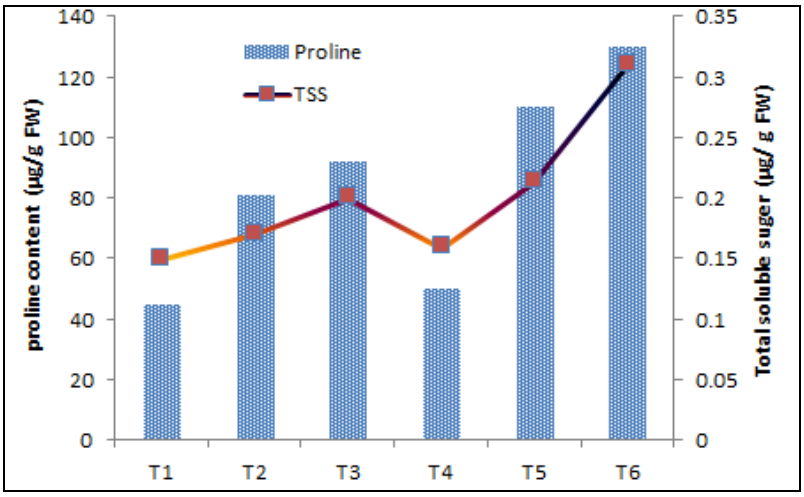
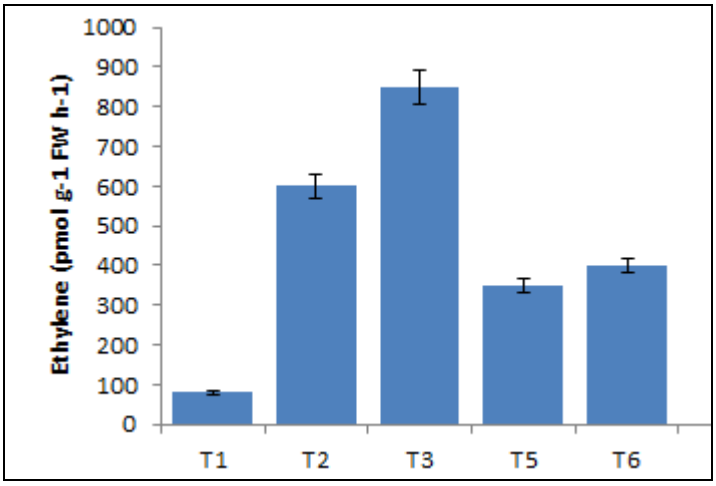


Fig.2 Effect of salinity stress on ethylene level in mungbean seedlings under different treatments. Where T1-control, T2- 100mM NaCl, T3- 200mM NaCl; T5- AU + 100mM NaCl; T6- AU inoculated + 200mM NaCl. Data were analyzed by One way ANOVA analysis followed by DMRT test at P=0.05. Values represent the means ±SD, n=6.



A high level of proline under abiotic stresse has been reported in the presence of beneficial bacteria such as, *Burkholderia* (Porcel and Ruiz-Lozano2004), *Arthrobacter* (Sziderics *et al*., 2007) and *Bacillus* (Gururani *et al*., 2013). Salinity stress induced the accumulation of soluble sugars in the plants that are contributed to the plant tolerance under salt stressed condition (Trotel-Aziz *et al*., 2000). In reference to above statement, bacterial treated seedlings showed increase TSS and proline content in their root during salt stressed condition (Fig.1). In this study ACC-deaminase eliminate the "salt stress imposed effects" on root and shoot growth were evident from the data documented. It was also observed that inoculation with

PGPB containing ACC-deaminase was effective in increasing water use efficiency in peas under drought stress conditions (Ali *et al*., 2014). This verifies that ACC-deaminase breakdown the ACC level which results in the lower level of ethylene, the best results were found with AU inoculation (Fig. 2). Belimov *et al*., (2002) and Penrose *et al*., (2001) also studied the ability of ACC-utilizing PGPB to improve plant growth inhibition caused by stress ethylene through decrease ACC content in plant tissue.

In conclusion, the overall data shows that plant tissues are highly influenced by bacterium AU by means of induced systemic tolerance mechanisms against salt stress. It seems that the AU enhances osmotic adjustment by the accumulation of osmolytes and inhibition of stress ethylene level in plant tissue which could contribute to maintaining water potential encouraging to the water movement from soil into the roots. So, the inoculation of *pseudomonas simiae* strain AU through may improve the salt tolerance and enhance plant growth.

Acknowledgments

The research was supported by SERB-Grant no. SR/FT/LS-129/2012 to DKC. Some of the research has partially been supported by DBT grant no. BT/PR1231/AGR/21/340 /2011 to DKC. Authors would also like to thanks to Dr. Ajay kumar, Advanced Instrumentation Research Facility (AIRF, JNU), New Delhi for GC analysis.

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How to cite this article:

Sarita Kumari, Anukool Vaishnav, Shekhar Jain, Devendra Kumar Choudhary and Kanti Prakash Sharma. 2016. Regulation of Ethylene Level in Mungbean (*Vigna radiata L.*) by 1-Aminocyclopropane-1-Carboxylic Acid (ACC)-Deaminase containing Bacterial Strain under Salt Stress. *Int.J.Curr.Microbiol.App.Sci.* 5(11): 275-283.
doi: http://dx.doi.org/10.20546/ijcmas.2016.511.030